CHARACTERIZATION OF THE *IN VITRO* IMMUNE FUNCTION OF REPTILE MACROPHAGES FROM *SCELOPORUS OCCIDENTALIS*

By

DAVID CARL MELSON

Bachelor of Science in Microbiology
Oklahoma State University
Stillwater, Oklahoma
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OCCIDENTALIS

Dissertation Approved:

Dr. D. Kim Burnham

Dr. Jeffrey Hadwiger

Dr. Anand Sukhan

Dr. Larry G. Talent

Dr. K. Darrell Berlin

Dr. A. Gordon Emslie

Dean of the Graduate College

PREFACE

Macrophages are instrumental in many physiological conditions, both beneficial and detrimental. Examples of these conditions include tissue remodeling, clearance of microbial pathogens, autoimmune disease and both enhancement and suppression of tumor growth and metastasis. As a cellular component of the innate branch of the immune system, they represent an evolutionarily conserved cell-type, the mononuclear phagocyte.

Ectotherms in general, and reptiles in particular, comprise a poorly represented niche in biological research, including immunity. The western fence lizard, *Sceloporus occidentalis* is a species similar in size to the classic mammalian model of immunity, the mouse. A strain of this species bred in captivity at Oklahoma State University was selected as a source of reptile macrophages to explore basic immune parameters of macrophage biology.

Peritoneal macrophages were harvested from experimental subjects and cultured for individual testing/challenge by common agonists. Such stimuli as lipopolysaccharide (LPS), Concanavilin A (ConA), phorbol myristic acetate (PMA) and formyl-Methionine-Leucine-Phenylalanine (fMLP) were chosen to activate immune mechanisms including, but not limited to, respiratory burst, chemotaxis, phagocytosis, and pinocytosis over a range of temperatures. Most of these parameters were detected and quantified by colorimetric assays.

As compared to RAW 264.7 murine macrophages, reptile peritoneal macrophages

produced significant quantities of superoxide radical though no detection of nitric oxide was observed. As might be expected, the reptile macrophages performed better at lower temperatures (27 ^{o}C). The lysosomal enzyme acid phosphatase was also easily detected in the activated macrophages.

Due to a consistently insufficient number of resident macrophages, chemotaxis in the reptile macrophages proved elusive. Based on literature observations of mammalian macrophages, elicited/activated macrophages have already migrated to a stimulus and cannot respond to a second signal.

Phagocytosis was initially observed in reptile macrophages by a novel method utilizing bacteria transformed to express green fluorescence protein (GFP). Detection was made by fluorescence microscopy. Further studies were performed using fluoroscein isothiocyanate (FITC) labeled bacterial particulate.

While additional information about general macrophage biology was gained, as was support for a standardized reptile laboratory model, and satisfactory results were achieved to determine reptile macrophage function, a reptile with the capacity for a higher yield of both resident and elicited macrophages is desired. When such a model can be determined, further comprehensive immune functions can be elucidated, including cells other than peritoneal macrophages, such as splenocytes, peripheral blood neutrophils, bone-marrow derived dendritic cells, and macrophages from other tissue sources.

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CHAPTER I

INTRODUCTION

Virtually all living organisms are susceptible to periodic invasions by various microbial parasites which often results in a pathological state. Eukaryotes in particular have developed defensive systems in order to minimize the damage incurred by these invaders and to clear the offending microbe completely. Vertebrates have long been recognized for their unique possession of a binary immune system consisting of both a germ-line encoded innate arm that recognizes highly conserved motifs in a relatively non-specific process as well as an adaptive arm that by somatic rearrangement of multiple genetic loci is able to adapt and respond to each invader in a highly selective manner. The outcome of a microbial infection is often dependent upon the speed and strength with which a defensive response is mounted. As such, the innate immune system plays a critical role during the early stages of an infective event.

The innate immune system is of particular interest for a variety of reasons. Firstly, the components can be studied in a more straightforward manner due to the independence from previous antigenic exposure. Also, being endowed at birth, experimental subjects require a shorter time to immunological maturity. Finally and most importantly for the purpose of evolutionary studies, the innate system possesses the most conserved elements and functions, some of which are seen throughout the entirety of eukaryotic life.

A cellular component of the innate system was first documented in the late 1800s by Elie Metchnikoff who observed the migration and response of cells in starfish larvae to inflammation mediated by thorns (Karnovsky and Bolis, 1982; Bibel, 1988; Gallin and Fauci, 1982; Espey, 2006; Kurtz, 2004; Silverstein, 1979) resulting in a Nobel Prize in Medicine in 1908 (Paul, 1984). Since that time, cells with similar functions have been identified in numerous multi-cellular species, spanning the diversity of eukaryotic organisms. These cells are most commonly phagocytes, having the capacity to engulf particulate matter and even whole cells.

Phagocytes, such as macrophages, constitute a prominent first line of defense. Derived from circulating monocytes, tissue bound macrophages perform basic homeostatic functions such as the removal of cellular debris from the microenvironment. These resting macrophages can be activated by direct contact with a microbe, cell surface receptor binding with a microbial ligand, or cytokines secreted by other host cells in response to tissue trauma. The macrophage subsequently responds by one or more classic functions, including but not limited to phagocytosis, production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), secretion of cytokines for the recruitment of other leukocytes, and antigen presentation to activate T-lymphocytes (thymus derived) to initiate an adaptive response and the direct and/or non-direct destruction of infected or aberrant host cells.

Macrophages are also of particular importance and interest in both research and health. With a diverse array of functions and capacities, macrophages can influence a wide variety of physiological activities. For the host organism, homeostasis via endocytic debris removal, wound healing by means of tissue remodeling functions, and

resolution of invasion by the destruction and clearance of microbial pathogens all serve to maintain the host's health. Of equal importance, however, are the implications that macrophages mediate numerous disease states, most notably auto-immune inflammatory conditions, and that macrophages can contribute to the survival and metastasis of tumors within a given tissue microenvironment.

Ectotherms represent an important biological group in multiple disciplines including evolution, developmental biology, toxicology, ecology, and molecular biology. Studies in embryonic development often utilize the eggs of frogs and salamanders based on the ease of microscopic observation and manipulation. The social behaviors of lizards have contributed to our general understanding of behavioral ecology. Salamanders are able to thermoregulate due to molecular mechanisms that led to the discovery of heat shock proteins. Most recently, reptiles have formally entered into the field of genomics. The green anole, *Anolis carolinensis*, has been selected as the first reptile for genomic sequencing and upon completion will be available for comparison with established genomic models. It is expected that this addition will be of great benefit based upon the earlier sequencing of the African clawed frog, *Xenopus laevis,* and the subsequent comparative research performed between various genomic models. In addition, there has been a concerted effort to establish a reptile model for laboratory studies in general and developmental biology (Talent lab, Oklahoma State University) immunology (Burnham lab, Oklahoma State University) and immunotoxicology (Rooney lab, University of Quebec).

Numerous and diverse studies have been performed on a wide variety of reptile species, however to date no extensive, multi-functional assays have been performed on a

single species. It is the intent of this project to perform such a study with the ultimate goals of furthering the understanding of the evolutionary development of macrophage function, expanding the limited immunological understanding of ectothermic vertebrates and establishing a baseline for a reptile macrophage model system.

While there are many attributes by which macrophages can be characterized, this project will focus on those most commonly accepted by the scientific community as being universal among the mononuclear phagocytes of many species. The hallmark functions of an activated macrophage are endocytosis, respiratory burst, and cytotoxicity. The study will also be extended to lesser noted but equally important functions such as chemotaxis and degradative enzyme production. To fully understand and quantify the results of this study, side by side comparisons were made when possible with either murine primary peritoneal macrophages or the murine macrophage-like RAW 264.7 cell line purchased from the American Type Culture Collection (ATCC). These mammalian cells served as both control cells and as a comparative contrast between the ectothermic reptile and the endothermic mammal.

CHAPTER II

REVIEW OF LITERATURE

There has been a resurgence in interest of innate immunity with the advent of molecular biology. As genomic and proteomic techniques have been developed, molecular components in the germ line have been discovered, most notably the Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b; Medzhitov and Janeway, 1998; Medzhitov and Janeway, 2000; McGettrick and O'Neill, 2004; Kopp and Medzhitov, 1999; Kopp and Medzhitov, 2003; Akira, 2003; Anderson, 2000; Krutzik, Sieling and Modlin, 2001; Underhill and Ozinsky, 2002, Hultmark, 2003; Medzhitov and Biron, 2003; Kabelitz and Medzhitov, 2007; O'Neill, 2006; Magor and Magor, 2001; Roach et. al., 2005; Flajnik and Du Pasquier, 2004). Homologues of Toll elements, first identified in *Drosophila*, have been found in numerous organisms and multiple cell types. Their function in higher vertebrates has been determined as being pattern recognition receptors (PRRs) for pathogen associated molecular patterns (PAMPs) derived from microbial pathogens. Much like other PRRs such as the Fc receptor which binds the crystallizable fragment of soluble antibodies (Anderson, 1989; Mellman, 1988; Jungi et. al., 1988), the mannose receptor that recognizes carbohydrate residues on pathogenic microbes (Artursson, Johansson and Sjoholm, 1988; Shepherd et. al., 1981), and the complement receptor which recognizes host serum proteins from the complement cascade (Artursson, Johansson and Sjoholm, 1988), TLRs recognize the

highly conserved motifs such as lipopolysaccharide (LPS), peptidoglycan (PGN), zymosan, or flagellin expressed by microbes. With the sequencing of additional organisms, studies into the comprehensive function of these molecular receptors have expanded rapidly.

Cellular elements of the innate immune system have been studied in numerous species; however, the vast majority of research performed involved mammals. Macrophages are a major cellular component of innate immunity and have been widely studied to elucidate the mechanisms and extent of their immune function. The response that macrophages mount against pathogens can be manifested by several means. The most common attack usually involves a direct cell-cell interaction culminating in the engulfment and/or destruction of the invader by chemical products (Sasada and Johnston, 1980; Vazquez-Torres et. al., 2000; Brummer, Morrison and Stevens, 1985; Brennan et. al., 2004). As key mediators in inflammation, macrophages often initiate the concerted effort of various leukocytes. Under optimal conditions, primarily the increased expression of surface Ia molecules and B7 co-stimulatory molecules, they may serve as antigen presenting cells (APCs) to recruit the adaptive immune response (van Furth, 1985; Sompayrac,1999; Paul, 1984). The various states in which they may exist have also drawn the attention of those interested in the process of differentiation (See Figure 1).

Published research includes comprehensive reviews covering the extensive list of macrophage secreted products. These include numerous enzymes such as elastase and lysozyme, cell growth factors and regulators such as interleukin 1 (IL-1), tumor necrosis factor alpha

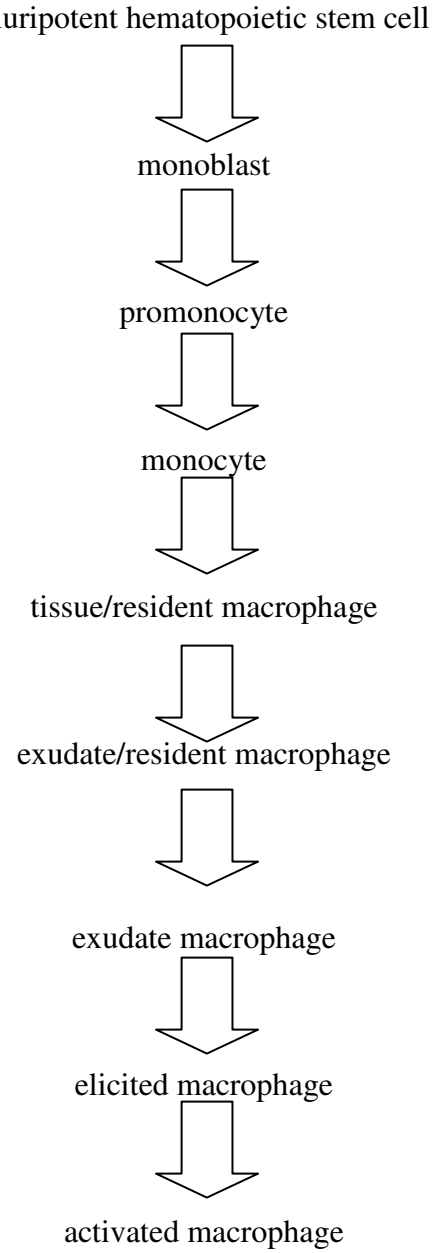

Figure 1. Simplified schematic diagram of macrophage maturation. There is considerable overlap of the functions and capacities at the final 3 steps of maturation.

(TNFα), cyclic adenosine monophosphate (cAMP), and cytostatic/cytocidal molecules such as interferons alpha (IFNα) and beta (IFNβ) and reactive metabolites such as superoxide, singlet oxygen, and hydrogen peroxide (Rappolee and Werb, 1989). Studies have also been performed to determine the differential secretion and membrane receptor expression under differing states of activation. For example, lysozyme production has been found to be constitutive, whereas complement protein C_5A receptor is up-regulated in resident macrophages but down-regulated in inflammatory macrophages, and reactive oxygen species (ROS) production is variable with the eliciting agent (Adams and Hamilton, 1984). This phenotypic variation makes the macrophage not only attractive as a model for differentiation but also allows for the wide array of functional capacities seen throughout the life cycle of the cell.

Multiple groups have studied the activation process to varying degrees and specificities. While some labs determined activation by more physiologically relevant "pathogenic" conditions (Adams and Hamilton, 1984) others investigated the state of activation under more stringent stimuli such as lectin activation (Kesherwani and Sodhi, 2007) or interleukin stimulation alone (Mosser, 2003). The system-wide effects of inflammatory stimuli were also investigated by determining the ontogeny of monocyte/macrophage production during under such conditions (van Furth, 1985).

The function(s) and characteristic(s) of activated macrophages lend them to even more extensive study. Commonly attributable functions like respiratory burst and endocytosis have been studied from a variety of perspectives.

Respiratory burst was first identified by Baldridge and Gerard in 1935 as an increase in mitochondrial consumption of oxygen. In 1959, Sbarra and Karnovsky

determined that respiratory burst was not affected by cyanide eliminating the possibility of mitochondrial contribution (Babior, 2000). Since that time, respiratory burst activity has been recognized as a notable increase in non-mitochondrial oxygen consumption at the cellular level with a subsequent production of one or more reactive/radical chemical species (Rossi et. al., 1985).

The initial product of this process is superoxide radical (O_2^-) mediated by membrane bound NADPH oxidase (Miller, et. al., 1995; Brubacher and Bols, 2001; Babior, 2000; Rossi, Bianca and De Togni, 1985; Badwey and Karnovsky, 1980; Lunardi, Lima and Assreuy, 2006). Based upon the stimulus, state of macrophage activation, and availability of further enzymes and chemical intermediates, formation of numerous other ROS may occur, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and peroxynitrite ($ONOO^-$) (Miller and Britigan, 1995; Nathan and Shiloh, 2000; Forman and Torres, 2001). The products and level of production is dependent on both the current state of activation in the macrophage as well as the tissue source of the cells.

The production of reactive nitrogen species (RNS) such as nitric oxide (NO) are associated with respiratory burst not only by shared stimuli but also by at least one shared product, peroxynitrite, produced by reaction between superoxide and nitric oxide (Forman and Torres, 2001; Babior, 2000). Nitric oxide is formed by the oxidation of the guanidino nitrogen of L-arginine to L-citrulline via the cytosolic enzyme nitric oxide synthase (NOS) (Miller and Britigan, 1995; Nathan and Shiloh, 2000). One of three isoforms of nitric oxide synthase, phagocytes utilize the inducible form (iNOS) in immune functions (Holstad, Jansson and Sandler, 1997; Kim et. al., 2005, Bae et. al., 2005). Long recognized as an important messenger molecule for neurons and as a

vasodilator in vascular endothelium, with extended emphasis upon the discovery of an immune function, nitric oxide was named “Molecule of the Year” by Science magazine in 1992 (Koshland, 1992).

The respiratory burst of phagocytes has been the subject of a wide array of investigations, ranging from the determination of radical products formed during the immune response to methods and materials capable of modulating the response. Further studies have elucidated the mechanisms and pathways by which the burst occurs, as well as the effect(s) mediated by the chemical species both in the target organism and the host. Interestingly, it has been determined that the unique interactions within a given micro-environment of the host can lead to a variety of consequences for the interacting cells and/or organisms, such as enhanced survival or destruction of tumors, host / self tissue damage, and even the initiation of pathogen survival systems.

Extensive reviews and experiments have outlined the extent of the radical products from phagocytic cells (Miller and Brittigan, 1995; Nathan and Shiloh, 2000; Fang, 2004; Forman and Torres, 2001; Badwey and Karnovsky, 1980) the underlying mechanisms and subsequent functions of these products (Rossi, et. al, 1985; Babior, 2000; Shepherd, 1986; Forman and Torres, 2001) and the enzymatic basis of respiratory burst (Rossi, et. al, 1985; Babior, 2000).

Many investigators have studied the distinct interaction between the phagocyte and PAMPs. Bacterial lipopolysaccharide (LPS) is a common activator of burst activity. A combination of LPS and tyrosine kinase inhibitors has been used to determine specific intermediates in burst production (Leu, et. al, 2005). Priming with sub threshold levels of LPS was shown to desensitize macrophage oxidative responses (Zhang and Morrison,

1993). LPS activated murine macrophages demonstrated increased production of phorbol myristate acetate (PMA) stimulated superoxide when compared to resident or unstimulated macrophages (Sasada, et. al, 1983). Multiple components of *Yersinia enterocolitica* were tested for the ability to induce a respiratory burst response in murine peritoneal macrophages. Cellular extracts and *Yersinia* outer proteins (Yops) showed control levels of nitric oxide and hydrogen peroxide production while LPS from the bacteria displayed a dose dependent production (Carlos, et. al, 2004). Interestingly, the combination of LPS and Yops suppressed NO production (Carlos, et. al, 2004). Pre-treatment with LPS or muramyl dipeptide (MDP) was also shown to enhance the production of superoxide upon stimulus by PMA (Pabst and Johnston, Jr, 1980)

Other PAMPs, such as lipoteichoic acid (LTA) and CpG oligodeoxynucleotides (a bacterial DNA motif lacking methylation) were shown to induce macrophage respiratory burst (Chang, et. al, 2006) and (Meng, et. al, 2003) respectively. Whole pathogen stimulation has been shown with microsporidian spores in rat macrophages (Leiro, et. al, 2001). As these parasitic organisms often mediate a pathologic event by disseminating throughout the host within macrophages, this study measured both extra cellular and intracellular radical production. Even gene encoded resistance mechanisms induced by exposure to radical species have been discovered in some strains of *Eschericia coli* (Nunoshiba, et. al, 1993). *Toxoplasma gondii* survival has been shown to be dependent on the ontogenic state of the host cell and the ability to commence respiratory burst. Monocytes were able to ingest Toxoplasma, initiate burst and destroy the organism, while macrophages ingested the organism but failed to trigger the burst pathway (Wilson, et. al, 1980).

Enhanced respiratory burst activity has been shown upon treatment with proteolytic enzymes such as trypsin and papain (Johnston, et. al, 1981) or by chemical elicitation with thioglycollate (Johnston, et. al, 1978). Increased cell density of *in vitro* cultures also enhanced nitric oxide production in murine macrophages (Jacobs and Ignarro, 2003). Catecholamines such as epinephrine and dopamine have been shown to enhance LPS stimulated nitric oxide production (Chi, et. al, 2002). Prostaglandin D (PGD) produced by activated macrophages also has a synergistic effect on nitric oxide production, however application of exogenous PGD has a suppressive effect (Bellows, et. al, 2006).

Examples of non-mammalian models for macrophage respiratory burst have been demonstrated. Chicken monocytes and heterophils and the avian macrophage cell line HD11 were compared side by side for nitric oxide production (Crippen, et. al, 2003). The monocytes demonstrated a more robust response as compared to the heterophils, while the cell line had an almost 10 fold increase over the monocytes. The HD11 cell line was also utilized to study signal transduction pathway inhibitors of nitric oxide production. Inhibitors of protein tyrosine kinase (PTK), the mitogen activated protein kinase (MAPK) p38, the regulatory molecule I kappa B (IκB), and nuclear factor kappa B (NF-κB) all demonstrated a dose dependent suppression of nitric oxide production (Crippen, 2006). These avian studies of nitric oxide were particularly relevant because they represented the first non-mammalian inducible nitric oxide synthase (iNOS) enzyme to be cloned (Crippen, 2006).

Respiratory burst parameters have also been useful in the determination of toxic effects by environmental contaminants. The fungicide chlorothalonil exerted a dose

dependent suppression of superoxide production in striped bass macrophages (Baier-Anderson and Anderson, 2000). The jet fuel JP-8 was shown to have a marginal effect on nitric oxide production (Keil, et. al, 2003). An estimated five billion gallons is used annually and it is expected that significant local contamination may occur. 3-Monochloro-1,2-propanediol, a by product of food product manufacture and known carcinogen in rats, had an inhibitory effect on ConA induced blastogenesis in murine splenocytes and in nitric oxide production in murine peritoneal macrophages (Byun, et. al, 2005). B-Chlorolactic acid, an intermediate in the formation of the above carcinogen, exhibited similar effects on murine splenocytes and macrophages (Lee, et. al, 2005). Nitrite and nitrate, end products of the short lived nitric oxide, were measured as indicators of nitric oxide production in response to exposure by food derived heterocyclic amine compounds. A dose dependent increase in nitrite/nitrate was seen, promoting the theory that the carcinogenic effects seen with these food compounds might be mediated by host nitric oxide (Yun, et. al, 2006).

Other deleterious effects have been attributed to the products of respiratory burst and the often resulting inflammation. Reviews have implicated nitric oxide in rheumatoid arthritis and systemic lupus erythematosus (Nagy, et. al, 2007) as well as Type I diabetes mellitus (Holstad, et. al, 1997). Studies have been performed that suggest a role in the DNA damage of liver cells by activated macrophages (Watanabe, et. al, 2001).

With the evidence of inducible respiratory burst and the potential for self/host damage resulting from the products formed, it is understandable from an evolutionary viewpoint that there would exist protective mechanisms. As potent producers of radical

species, macrophages have been shown to be protected from oxidative stress by a cytosolic NADP+-dependent isocitrate dehydrogenase (Maeng, et.al, 2004).

One of the most wide-spread areas of research in respiratory burst biology is the search for medically relevant means to suppress inflammation intentionally. The RAW 264.7 macrophage cell line was used to determine the basis of the anti-inflammatory action of azathioprine. A dose dependent decrease was seen in iNOS mRNA, iNOS protein levels and nitric oxide production (Moeslinger, et. al, 2006). Ketamine is a common anesthetic agent and was also studied in the RAW cell line. Oxidative capacity was both time and dose dependent on exposure to ketamine (Chang, et. al, 2005).

Numerous plant derived factors have also been examined for their potential to reduce or inhibit burst mediated inflammation. Phytochemicals from a wide variety of sources have shown to ameliorate inflammation. Soybean saponins suppressed production of iNOS mRNA, protein and nitric oxide (Kang, et. al, 2005). Cardamonin from *Alpinia rafflesiana* inhibited the expression of iNOS in the RAW cell line (Israf, et. al, 2007). A flavonoid from *Tamarindus indica* was shown to reduce the production of nitric oxide both *in vitro* and *in vivo* (Komutarin, et. al, 2004). Numerous carotenoids were tested for the capacity to inhibit superoxide and nitric oxide production in RAW cells. Generally, the compounds had almost a 50% reduction of the normal stimulated production with little or no cytotoxic effects on the cells (Murakami, et. al, 2000). Tetrahydrocannabinol, the primary psychoactive compound in marijuana, has shown a similar nearly linear dose dependent inhibition of nitric oxide production in murine macrophages (Coffey, et. al, 1996).

Some natural compounds have the opposite or at least partially opposite effect. Water soluble products from the mushroom *Hericium erinaceum* activates the production of nitric oxide without LPS and exhibits a dose dependent effect in RAW cells (Son, et. al, 2006). Likewise, Canova, a homeopathic compound of Brazilian origin, promoted an increase in both superoxide and nitric oxide production in murine peritoneal macrophages (de Oliveira, et. al, 2006).

Each of the previous examples of respiratory burst research can be expanded upon greatly. From such varied hosts as avians and mammals to the tissue origin/distribution of the actual macrophages of interest, the activation, function and activity of these immune cells are of vast interest for not only immunologists and health care practitioners, but also to bacterial microbiologists and biochemists due to the variable production and effects on both host and pathogen. The implications in chronic inflammatory diseases and tumor survival also demand continued emphasis to clearly identify the role of macrophages and respiratory burst in these conditions.

Much like respiratory burst, endocytosis is a central immune function of macrophages and there is a variety of perspectives for the study of this event and much like the dual nature of oxidative metabolism, the process of endocytosis also lends itself to studies of host defense as well as pathogenesis. From the simplest application of this function, phagocytosis can even aid in the purification/isolation of mixed cell preparations.

Endocytosis can occur in two distinct fashions, phagocytosis or “cell eating” and pinocytosis or “cell drinking”. Quite simply the distinction deals with whether the substance being ingested is particulate/solid or liquid. As previously mentioned,

macrophages have the capacity to ingest debris as a homeostatic function, and some primitive cells, such as amoeba actually perform phagocytosis for nourishment. In activated vertebrate macrophages however, the function seems to be purely for the destruction of pathogenic invaders.

The complex pathway by which phagocytosis occurs has been thoroughly studied (Underhill and Ozinsky, 2002; Aderem and Underhill, 1999; Harding, 1995; Greenburg, 1995; Swanson and Baer, 1995). The initial step is attachment of the particle/cell to the phagocyte. This is mediated by either non-specific (such as mannose receptor) or specific (such as FcR) ligand binding. A cytoskeletal rearrangement follows that forms an endocytic "cup" or invagination in the phagocyte membrane around the target. As the "cup" encloses to completely engulf the target, phagosome formation is complete. The remaining steps are dependent on the nature of the particle/cell being internalized. In most cases, the phagosome containing a cellular invader will fuse with a lysosome to become a mature phagolysosome. Fusion results in the release of the lysosomal contents, a variety of cytocidal and/or degradative chemicals, into the phagosome, with subsequent destruction of the microbial invader. Dependent on the ingested material(s), other immune functions may be initiated to promote clearance and removal of additional invaders, including induction of respiratory burst and activation of T-lymphocytes.

In the case of certain pathogenic microbes, however, internalization is the method by which the infection propagates in the host. To subvert phagocytic destruction, microbes have developed the means to interrupt destruction at a variety of junctures. Chlamydia and Toxoplasma are able to inhibit fusion with the lysosomal vesicle and replicate within the endocytic vesicle (Underhill and Ozinsky, 2002). Depending on the

status of the target upon attachment phase, *Trypanosoma cruzi* are able to escape the phagosome and enter the host cell cytoplasm where it replicates (Nathan, et. al, 1979). Finally, there are also pathogens such as *Brucella, Listeria monocytogenes and* tubercle bacilli that can survive within the mature phagolysosome and multiply (Silverstein et. al, 1977). In this manner, phagocytosis becomes detrimental to the host and mediates pathogenesis.

Many techniques have been developed to quantify the act of phagocytosis from microscopic examination of fixed/stained complexes to colorimetric/fluorometric measurement to flow cytometry. Equally, a variety of organisms such as *Candida albicans* (Ortega et. al, 2001), *Cryptococcus neoformans* (Walenkamp et. al, 2000), *Eschericia coli* (Wan et. al, 1993) and *Listeria monocytogenes* (Drevets and Campbell, 1991) and particles such as polystyrene beads (Olivier et. al, 2003) have proven susceptible to uptake by macrophages.

As mentioned previously, the act of phagocytosis is one means by which respiratory burst can be initiated. The concomitant activity of the two functions can result in enhanced killing of the invader (Djaldetti et. al, 2002). As such, the two functions are often studied in concert (Rouzer et. al, 1982; Long et. al, 2005) and as with respiratory burst, chemical/pharmaceutical methods of inhibition and enhancement have been discovered and studied.

Statin medications, used in treatment of hypercholesteremia, were studied based on the cholesterol content of cell membranes. In murine peritoneal macrophages, a variety of statins were shown to increase the percentage of phagocytic cells and to enhance the overall phagocytic activity of those cells (Djaldett et. al, 2006). Histamine

produced by basophils and mast cells during allergic responses displayed inhibitory action on both phagocytosis and respiratory burst in Wistar rat peritoneal macrophages (Azuma et. al, 2001). 2-deoxyglucose, a mannose analog, was shown to selectively inhibit Fc and complement receptor mediated endocytosis (Michl et. al, 1976). Steroids are common targets of research and corticosteroids were shown to inhibit superoxide production and phagocytosis in murine macrophages (Long et. al, 2005).

Cell-free extracts from probiotic bacteria were examined for their effects on macrophage phagocytosis. While *Lactobacillus acidophilus* extracts had a negligible effect on the production of lysozyme in the J774 macrophage-like cell line, there was a significant increase in phagocytic activity from the Lactobacilli as well as from the extract of *Bifidobacterium longum* (Hatcher and Lambrecht, 1993).

Aspects of the phases of phagocytosis have also been the focus of investigation. Being the critical first step, attachment and the disassociation of the attachment phase has been studied (Rabinovitch, 1967; Allen and Cook, 1970).

Temperature also plays an important role in biological functions. To that end, many tests of phagocytic efficiency have included temperature as a test parameter. There was a direct temperature dependent decrease in phagocytosis of fluorescein labeled *Saccharomyces cerevisiae* in both opsonized and non-opsonized yeast (Miliukiene et. al, 2003) and there was a decrease in superoxide production, percent phagocytosis and phagocytic index (of latex particles) with increasingly lower temperatures among rat macrophages (Salman et. al, 2000).

Currently, there is a strong focus on the mechanisms by which pathogens avoid destruction by phagocytic host cells. As mentioned earlier, many microbes are able to

inhibit the formation of a mature phagolysosome while others can avoid the endocytic process itself. *Coxiella burnetii* have been shown to interfere with complement receptor three (CR3) activity on the surface of the monocyte cell line THP-1, resulting in failure to bind and internalize the microbe (Capo et. al, 2003).

There are many fewer publications relating the study of pinocytosis, presumably because it is much more common for macrophages to encounter particulate antigenic material or whole cells. However, there have been several fluid phase reagents utilized to quantify pinocytic uptake. Horse radish peroxidase (HRP) has commonly been studied as a target of pinocytosis (Edelson et. al, 1975; Sung et. al, 1983). A wide variety of basic to neutral dyes such as acridine orange and eosin were investigated as indicators of pinocytic activity in rhesus monkey kidney cells (Allison and Young, 1964). One particular group has participated in multiple comparative studies of pinocytosis in different species. Sea urchin, mouse, fish and the HL-60 cell line cells were evaluated for uptake using neutral red (Plytycz et. al, 1992; Plytycz and Jozkowicz, 1994). Finally, a comparative study of the uptake of neutral red was performed among three species of estuarine fish (Weeks et. al, 1987).

Macrophages have been implicated in numerous investigations for the dichotomous interaction(s) with transformed cells. It has been theorized that the first stimulus received by a resting macrophage determines the function and activity of the cell. Under this theory, macrophages that are stimulated by healthy host cells within the micro-environment will assume a defensive role and release factors to inhibit or suppress the tumor. However, if the macrophage is stimulated by cells of the tumor mass itself, the macrophage will secrete factors to enhance tumor survival and in some cases

metastasis (Condeelis and Pollard, 2006). Interestingly, some of the effector molecules released are the same in both scenarios. While nitric oxide has been shown to cause tissue damage to both healthy and transformed host cells, it also induces mutations that can result in transformed cells and can induce angiogenesis which can provide the tumor with vascular nourishment (Lechner et. al, 2005). Neoplasms also benefit from the release of matrix metalloproteinase by tumor associated macrophages (TAM) to mediate invasion of neighboring tissues (Coussens and Werb, 2002). Macrophages also secrete prostaglandins which can suppress immune function in the area of the tumor mass (Lewis and Pollard, 2006; Young and Newby, 1986).

To demonstrate the complexity of the cellular interactions within the tumor micro-environment, it has been reported that nitric oxide produced by macrophages exerted a cytotoxic effect on Meth A fibro sarcoma murine tumor cells (Sveinbjornsson et. al, 1996) and conversely that Meth A cells can suppress the production of nitric oxide from macrophages (Kwon et. al, 2003).

Much like the case of identifying means by which to temper the inflammatory process previously mentioned, studies to determine means to therapeutically modulate TAM expression are of great importance in medical and clinical research. Interestingly, there is the possibility of identical targets and/or therapies due to the strong implication of inflammatory effects on tumor growth and suppression.

The literature covering reptiles and their immune functions is sparse. Some effort has been made to establish the ontogeny of the reptilian immune system (El Deeb and Saad, 1990; El Ridi et. al, 1988) as well as a phylogenetic perspective (Plytycz and Seljelid, 1996; Cuchens and Clem, 1979). Basic cellular functions have been studied in a

few cases. Leukocyte proliferation (Munoz and De la Fuente, 2003) respiratory burst (Pasmans et. al, 2001) respiratory burst and phagocytosis (Mondal and Rai, 2002) and finally T-cell like proliferation (Burnham et. al, 2005) have been formally studied and published.

Humoral immunity has been identified in various reptile species. In contrast to mammalian antibody types, only three have been identified in reptiles, an IgM like and two IgY like (Jacobson et. al, 2002). Bovine red blood cells (BRBCs) were used to stimulate antibodies for hemagglutination (Burnham et. al, 2003). The hemolytic plaque assay was used to verify antibody production against rat red blood cells (RRBCs) (Saad and Ridi, 1988).

Serum components of immunity have also been found in reptiles. A borreliacidal agent has been identified in the blood of the western fence lizard, *Sceloporus occidentalis* (Lane and Quistad, 1998). Antibacterial activity was seen in the serum of the American alligator, *Alligator mississippiensis* (Merchant et. al, 2003) and a factor that was shown to have antiviral activity as well (Merchant et. al, 2005).

Mononuclear phagocytes have been isolated from multiple tissue sources in reptiles. Splenic phagocytes expressed both phagocytic and cytotoxic properties in the wall lizard *Hemidactylus flaviviridis* (Mondal and Rai, 2001). Peritoneal phagocytes from the turtle *Trachemys scripta scripta* exhibited both phagocytic and respiratory burst functions (Pasmans et. al, 2002; Pasmans et. al, 2001).

Probably the most well characterized immune parameter in reptiles has been T-cell like lymphocytes. A mixed lymphocyte reaction (MLR) proliferative response was seen in splenic lymphocytes from *Sceloporus occidentalis* (Burnham et. al, 2003).

Lymphocytes from blood, spleen and thymus exhibited differential proliferation to ConA, LPS, pokeweed mitogen (PWM) and phytohaemagglutinin (PHA) in the turtle *Mauremys caspica* (Munoz and De la Fuente, 2003). T-like cells have also been characterized in the tuatara *Sphenodon punctatus* the only surviving member of an ancient order of reptiles. These also demonstrated a proliferative response to ConA and PHA with weak response to the MLR (Burnham et. al, 2005). T-like lymphocytes were also the subject of a study to determine the immunomodulatory effect(s) of hydrocortisone in the lizard *Chalcides ocellatus* (Saad et. al, 1983). Peripheral blood lymphocytes from the Florida alligator were equally responsive to ConA and PHA (Cuchens and Clem, 1979).

An equally well studied factor in reptile immunity appears to be the effects of temperature and cyclic, possibly hormonal dependent, changes in immune function. These effects have been studied in the lizard *Scincus scincus* (Hussein et. al, 1979) and the lizard *Chalcides ocellatus* and turtle *Mauremys caspica* (Zapata et. al, 1992). Seasonal/temperature dependent effects were fairly consistent in all cases. The ability to produce antibodies was highest in the summer or warm season with an appreciable impairment in humoral immunity during winter/cold seasons (Saad and Plytycz, 1994).

There is also evidence of a major histocompatability complex (MHC)-like component encoded in reptiles. This is indicated by allograft rejection and by graft-versus-host (GVH) rejection (Zapata et. al, 1992).

With increased interest in reptiles as household pets, there has been an increase in the study of the micro-organisms common to these animals. Specifically, the impact of *Salmonella spp.* has been studied by two labs (Pasmans et. al, 2002; Pasmans et. al, 2005; Mitchell et. al, 2001; Mitchell and Shane, 2000).

As demonstrated in the previous passage, there is an obvious interest in reptiles, but very limited and varied research in this area. Likewise, innate immunity and macrophage function in particular are of great significance. A comprehensive study focusing on the major aspects of reptile macrophage function would be of value to all the disciplines mentioned. It would also provide a more complete picture of the evolution and development on the innate immune system. The establishment of a macrophage model that is able to function optimally outside mammalian model parameters will greatly enhance our ability to understand macrophage function and host-pathogen interactions under diverse environmental conditions.

Based on the longevity with which reptiles have inhabited the earth, their immune system must be sufficient to provide that survival. It has been shown that the reptile adaptive immune system, at least in part, is slower to respond than the mammalian counterpart (Burnham et. al, 2003). These facts when taken together likely indicate a more prolific or rapid innate response to compensate for the lag time of the humoral response and the seasonal dependent decrease in acquired immunity. By the use of peritoneal mononuclear phagocytes from the western fence lizard, *Sceloporus occidentalis,* this theory will be addressed by *in vitro* assays for common functions.

CHAPTER III

MATERIALS AND METHODS

CULTURE MEDIA

REPTILE PERITONEAL EXUDATE CELL (RPEC) MEDIUM

Leibowitz (L-15) 2X prepared basal media without phenol red or L–glutamine (Biowhittaker) was reconstituted with 100 mL sterile H_2O and supplemented with 1.0 mM sodium pyruvate (Sigma), 2.0 mM L-glutamine (Sigma), 1X MEM non-essential amino acids (Sigma), 200 U / mL Penicillin and 200 µg / mL Streptomycin (Sigma), 5 % bovine growth serum (BGS) (Cellgro), 1X amphotericin B (Sigma), and 5.0 x 10^{-5}M 2-mercaptoethanol (Kodak).

RAW 264.7 CELL-LINE MEDIUM

RPMI 1640 1X prepared basal media with phenol red was supplemented with 1.0 mM sodium pyruvate (Sigma), 2.0 mM L-glutamine (Sigma), 200 U/mL Penicillin and 200 µg/mL Streptomycin (Sigma), and 10 % bovine growth serum (BGS).

CRYOPRESERVATION/FREEZE MEDIUM

Fresh, sterile bovine growth serum (BGS) was supplemented with 5% sterile-filtered DMSO).

TRYPTIC SOY BROTH

Tryptic soy broth was prepared by dissolving 30.0 grams of dry TSB (EMD) in 1.0 liters nanopure, deionized water and sterilization by autoclaving for 20 minutes @ 15 psi.

REPTILES

Sexually mature male western fence lizards, *Sceloporus occidentalis,* were obtained from a captive colony at Oklahoma State University. The current colony was derived from lizards that were collected from the San Joaquin Valley in California (Talent et al., 2002). Lizards were housed on corncob substrate in 7.2 liter glass aquaria that were covered with a 3-mm steel mesh lid. Heat and light were provided by a 60-W incandescent light bulb that was positioned over one end of each cage to permit thermoregulation across a temperature gradient of ~26 – 40 ^{o}C. Ambient room temperature was maintained at ~22 ^{o}C and a 14:10 hour light:dark cycle was provided. Lizards were provided with a water source and fed daily with house crickets, *Acheta domesticus*, *ad libitum.* Crickets were dusted with Herptivite® and Rep-Cal® (Rep-Cal Research Labs, Los Gatos, CA, USA).

CELLS AND CELL-LINES

Primary peritoneal exudate macrophages were elicited by intraperitoneal injection of 4.0 mL sterile thioglycollate and harvested by peritoneal lavage with sterile, ice-cold

phosphate buffered saline (PBS) 3–5 days after thioglycollate injection. Harvest of resident (non-elicited) peritoneal macrophages was attempted, however, the resulting numbers were unsatisfactory for the purposes of this study.

The murine macrophage-like cell line RAW 264.7 (designated TIB-71) was obtained from the American Type Culture Collection (ATCC) for use as a positive control for typical macrophage function(s) and as a comparison for reptile vs. mammal macrophage potentials.

BACTERIAL STOCKS

All bacterial stocks were prepared from pure streak plate cultures generously provided by Connie Budd. Freezer stocks of *Staphylococcus aureus, Salmonella typhimerium, Escherichia coli, Bacillus megaterium, and Micrococcus luteus* were made for potential macrophage agonist assays or phagocytosis assay.

REAGENTS / SOLUTIONS

Sterile phosphate buffered saline (PBS) 0.15 M was prepared by adding 1.15 grams of anhydrous, dibasic sodium phosphate (Na_2HPO_4) (Fisher), 8.0 grams reagent grade sodium chloride (NaCl) (VWR), 0.2 grams monobasic potassium phosphate ($K_2 HPO_4$) (Fisher) and 0.2 grams potassium chloride (KCl) (Fisher) to nanopure, deionized water to a final volume of 1.0 liter, adjusting pH to 7.2 and autoclaving for 20 minutes @ 15 psi.

Sterile thioglycollate medium was prepared by adding 29.0 grams of sterility test broth (EMD) to nanopure, deionized water to a final volume of 1.0 liter and sterilization by autoclaving for 20 minutes @ 15 psi.

Phorbol-myristate-acetate (PMA) (Sigma) was prepared by dissolving 1 milligram of PMA in 1 milliliter sterile-filtered ethanol (EtOH) then diluting in 24.0 mL sterile PBS to a final working concentration of 40 micrograms PMA / milliliter.

Lipopolysaccharide (LPS) (Sigma) was prepared by dissolving 2 milligrams of LPS in 50 mL of sterile PBS and sterile filtering resulting solution for a final, working concentration of 1 microgram of LPS per 25 micro liters.

Crystal violet (for quantification of adherence) was prepared by dissolving dry crystal violet (Sigma) in sterile PBS for a final concentration of 0.1 % w/w, followed by sterile-filtering to remove any undissolved dye.

Non-specific esterase fixative was prepared by addition of 40 mL of 10% buffered formalin to 60 mL acetone.

Non-specific esterase phosphate buffer was prepared by dissolving 8.4 gms of sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) in 100 mL of double distilled water and dissolving 7.1 gms of sodium phosphate dibasic (Na_2HPO_4) in a separate volume of 100 mL of

double distilled water. The two volumes were mixed to obtain a pH of 6.5 and diluted with 6 volumes double distilled water for a final concentration of 70 mM.

Non-specific esterase reaction buffer was prepared by dissolving 0.2 gms of alpha naphtyl butyrate, 0.15 gms of fast garnet GBC salt and 1.0 mL of ethylene glycol monomethyl ether in 100 mL of the 70 mM phosphate buffer (above).

Hematoxylin was used at manufacturer's concentration as a counter-stain for non-specific esterase activity.

Glycerol/PBS was prepared by adding 1.0 mL of PBS to 9.0 mL of glycerol.

Acid phosphatase substrate (para-nitro-phenol-phosphate (pNPP)) (Sigma) buffer consisted of 0.1 M glycine buffer, 1.0 mM $MgCl_2$, and 1.0 mM $ZnCl_2$ adjusted to a pH of 10.4 with 1.0 M NaOH.

Acid phosphatase substrate was prepared by dissolving pNPP tablets in substrate buffer to a final concentration of 1 milligram per milliliter.

N-Formyl-Met-Leu-Phe (fMLP) (Sigma) was prepared as a chemoattractant by dissolving 10 mg in 1.0 mL ethanol (EtOH) and further diluting in sterile PBS to a final concentration of 0.1 μM.

Amino guanidine was prepared by dissolving 0.4 g in 4.0 mL of 1.0 M HCl and further diluting in sterile PBS to a final, working concentration of 0.1 mM.

Concanavalin A (ConA) (Sigma) was prepared by dissolving 5 milligrams of ConA in 25 mL of 1.0 M NaCl and sterile filtering for a final, working concentration of 1 microgram of ConA per 5 micro liters.

Autologous pooled reptile serum was obtained by retro orbital blood collection from 3 or more *Sceloporus occidentalis* with heparinized capillary tubes. Whole blood was then centrifuged for 5 minutes @ 11,500 rpm. Serum (straw colored supernatant) was then aseptically removed and stored at -20 ^{o}C until needed.

Griess reagent (for nitrite quantification) consisted of two solutions. Sulfanilamide was prepared by dissolving 1% sulfanilamide (Sigma) w/v in a 5% phosphoric acid (H_3PO_4) / 95% sterile water solution. N-1-Napthylethylenediamine dihydrochloride (NED) was prepared by dissolving 0.1% NED (Sigma) w/v in sterile water.

Nitro-blue tetrazolium (NBT) (Sigma) solution was prepared by dissolving 1 NBT tablet in 1.0 milliliter sterile water and adding the resulting solution to 30 mL of acid phosphatase substrate buffer (as above).

Acid alcohol consisted of 3% v/v HCl in ethanol (EtOH).

Neutral red solution was prepared by dissolving Neutral Red (Sigma) in sterile water for a final concentration of 1 mg per milliliter.

Citrate buffer was prepared by dissolving sodium citrate ($Na_3C_6H_5O_7$) in sterile water for a final concentration of 0.1M.

METHODS

TYPICAL RPEC HARVEST

Lizards were injected with 4.0 mL of sterile thioglycollate medium intraperitoneally 3-5 days prior to harvest by peritoneal lavage. Lavage was performed by injecting 4.0 mL of ice-cold, sterile PBS intraperitoneally, followed by brief, gentle manipulation of the abdominal area of the lizard and finally withdrawing the resulting exudate solution and transferring to a sterile 15-mL, screw-cap centrifuge tube. This step was performed multiple times to obtain complete exudate sample from the experimental subject. Sterile PBS was added to the tube(s) for a final volume of 14.0 mL, and the tube(s) were centrifuged @ 1500 rpm and 4 ^{o}C for 9 minutes. The supernatant was discarded and the remaining cell pellet was resuspended in 14.0 mL of sterile PBS and centrifuged again. The preceding step was repeated for a total of three wash/centrifuge cycles. The final pellet was resuspended in 1-2 mL of sterile L-15 complete medium. Cell density was determined by trypan blue exclusion with a hemacytometer and adjusted to 1.0 x 10^{6} cells per milliliter of media. Cells were then plated in either 24-well or 96-well tissue culture plates and allowed to acclimate to media and incubator conditions (27 ^{o}C) for 48 hours.

SPLENOCYTE SUSPENSION

Reptile splenocytes were obtained by removing the spleen from a recently euthanized lizard and placing the spleen in a Petri dish with sterile PBS. Using 2 scalpels simultaneously, the spleen was minced repeatedly until all large portions of the spleen had been thoroughly dissected. The liquid suspension and the tissue clumps were transferred to a sterile centrifuge tube and placed on ice until all visible tissue clumps had settled. The liquid suspension was then carefully removed and transferred to another sterile centrifuge tube. The tube was centrifuged at 650 g and 4 ^{o}C for 9 minutes to pellet the splenocytes. Cell density was then determined via trypan blue exclusion and hemacytometer count.

CELL DENSITY ASSAY (TRYPAN BLUE EXCLUSION)

A 10-microliter sample from the final pellet resuspension (typical harvest, above) was added to 40 microliters PBS and 50 microliters 0.1% trypan blue and mixed thoroughly. Approximately 10 microliters was applied to the counting chamber of a hemacytometer and the counts of viable cells in 4 quadrants were averaged. Cells staining blue were designated non-viable. The resulting average of viable cells was adjusted by multiplying by 10^5 to obtain the final cell density per milliliter.

PURIFICATION/ISOLATION PROCEDURE

In the event of obvious significant erythrocyte contamination of the exudate suspension, the first pellet obtained was resuspended in a minimal volume of sterile water for 2 minutes to lyse the erythrocytes, followed by immediately adding PBS to a final volume

of 14.0 mL to reestablish an acceptable osmolarity. Upon trypan blue exclusion, if erythrocyte contamination still appears significant, further purification can be obtained after plating the cells by allowing the adherent fraction to attach to the substrate for 2 hours, then gently agitating the plate(s) for 1 minute and flicking out the non-adherent cell suspension. If necessary, sterile PBS can be added to each well and the agitation step can be repeated as needed. Upon clearance of erythrocytes, sterile L-15 complete medium is reapplied to each well and the 48 hour acclimation can proceed.

ADHERENCE ENHANCEMENT PROCEDURE

To enhance attachment of the adherent fraction of the exudate cells, tissue culture plates were pre-treated with heat inactivated BGS. Briefly, BGS was heated @ 56 ^{o}C for 30 minutes. An appropriate volume (based on well size) was applied to the wells of tissue culture plates and the plates were incubated overnight @ 4 ^{o}C. Prior to plating the exudate cells, wells were washed 3X with room temperature sterile PBS.

SPREADING ASSAY

Peritoneal exudate cells plated in 24-well plates and incubated for 48 hours were observed via inverted phase-contrast microscopy. Mononuclear phagocytes in suspension are typically round/spherical in morphology. Upon attachment, pseudopodia and/or ruffling occur to mediate attachment/adherence. Cells that exhibited multiple extensions were counted in a given microscopic field and divided by the total number of macrophages (attached and suspended) to obtain a spreading index. The resulting fraction was multiplied by 100 to determine the % adherent.

SUPEROXIDE ASSAY

A typical peritoneal harvest was performed through the 48 hour incubation in 96-well plates. Media was removed by flicking plates briskly and fresh L-15 complete supplemented with NBT substrate 100 microliters per mL and PMA 1 microgram per mL and plates were further incubated for 1 hour at 27 ^{o}C. 200 micro liters of warm PBS was then added to each well and the plates shaken gently for 5 minutes. Plates were flicked out to remove liquid and 200 microliters of methanol was added to each well briefly and removed by flicking the plates and the plates were allowed to air dry. Cell membranes were solubilized by addition of 120 microliters of 2.0 M potassium hydroxide (KOH) and blue formazan product was dissolved by addition of 140 microliters of DMSO. Wells were mixed thoroughly by pipetting and the resulting solutions were read in a plate reader at 630 nm. Results were reported as relative amounts of formazan converted by superoxide.

NITRIC OXIDE ASSAY

A typical peritoneal harvest was performed through the 48 hour incubation in 96-well plates. Media was removed by flicking plates briskly and replaced with fresh L-15 complete supplemented with macrophage agonists. Plates were further incubated for 24 and 48 hours prior to Griess assay for quantification of nitrite, the major product of the short lived nitric oxide (NO), in the cellular supernatant. Briefly, 100 microliters of cell free supernatant was removed from each well and transferred to a corresponding well in a 96-well flat-bottomed plate. Sulfanilamide and NED were allowed to reach room

temperature and 100 microliters of each solution was added to each well containing the cell free supernatant. The samples were than read at 450 nm in a plate reader and compared to sodium nitrite ($NaNO_2$) standards.

ACID PHOSPHATASE ASSAY

A typical peritoneal harvest was performed through the 48 hour incubation in 96-well plates. Media was removed by flicking plates briskly and replaced with fresh L-15 complete supplemented with macrophage agonists, followed by further incubation for 24 hours. Media was removed by flicking the plates briskly and 25 microliters of 0.1% Triton X-100 was added to each well to lyse the cells. A 3:1 solution of complete para-Nitro-Phenol-Phosphate substrate (substrate dissolved in buffer) in 0.1 M citrate buffer was made immediately prior to applying 180 microliters to each well. Incubation continued for 1 hour and the reaction was interrupted by addition of 60 microliters of 0.2 M borate buffer and the plates were read at 405 nm in a plate reader. A 10 mM solution of para-nitrophenol (pNP) was serial diluted to form a standard curve for quantification of sample acid phosphatase activity.

PHAGOCYTOSIS ASSAY

A typical peritoneal harvest was performed through the adjustment of cell density step. Cells were then plated in a 24-well plate and macrophage agonists were added followed by addition of GFP-expressing bacteria. Plates were incubated for 90 minutes and wells were then gently scraped with a cell scraper or rubber policeman to collect all cells present in the wells. Cell suspension was transferred to an eppendorf tube and pulsed in a

centrifuge to pellet the cells. The pellet was washed 2X in sterile PBS to remove debris and external bacteria. The final pellet was resuspended in PBS and a 20 microliter sample was applied to a clean glass microscope slide and covered with a glass coverslip. The slide was then viewed with fluorescent/light microscopy, with total cells per field being counted under normal white light and cells that have internalized bacteria being counted in the same field under fluorescent filtered light. A minimum of 4 fields and/or 100 cells per assay was counted and the number of phagocytic (containing bacteria) cells was divided by the total number and multiplied by 100 to determine the % of phagocytic cells.

MIXED LEUKOCYTE REACTION (MLR) ASSAY

A typical peritoneal harvest was performed to the adjustment of cell density step, but cells were adjusted to a density of 2.5 X 10^5 cells per mL. A freshly prepared suspension of splenocytes was prepared and likewise adjusted to 2.5 X 10^5 cells per mL. In a 96-well plate, 100 microliters of the macrophage suspension was applied to each well in a single row, 100 microliters of the splenocytes suspension was applied to each well in a second single row, and in a third row, 100 microliters of each cell type was added to each well. For a control, 100 microliters of the splenocytes suspension was added to each well of a fourth row with ConA added to the wells at a concentration of 5 micrograms per mL. Plates were incubated for 6 days at which time fresh media was added to each well. On day 8, an MTT assay was performed to quantify splenocyte proliferation.

CRYOPRESERVATION OF EUKARYOTIC CELL-LINES

Cells were grown to near confluence in sterile tissue culture flasks as determined by microscopic observation. Media is removed and replaced with fresh complete media followed by detachment of adherent cell monolayer. First, the flasks were jarred briskly with the heel/palm of the hand, and then the monolayer was scraped with a sterile cell scraper repeatedly. Cell suspension was removed by pipetting with brief washing of entirety of the internal environment of the flask, finally transferring all liquid to a conical centrifuge tube. Suspension was then centrifuged at 650 g and 4 ^{o}C for 7 minutes to form a soft pellet. Supernatant was discarded and the pellet was gently resuspended in freezing medium with 1.0 mL aliquots transferred to cryotubes with tubes/cells stored at -80 ^{o}C. When needed, a single cryotube was removed from the freezer and rapidly thawed (1-2 minutes in a 37 ^{o}C water bath. The thawed solution was then transferred to a sterile tissue culture flask containing 15.0 mL of sterile culture media and incubated at 37 ^{o}C for 4-8 hours to allow attachment at which time the media was discarded to remove the components of the freeze medium. Fresh sterile culture media was then added to the growing monolayer, and incubation resumed until growth was sufficient for assay(s).

PREPARATION OF BACTERIAL FREEZER STOCKS

A single, isolated colony of each bacterial strain was aseptically obtained from streak plates and transferred to 15 mL of tryptic soy broth (TSB) in a sterile flask. Flasks were then shaken at 37 ^{o}C for 18-24 hours until sufficient turbidity is evident. A 0.5 mL sample was taken from each flask and transferred to a cryo-tube containing 0.5 mL of sterile glycerol. Cryo-tubes were then stored in a -80 ^{o}C freezer. Revival of the cultures

was performed by obtaining a small sample of the viscous stored stock(s) on a sterile toothpick and suspending in 10-15 mL of sterile tryptic soy broth followed by shaking at 37 ^{o}C until a sufficiently turbid suspension was gained.

CHAPTER IV

FINDINGS

The initial objective of this project was comprised of four specific goals. Foremost of these was the elucidation of comprehensive immune function in a reptile macrophage. The successful completion of this aspect would furnish the necessary data to achieve two of the remaining goals, namely the enhancement of our broad understanding of macrophage biology and innate immunity and support for the formal establishment of a reptile laboratory model by expanding the role of reptiles beyond the present scope. Finally the data gained from the study was to be compared to an acceptable mammalian counterpart to validate the use of reptile macrophages as physiologically relevant models for innate immune function.

The selection of the particular innate immune parameters that would be investigated was based on the topics with the most widespread coverage in the available relevant literature. By far, the most numerous publications dealt with one or more aspects of either respiratory burst or endocytosis.

As noted before, respiratory burst involves the production of reactive chemical species in response to one of many signals indicating a threat to the well-being and/or homeostasis of the host. The purpose of the chemicals generated is to destroy the source

of said agonist, to inhibit the growth/expansion of the agonist, or to suppress the harmful effects of the agonist.

Also mentioned earlier, endocytosis is the uptake of insoluble particles (phagocytosis) or liquid-phase materials (pinocytosis) in order to sequester them from other, healthy cells and to destroy/neutralize those agents that were engulfed.

Due to the integral nature of chemotaxis in mediating an immune response, this function was also deemed worthy of exploration. Chemotaxis is the unidirectional movement across a concentration gradient generated by a pathogen, a pathological condition, or another host cell.

Through well-documented assays from mammalian and other models, the noted functions were explored within the limits of our available resources. Modifications of existing protocols were made as needed.

All procedures were performed on a minimum of three test subjects on two or more occasions with each subject's assay in triplicate format. The data reported represents the pooled mean values ± the standard error of the mean. The student t-test was performed to determine any significant difference of treatment values from the control.

ELICITATION OF OPTIMAL MACROPHAGE DENSITY

For the most effective experimental methodology, a variety of conditions were compared to determine the method that would yield the highest mean yield of RPEC. Although a variety of inflammatory agents have been used to produce murine peritoneal exudates having sufficient macrophages for functional assays, it was decided to limit the

scope of this research to the most common of these agents, thioglycollate medium. Typical variations in murine macrophage elicitation include; the number and interval of TGM injections for a given test subject, as well as the duration of *in vivo* migration into the peritoneal cavity prior to harvest (Meltzer, 1981). After numerous trials comparing the mean RPEC harvest per reptile subject, it was determined that the optimal cell density was produced by a single i.p. injection of 4.0 mL TGM 3-5 days prior to harvest (See Table 1). As the single injection 3 days prior proved superior, every effort was made to perform harvests based on this procedure, but the single injections on 4 and 5 days prior were utilized when necessary. Multiple injections yielded not only fewer cells but also produced an experimentally unsatisfactory level of red blood cell (RBC) contamination.

CONFIRMATION OF MACROPHAGE IDENTITY

The first genuine obstacle of this undertaking was to verify that our experimental cells were actually macrophages. As a consequence of the heterogeneous nature of a peritoneal exudate suspension of cells, it is necessary to make reasonable attempts to purify and qualify the existence of a high percentage of the cell-type of interest, in this case macrophages.

Under some applications whereby a particular subset of cells is more recognized, a single function or characteristic may be a sufficient determinant of cellular identity. For example, substrate adherence may in some situations be suitable for presumptive identification of peritoneal macrophages (Nathan and Root, 1977; Stewart, et. al., 1975). Ideally, a distinct, conserved cell-surface marker would be utilized for absolute confirmation of identity. However, as yet, no such characterization of reptile markers has

Conditions	Mean Cellular Yield (viable cells)
Single injection 3 days prior to harvest	9.3×10^6
Single injection 4 days prior to harvest	8.9×10^6
Single injection 5 days prior to harvest	8.8×10^6
Dual injections 3 and 2 days prior to harvest	7.3×10^6 w / RBC contamination
Triple injections 3, 2 and 1 days prior to harvest	4.2×10^6 w / RBC contamination

Table 1. Determination of best conditions for optimal RPEC yield. Yields are reported as the crude means of three experiments with no standard error(s) reported.

been made and due to the novelty of reptile macrophages in general and from *Sceloporus occidentalis* in particular, a more rigorous regimen is warranted for the qualification of these particular mononuclear phagocytes. Furthermore, it should be noted that the exudate cell suspension not only consists of a mixed population of cell types, but almost certainly a variety of macrophages based upon diverse states of differentiation and/or activation, and that in common mammal models, such an array of macrophages will result in the expression of at least slightly different properties.

Based upon the gross, microscopic morphology, such as relative size and shape, of freshly harvested RPEC, and the spreading/adherence index of suspensions incubated for forty-eight hours, greater than 80% of the RPEC were determined to be macrophages. As shown in Figure 2, approximately 84.4% of the exudate cells exhibited spreading/adherence as compared to only 34.15% of the RAW cells. Figures 3 and 4 provide photographic evidence of the spreading phenomena in the RPEC and RAW cells respectively. Figure 5 demonstrates photographically the locally dense regions of nearly 100% RPEC spreading . The notable difference between the two cell types may be attributable to vital secretions by accessory cells within the mixed cell population of the RPEC. There was also an apparent density-dependent effect on spreading. RPEC plated at significantly less than 1 X 10^6 cells/mL displayed a near complete absence of spreading (not shown).

Another macrophage-associated capacity by which identification is often made is non-specific esterase (NSE) activity. Briefly, a reaction mixture containing a suitable substrate for esterase activity, such as α-naphthyl acetate or butyrate, is incubated with a

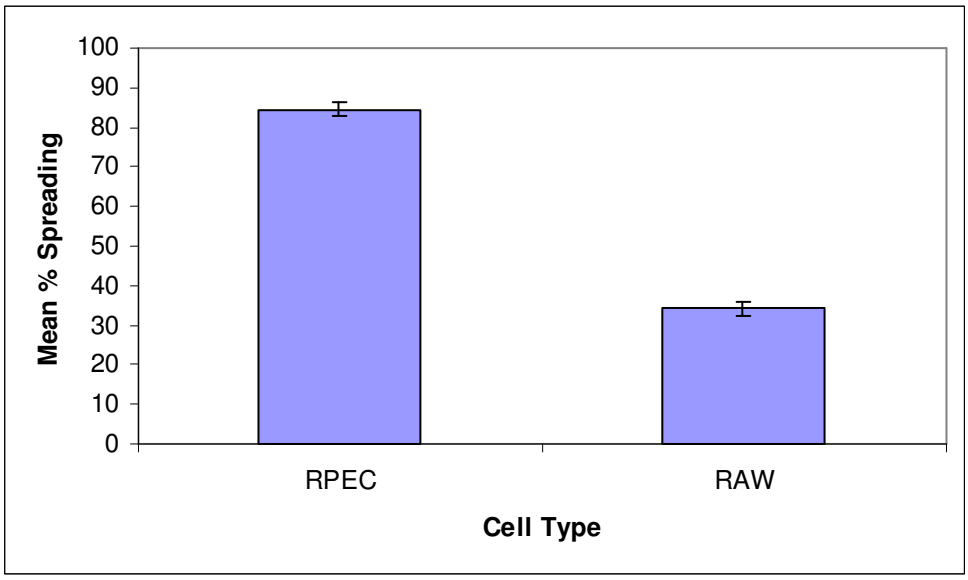

Figure 2. Mean % of cells spreading as an indicator of macrophage identity. Reptile cells were compared to the RAW 264.7 cell line. All data represent the pooled mean of two or more experiments with a sample size of 4 or greater ($n \geq 4$) ± the standard error of the mean.

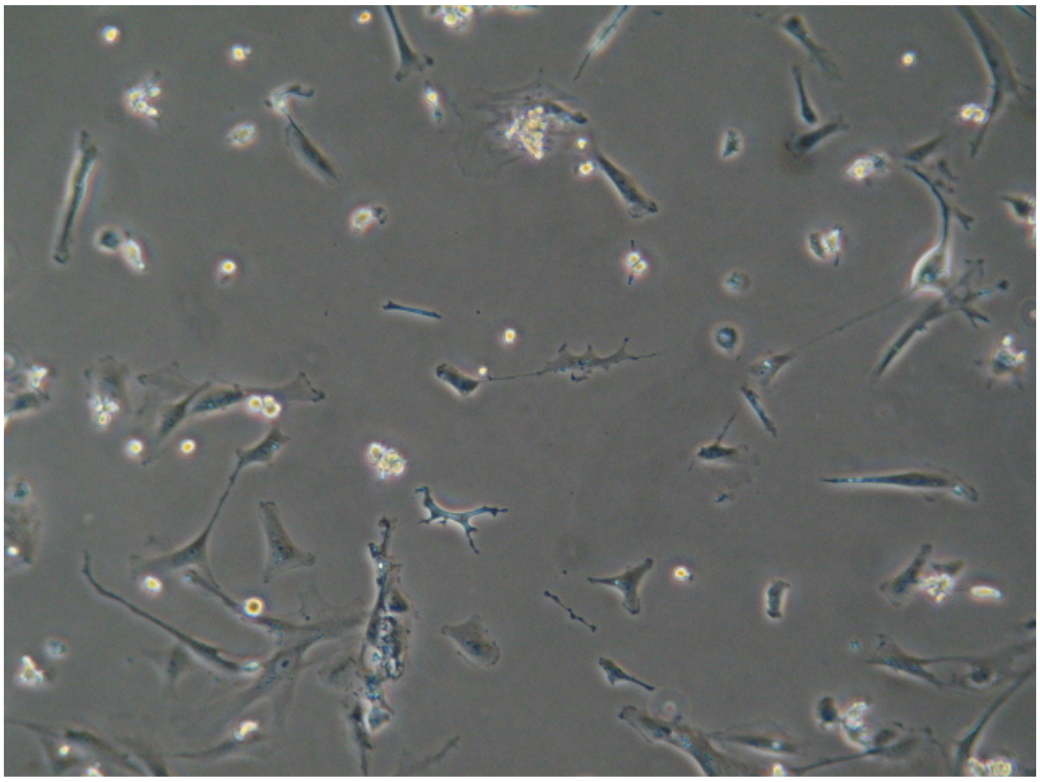

Figure 3. Representative RPEC exhibiting spreading activity after 48 hours in culture.

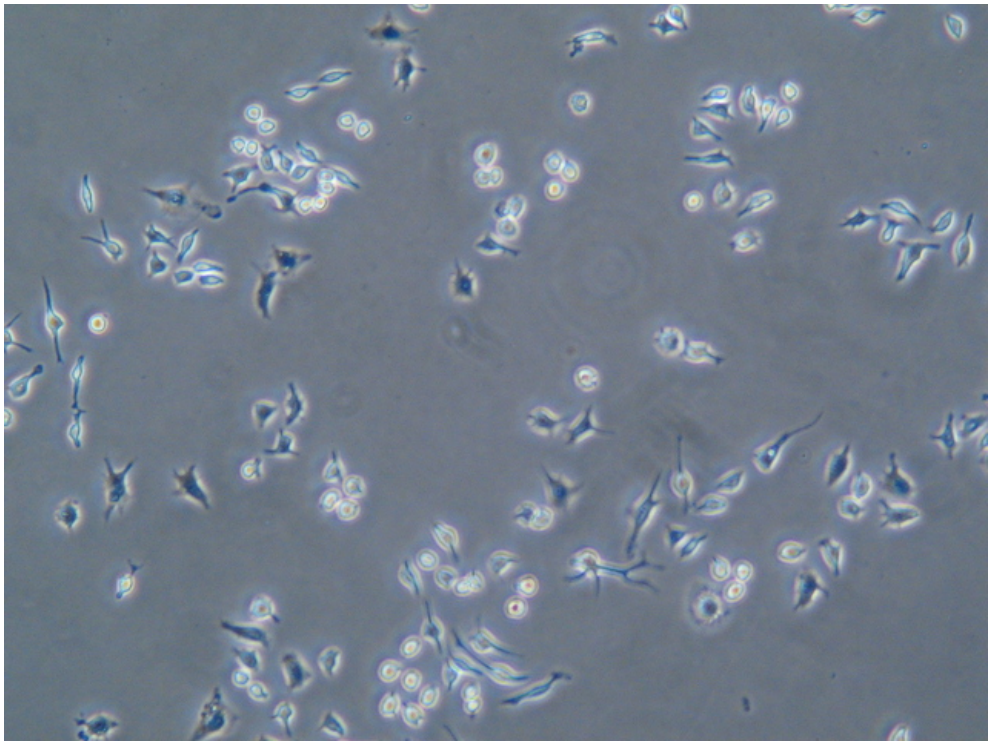

Figure 4. RAW 264.7 murine cell-line macrophages exhibiting spreading activity after 48 hours in culture.

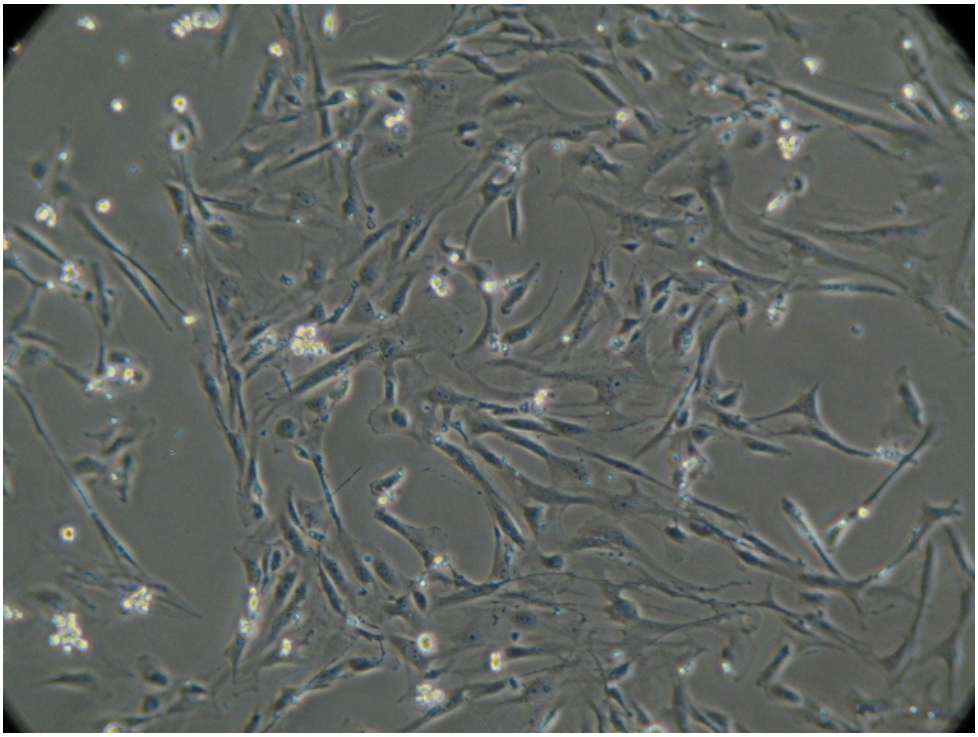

Figure 5. RPEC exhibiting locally dense spreading activity after 48 hours in culture.

fixed monolayer of macrophages. Hydrolysis of the ester group yields a naphthol that forms an insoluble azo-dye when coupled to a diazonium salt. Counterstaining distinguishes cells with no esterase activity and NSE+ percentages can be calculated by differential microscopic counts.

In the case of the RPEC, numerous attempts were made by three different investigators without satisfactory results. The principal complication was the microscopic examination of the monolayer after counterstaining. There was a poorly distinguishable difference between the reddish-brown coloration expected from the NSE+ cells and the deep red of the NSE- cells.

It should be noted that differential expression of NSE activity has been documented, related to the model organism utilized, the tissue of origin of the mononuclear phagocytes and the activation state of the cells (Miller and Morahan, 1981). Likewise, some reports indicate that false positive staining patterns have been observed in other cell types (Miller and Morahan, 1981).

A final attempt to establish uniformly quantifiable uptake of neutral red was performed and uptake was achieved under multiple cell densities and various concentrations of the neutral red. However, uniformity of uptake was not apparent and there was no obvious linear correlation between time of incubation, cell density and/or neutral red concentration (data not shown).

The final method employed for verification of the RPEC as predominantly macrophages was the capacity to phagocytose bacteria as evidenced by microscopic examination. Satisfactory evidence of phagocytic activity was acquired and as this

represents a major portion of a later section (endocytosis) of our results, the data will be presented in that section.

RESPIRATORY BURST

Macrophage respiratory burst generates numerous radical/reactive chemical species, such as superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and nitric oxide with other reactive species forming from reactions with those previously mentioned such as hypochlorous acid and peroxynitrite. Those best represented in the scientific literature are superoxide and nitric oxide. These are both produced directly by cellular enzymes without intermediates and are therefore more easily monitored from agonist signal to completion.

There are numerous ligands that trigger respiratory burst including microbial products such as zymosan, LPS, PGN and synthetic analogs such as fMLP and PMA. The first action to determine activity in the RPEC was to stimulate the cells in culture with a variety of triggers for an appropriate duration followed by assay with existing mammalian protocols for a response. Once the conditions had been determined that yielded a consistent satisfactory production of the desired chemical species, different temperatures were assayed for differential activity and comparisons with the RAW 264.7 cell-line were made.

To assay generation of superoxide anion, cellular lysates were subjected to a colorimetric protocol that quantifies superoxide indirectly by the measurement of the production of an insoluble formazan, the product of NBT reduction. Data was reported as relative amounts of the reduction product.

In agreement with the procedure outlined in materials and methods, media was removed from RPEC that had acclimated to their *in vitro* environment for 48 hours and fresh media supplemented with the desired treatments and the NBT substrate was applied in triplicate wells. Negative controls consisted of replacement with untreated media supplemented with the NBT substrate. After incubating for 1 hour at the noted temperatures, warm PBS was added to each well and the plates were gently shaken for 5 minutes, followed by briskly flicking out the plates to remove all liquid. Wells were then briefly rinsed with methanol, followed by flicking out the fluid and air drying the plates. Cells were solubilized with potassium hydroxide and the blue formazan product of the NBT reduction was liberated by vigorously pipetting DMSO in the wells, followed by quantifying with a plate reader at 630nm.

As seen in Figures 6 and 7, RPEC perform at a higher level at lower temperatures and RAW 264.7 cells perform at a higher level at higher temperatures. This seems obvious given the physiology of reptiles and mammals. However, it is interesting that the reptile macrophages produce more superoxide at their optimal temperature than the mammalian cells do at their optimal temperature and likewise, the reptile macrophages perform better at their sub-optimal temperature as compared to the same in the mammalian macrophages. The standard protocol for NBT reduction calls for treatment with PMA with the NBT substrate, but if the error bars are disregarded, LPS seems to evoke a better response in both the RPEC and the RAW cells. It is also interesting to note the consistent reduction within the RAW cells at low temperature. This might indicate a baseline constitutive production of superoxide with inductive effects being inhibited at the lower temperature. The student t-test also indicated a significant

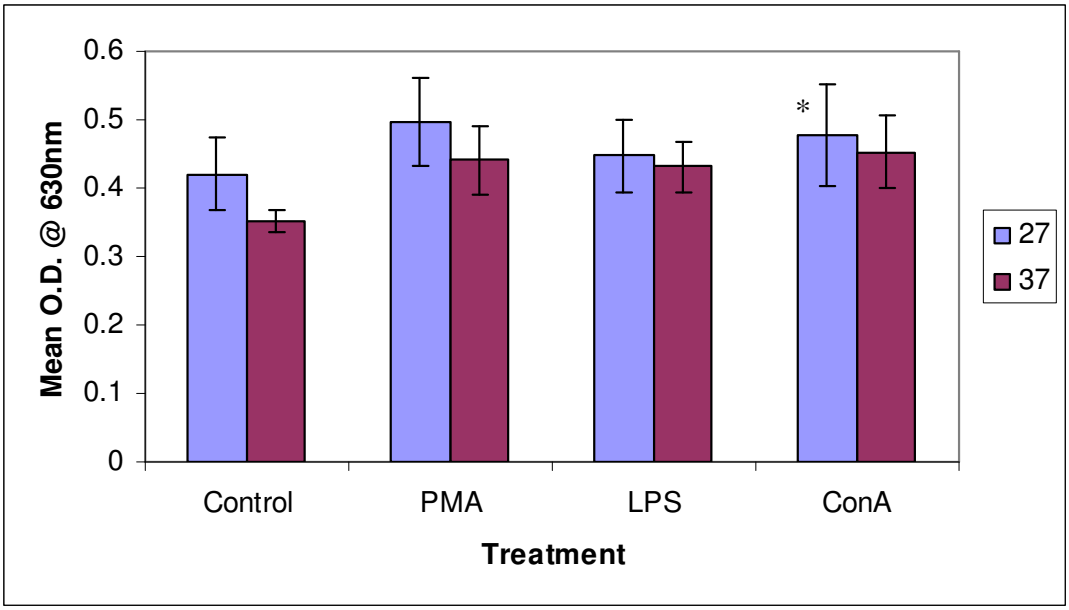

Figure 6. RPEC superoxide production as measured indirectly by the reduction of NBT substrate to formazan. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C) (* indicates a significant difference as compared to the control by student t-test with $p \leq 0.05$).

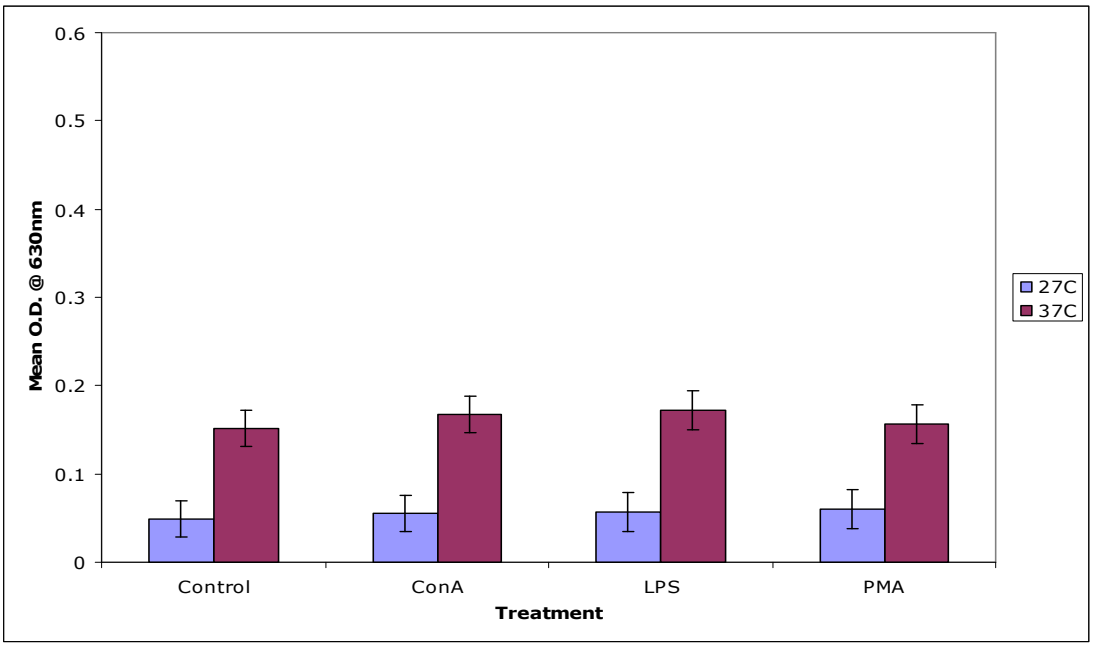

Figure 7. RAW 264.7 murine macrophage cell-line superoxide production as measured indirectly by the reduction of NBT substrate to formazan. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C)

difference between the RPEC 27 ^{o}C ConA treated cells and the corresponding control cells.

Nitric oxide is an extremely important molecule despite its amazingly small, simple structure. Numerous pharmaceuticals have been developed based on the wide range of physiological function of nitric oxide. A large volume of patents has been applied for based on these same characteristics (Stix, 2001) and Science magazine named nitric oxide "Molecule of the Year" in 1992.

As a component of innate immune response, NO has been implicated as a potent defensive mechanism capable of rendering an invading microbe inert, but equally regarded as a major source of tissue damage due to inflammation, especially in autoimmune disease.

The long accepted method for quantifying the production of NO is to measure the biological fluid, be it serum, urine or cellular supernatant for nitrites and nitrates, the stable end-product of the extremely short-lived radical species. Many kits are available based on the Griess assay which measures nitrite in fluids.

The Griess assay is a colorimetric procedure whereby sulfanilamide creates an acidic environment within the culture wells and competes for nitrite in the culture supernatant and NED reacts to form an azo compound that can be measured in a plate reader. This is an indirect but well-accepted method for quantifying NO.

In accordance with the listed protocol in the materials and methods section, media is discarded from plates in which RPEC have acclimated for 48 hours and replaced with fresh media supplemented with stimuli to induce the production of nitric oxide, or media alone as a negative control. Plates require an additional 24-48 hours incubation time

before the Griess reagents can be applied. Upon completion of the additional incubation, 100 µL of cell-free supernatant is transferred in triplicate to a 96-well flat bottom tissue culture plate. Equal volumes of the Griess reagents at room temperature are added sequentially at 5-minute intervals, sulfanilamide first then NED, with the plates incubated in the dark at room temperature during the intervening 5 minute spans. An obvious pink response is visible in the presence of nitrite indicating the production of NO. Plates read at 450 nm can be quantified by creating a serial dilution of sodium nitrite standards and performing the prescribed procedure on the standards.

Numerous attempts were made to assay nitric oxide in RPEC cultures. Different percentages of serum were tried as well as different temperatures and incubation times. Griess reagents from a commercial kit yielded the same results as reagents prepared in the laboratory.

In some mammalian systems, an LPS binding protein has been implicated as critical for the induction of NO production. To try to compensate for this, pooled reptile serum was collected to provide soluble factors that might complete the signal/pathway in this process.

Although in each modification measurable levels of NO were detected in the RAW control cells, all efforts to detect nitric oxide in the RPEC were unsuccessful (See Figures 8 – 10).

It has already been mentioned that the tissue origin of macrophages can have a profound effect on their functions/capacities, and this might be the case with NO. While nitric oxide has been detected in reptile macrophages, these were splenic macrophages from another species of reptile (Mondal and Rai, 2001; Mondal and Rai, 2002).

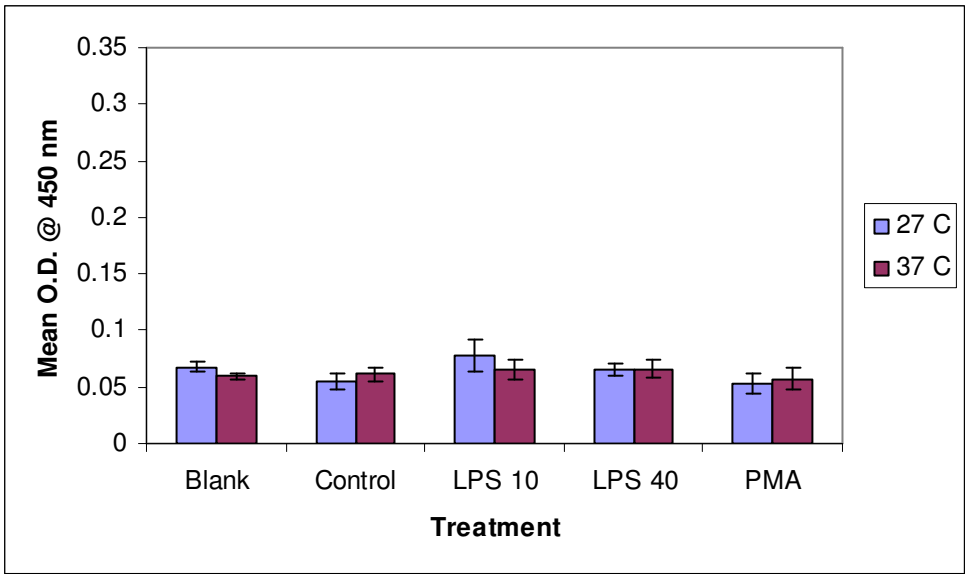

Figure 8. Lack of nitric oxide expression in RPEC as determined by the Griess assay. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C)

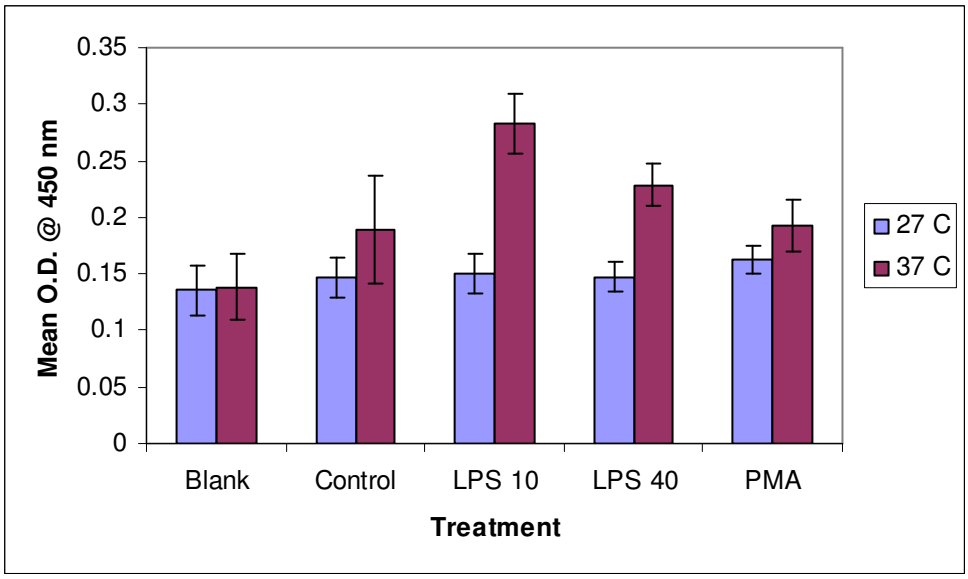

Figure 9. Nitric oxide expression in the RAW 264.7 murine macrophage cell-line as determined by the Griess assay. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C)

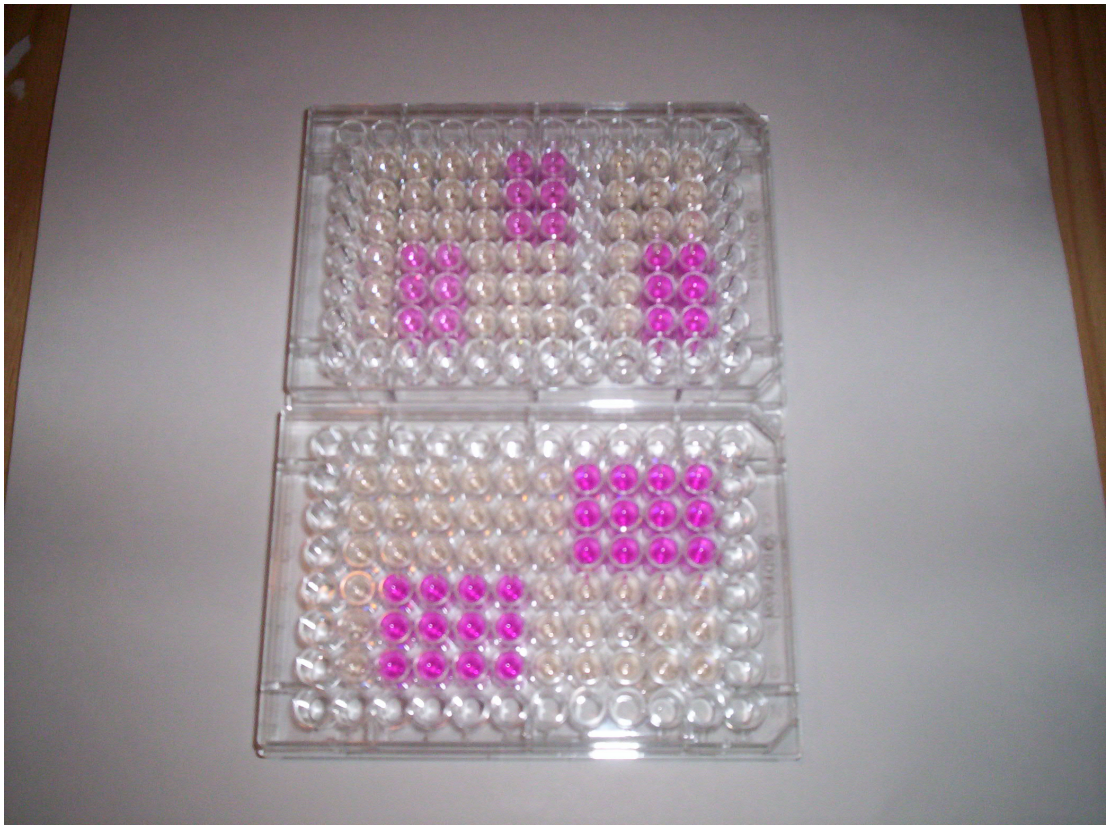

Figure 10. 96-well flat-bottomed micro-titer plates representing the results from the colorimetric Griess assay for nitric oxide production in RAW 264.7 murine macrophage cell-line with positive wells displaying deep pink and control wells displaying media colored fluid.

Given the highly conserved nature of nitric oxide and with multiple physiological functions identified in vertebrates, it is highly unlikely that RPEC do not produce NO and certainly, the entirety of possible culture conditions has not been exhausted. Still, with other physiological differences between reptiles and mammals, it is possible that nitric oxide plays a smaller role in host defense than commonly seen in established mammal models and/or the level of detection with the Griess assay is insufficient for RPEC.

A final note concerning the discrepancy in NO expression involves the comparison between two mammalian species, human and mouse. The iNOS gene has been well characterized in many common immunological models, and numerous publications have focused on the production and quantitation of nitric oxide in biological fluids. However, although the iNOS gene has been identified in humans and "iNOS like genes" have likewise been determined, the induction of human iNOS has been problematic (Wang and Marsden, 1995). Multiple human cell-types have been shown to express the messenger RNA of the iNOS gene, but the level of activity is less vigorous than that seen in other mammalian species, especially the mouse (Wang and Marsden, 1995).

Given the differences seen between these two "similar" species, it may be difficult to quantify the production of NO in the proposed model as compared to a reportedly robust producer of nitric oxide in the mouse model.

Although not implicitly a component of respiratory burst, the concomitant production of lysosomal enzymes is regularly identified with the same stimuli in macrophage cells. Recognized as the "classic" lysosomal enzyme, acid phosphatase

lends itself well to colorimetric assays for quantification (Dannenberg Jr. and Suga, 1981).

Numerous substrates have been used to quantify acid phosphatase activity, including glycerophosphate and naphthyl phosphate which require microscopy for both verification and quantification. A suitable substrate was found which allowed both the convenience and increased reliability of spectrophotometric measurement in a micro-titer assay.

As with the previous burst assays, a cell monolayer was established from the RPEC and allowed to acclimate to the culture media for 48 hours. After the 48 hour incubation period, media was removed by vigorous flicking of the plate(s) and fresh complete L-15 supplemented with the appropriate agonists was applied and incubation for a further 24 hours was carried out. Triton X-100 was then administered to the monolayers to mediate cell lysis and para-nitro-phenol phosphate (pNPP) was added as substrate for the enzyme activity. A colorless substrate, upon enzymatic activity the phosphate is liberated yielding para-nitro-phenol a yellow end-product. Measurement of the acid phosphatase activity is therefore measured indirectly by the formation of the end-product.

The reptile macrophages demonstrated a high level of enzymatic activity at both temperatures investigated previously (Figure 11). The RAW 264.7 murine macrophage cell-line exhibited only minimal activity at 37 ^{o}C and negligible activity at 27 ^{o}C (Figure 12), however there was no literature located to support this particular capacity in the cell-line utilized. The relatively high activity seen in the RPEC (including the untreated

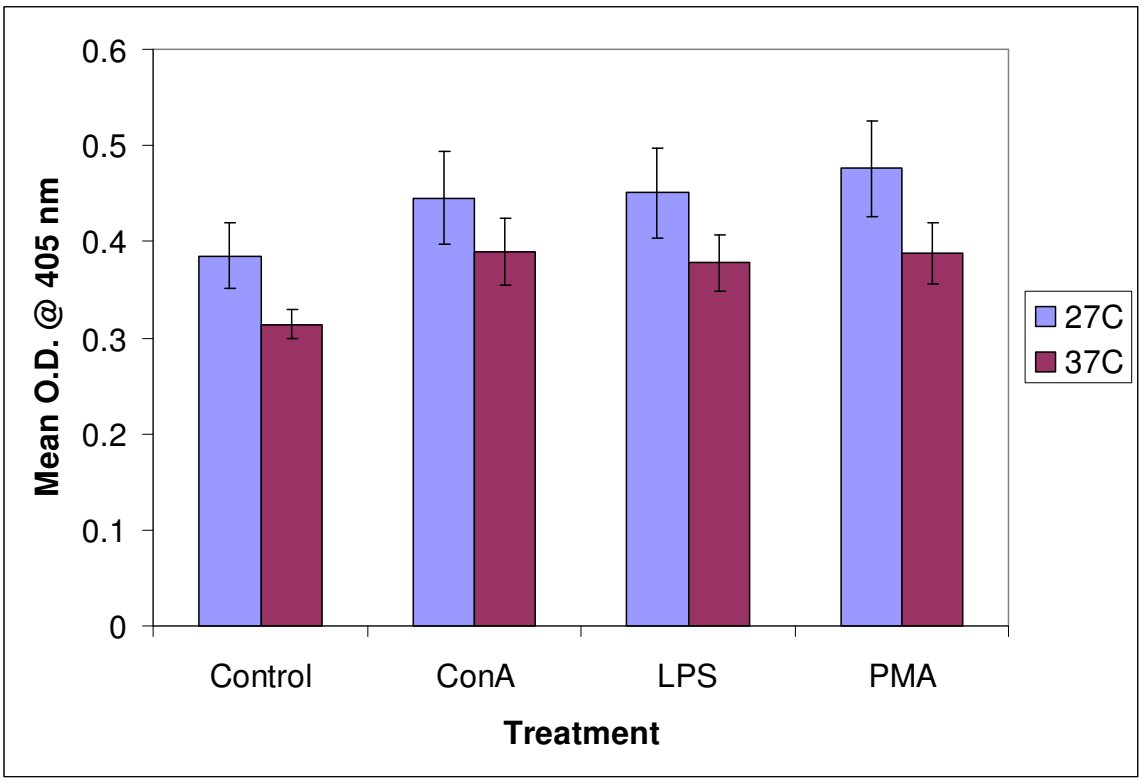

Figure 11. Lysosomal enzyme (acid phosphatase) activity in RPEC as measured indirectly by the appearance of a colored end-product from a colorless substrate. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C)

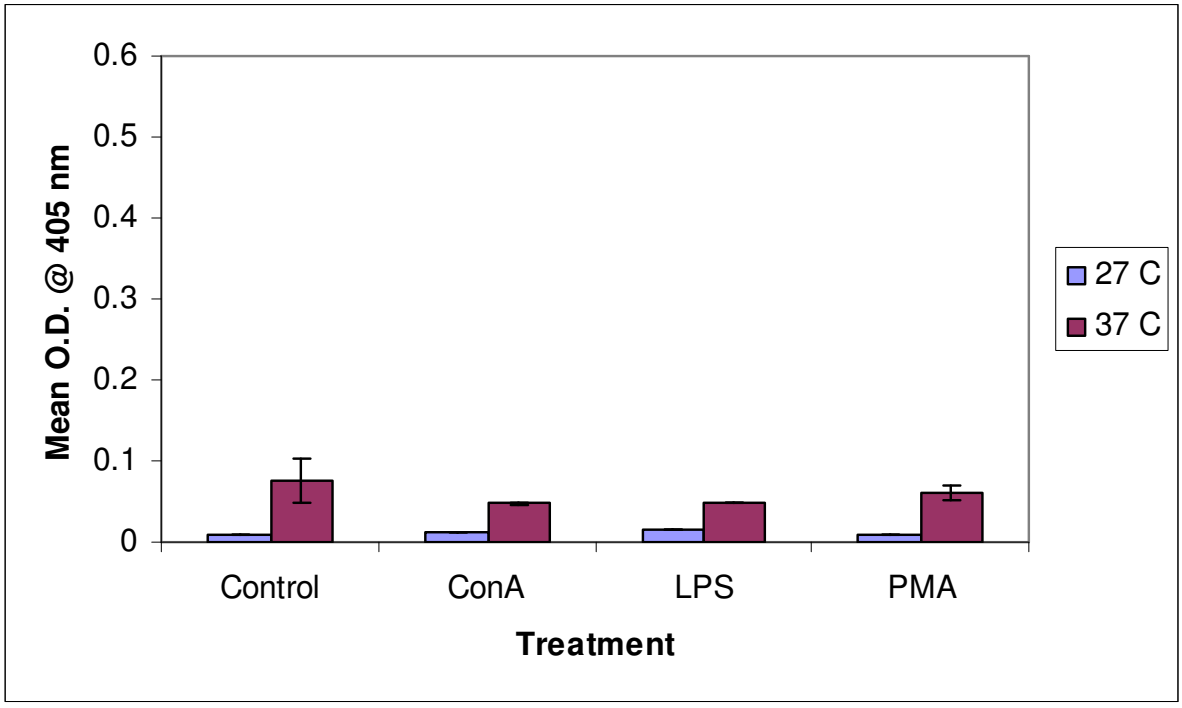

Figure 12. Lysosomal enzyme (acid phosphatase) activity in the RAW 264.7 murine macrophage cell-line as measured indirectly by the appearance of a colored end-product from a colorless substrate. All data represent the pooled mean of triplicate samples from a sample size of 4 or greater ($n \geq 4$) from two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean. (Chart legend indicates the temperature at which the assay was performed, eg. 27 ^{o}C and 37 ^{o}C)

control cells) may be due to the level of activation from the elicited cell population or to the possibility of accessory cell activity among the mixed cell population.

As a major component of the innate immune response mediated by macrophages and as an equally important indicator of the soundness of said macrophages, respiratory burst plays a major role in the control and clearance of microbial invaders. As such, the establishment of any model of macrophage function quite clearly should include the basic parameters for an effective respiratory burst as well as extended parameters where possible. This might include assays for hydroxyl radical and hydrogen peroxide production.

Due to the limitations of facility equipment and resources, exploration of such extended criteria was not possible within the scope of this project. However, the information garnered about RPEC macrophages is both valuable and interesting. The effects of temperature, while not surprising when considering reptile physiology, were compelling when considering the production of superoxide and acid phosphatase.

An alternate cell/cell-type might be considered for control/comparison of the acid phosphatase activity due to the poor results from the RAW cell-line. Certainly, further investigation is warranted concerning the production of nitric oxide by reptile macrophages.

ENDOCYTOSIS

As seen in Figure 13, control cells and treated cells were both able to phagocytose the bacteria. In numerous trials under the listed optimal conditions, RPEC treated with PMA outperformed the controls and other treatments. Cells treated with ConA were not

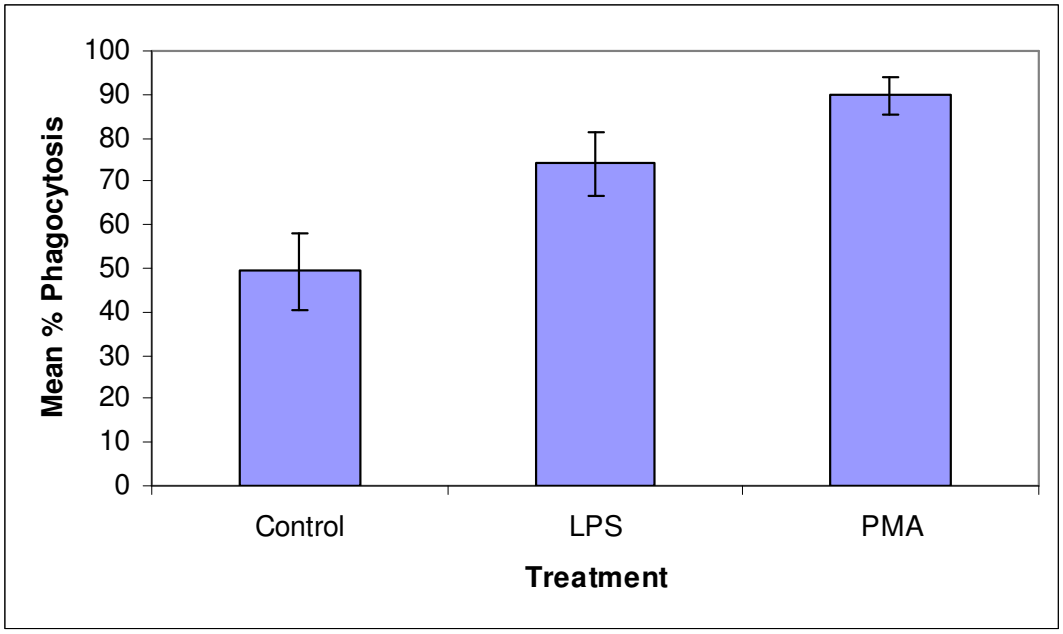

Figure 13. RPEC phagocytic activity expressed as the % of cells performing phagocytosis as determined by microscopic examination of ingested bacteria. A minimum of four fields and/or 100 cells was counted in each trial. All data represent the pooled mean of two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between each treatment mean and the control mean.

countable due to clumping of the RPEC and reptile erythrocytes. Figures 14a and 14b provide photographic evidence of said phagocytosis. Reptile erythrocytes can be seen in both the white light and fluorescent photos as reference markers of the microscopic field.

Concerns about the possibility of bound but un-ingested bacteria prompted the use of a Gentamicin assay to kill any bound bacteria and exclude fluorescence from these as well. Basically, Gentamicin was applied to the co-cultures at the completion of the 90 minute incubation in order to kill any extra-cellular bacteria. The aforementioned wash steps were then performed followed by the microscopic examination. Results were similar to those shown in Figure 13.

To further verify and quantify the phagocytic capacity of the RPEC, a second method was utilized. Overnight shaking cultures of selected Gram (-) and Gram (+) bacteria were labeled with FITC, agitated to create labeled particulate and added to the RPEC cultures as described above. To eliminate the possibility of attached bacteria, trypan blue was used to quench the FITC fluorescence of any extra-cellular particles.

Results are shown in Figure 15. As in the previous phagocytosis assay, a minimum of four fields and/or 100 cells were counted to establish the percent phagocytosis of the RPEC.

It was interesting to note that RPEC phagocytosis was similar for both the Gram (-) bacteria, *Escherichia coli*, and the Gram (+) bacteria, *Staphylococcus aureus.* While a relatively strong response was seen in Figure 14 to LPS, a derivative of Gram (-) species, no Gram (+) specific agonist has been employed for this study. It seems unlikely that the reptile macrophages would respond "equally" to these diverse organisms,

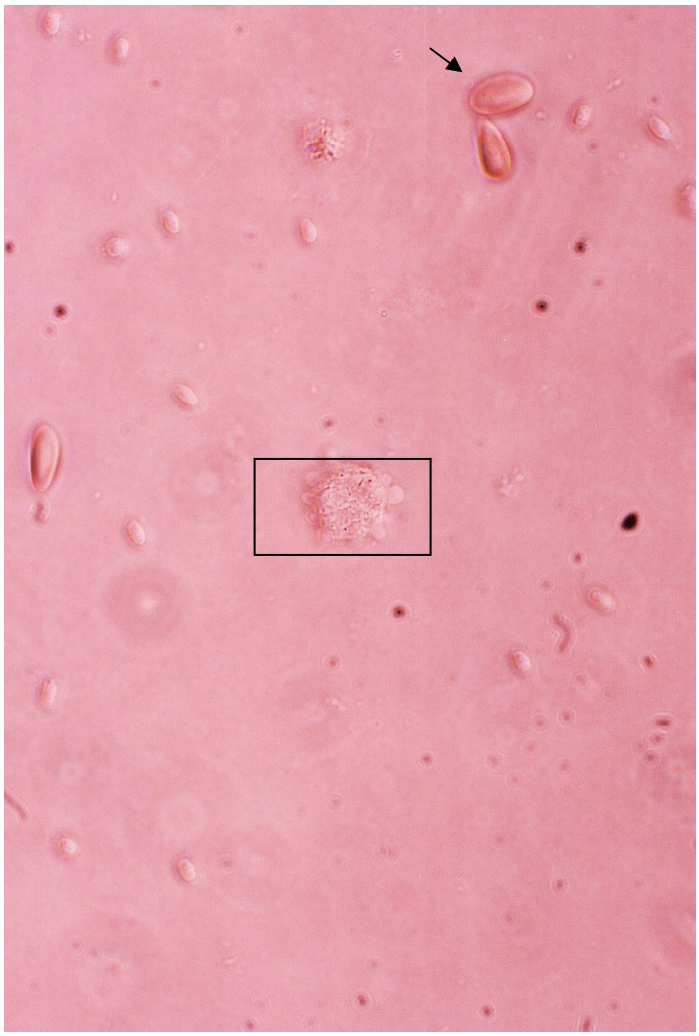

Figure 14a. Photographic depiction of a representative RPEC under white light. The arrow indicates reptile erythrocytes for reference to Figure 14b. The RPEC is located within the box.

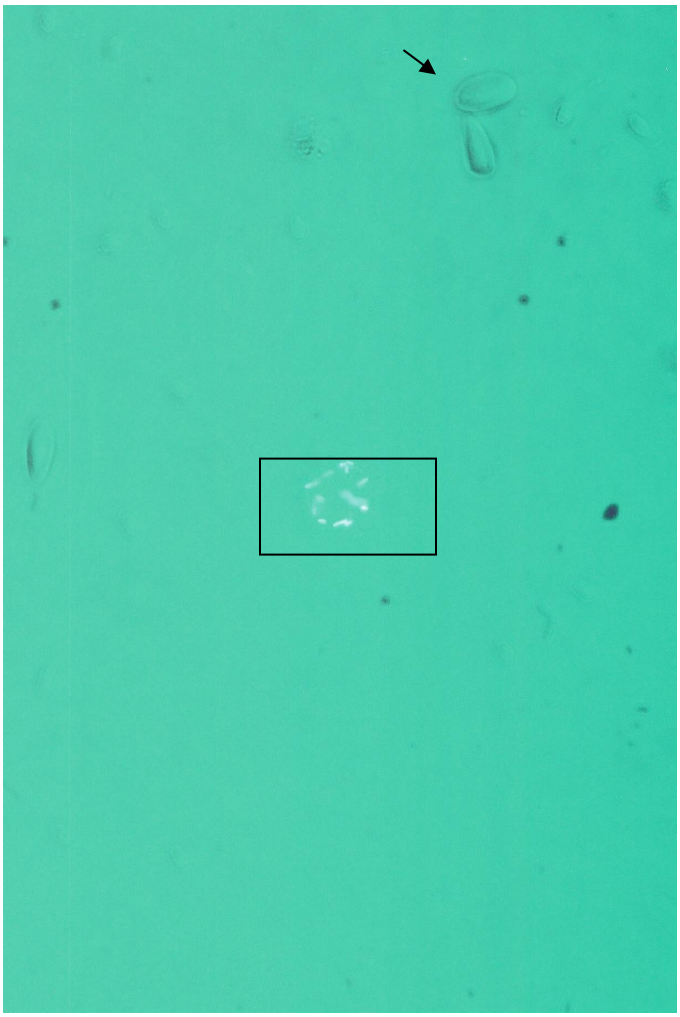

Figure 14b. Photographic depiction of a representative RPEC with ingested GFP bacteria under fluorescence. The arrow indicates reptile erythrocytes for reference to Figure 14a. The RPEC is located within the box.

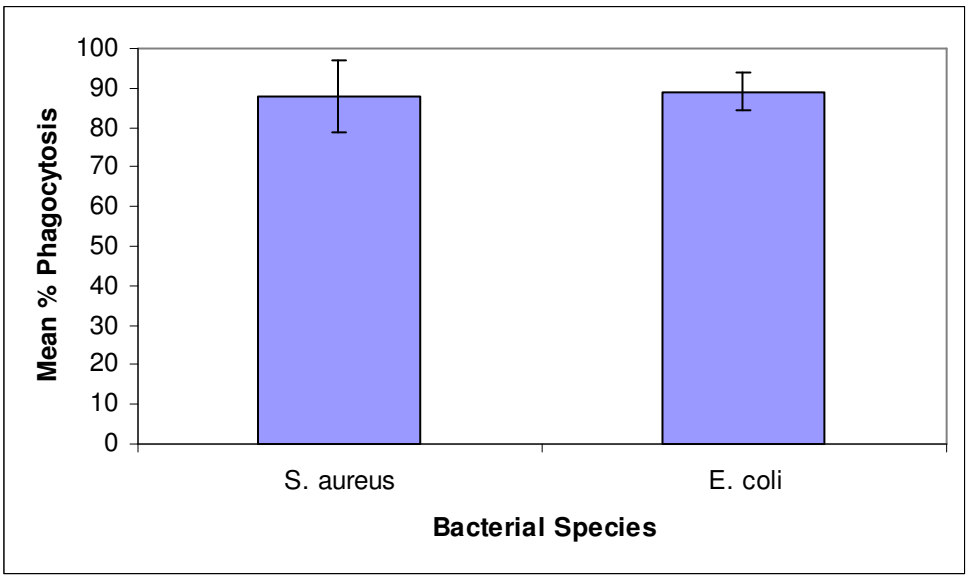

Figure 15. RPEC phagocytic activity expressed as the % of cells performing phagocytosis as determined by microscopic examination of ingested bacterial particulate. A minimum of four fields and/or 100 cells was counted in each trial. All data represent the pooled mean of two or more experiments ± the standard error of the mean. The student t-test was performed to determine any significant difference(s) between the species chosen.

however the original supposition for this study included an enhanced innate immune response to provide the evident success of reptiles as a taxon. Possibly, there is a less specific cell-surface receptor in reptile macrophages that allows for the ready recognition of a variety of microbes as yet unseen in established models.

CHEMOTAXIS

Chemotaxis is the directed cellular migration along a stimulus gradient. In higher organisms, immune cells migrate toward a variety of signals produced by either host cells or microbial invaders (Firtel and Chung, 2000). In more primitive organisms such as *Dictyostelium discoideum,* chemotaxis is a means for feeding as well as aggregation to form a multi-cellular organism to survive times of starvation (Firtel and Chung, 2000; Chen, et. al., 2007).

Chemotaxis proved a more elusive function/capacity of the reptile macrophages used in the current study. Numerous attempts were made to verify the ability of the RPEC to respond to a signal, including a "spot-drop" method, transwell inserts and finally an under-agar assay. For each trial and type of trial, data was either unsatisfactory or non-existent. After numerous searches in the available literature to locate more appropriate conditions by which chemotaxis could be quantified or at least verified, a reference was found that offered a plausible explanation. It has been determined that elicited macrophages have been primed or "programmed" to migrate into the serous cavity from which they are subsequently harvested (Wilkinson, 1976). This seems to preclude any further migratory response(s).

The obvious action to resolve this problem would be to utilize resident macrophages for chemotaxis studies. Unfortunately, enumeration of resident macrophages was not considered critical in the early stages of the project and when it was determined to be necessary to complete the migration work, a satisfactory harvest of resident macrophages failed to materialize.

While it was recognized early on that *Sceloporus occidentalis* produced far fewer elicited peritoneal exudate cells as compared to typical murine species, there was sufficient cells to carry out the selected assays of immune function. In the case of resident cells and chemotaxis, a more thorough literature search and planning might have prevented the lack of success.

To detail the discrepancy between cellular yields between the reptile and murine models, experimental data from this project was compared to literature data for murine resident and elicited macrophage yields (Table 2). Although the reptile yields were lower in both resident and elicited, it should be noted that in both species the elicited yield was a fairly uniform and consistent ten-fold increase from the resident yield.

REPTILE SPECIFIC PRELIMINARY FINDINGS

To address the establishment of a reptile model, data was obtained concerning reptile specific characteristics such as endocrine effects (exhibited in crowding) and hibernation. It should be noted that the data presented is only considered preliminary and further assays will/would need to be performed to make appropriate conclusions as to the value of said data and what it might imply for the development of a reptile model

	Reptile	Murine
Resident	8.75×10^5	4.0×10^6
Thioglycollate elicited	9.0×10^6	3.0×10^7

Table 2. Mean peritoneal exudate cellular yield per animal for reptile and murine models. Reptile yields are directly from experimental procedures with *Sceloporus occidentalis.* Murine yields are taken from Adams, Edelson and Koren.

As reptiles have been shown to be more susceptible to hormonal effects, and immune function in mammals has been shown to be down-regulated by cortisol, assays for basic immune function were performed under "crowding" conditions. Basically, it was considered that the number of subjects housed in a single aquarium, the crowding effect, might contribute to a stress-induced increase in serum cortisol resulting in a depression of immune function. Aquaria were established with one, two, four and eight subjects for a period of two weeks. Sexually mature females were used for the purposes of these preliminary findings.

The first parameter investigated was antibody production. A hemagglutination assay to quantify the anti-bovine red blood cell (BRBC) production in the test subjects after i.p. injection with BRBCs at one week post-injection and two weeks post-injection. Such a study has already been published with this same model and satisfactory antibody production (Burnham, et. al., 2003).

In the first assay, it was quite evident that the less-crowded reptiles produced higher titers of antibody to the BRBCs (Figure 16a) and in the second week post-injection, although the two-subject aquaria was more efficient than the one-subject aquaria, they both performed better than the more crowded groups (Figure 16b). These results indicate the need for further exploration of the crowding effect in this reptile species and possibly other reptiles as well.

An assay of the elicitation/migration of RPEC for mean cell-harvest yield was also performed as a function of crowding. With the same experimental conditions as the hemagglutination study, mean yield via TGM elicitation was determined as outlined in the typical RPEC harvest procedure documented previously, however the duration of

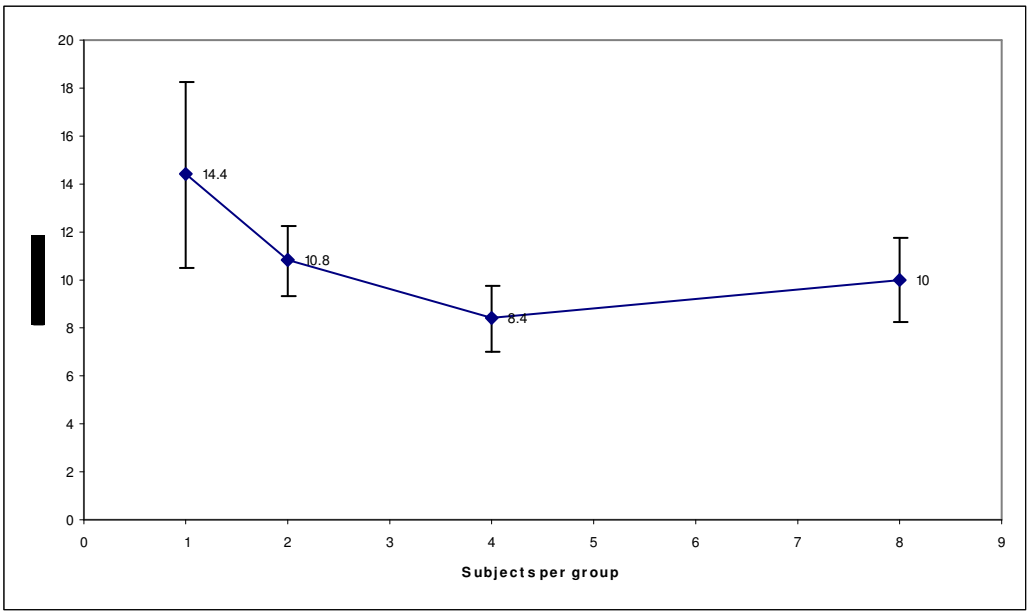

Figure 16a. Antibody production to BRBCs one week post-injection as assayed against crowding in reptile subjects. Data is represented as the mean titer ± the standard error of the mean of triplicate samples of a single experiment. Offered as preliminary data only.

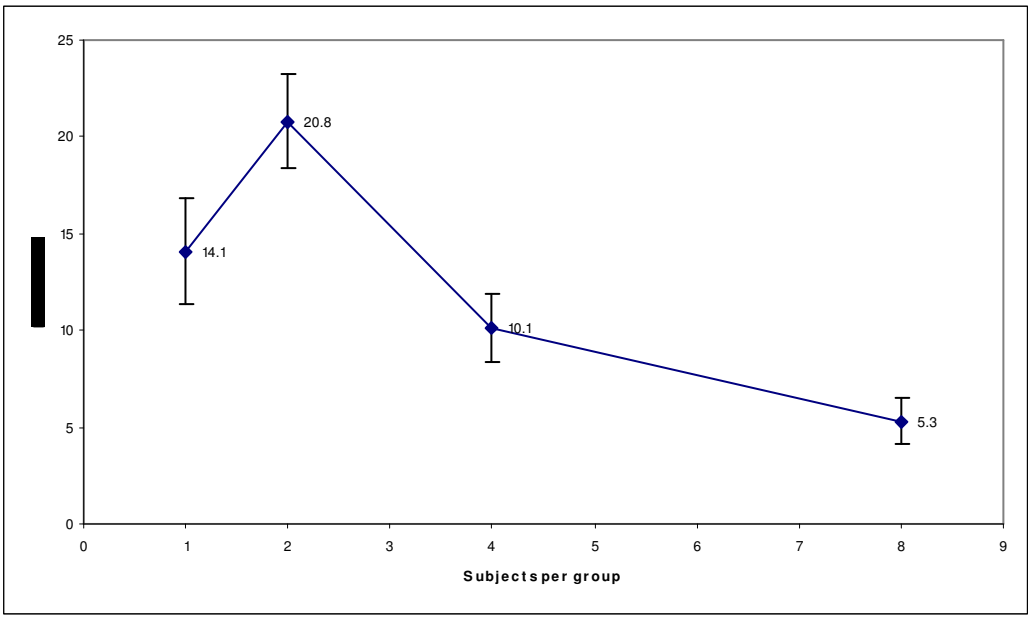

Figure 16b. Antibody production to BRBCs two weeks post-injection as assayed against crowding in reptile subjects. Data is represented as the mean titer ± the standard error of the mean of triplicate samples of a single experiment. Offered as preliminary data only.

crowding was only assayed for a two-week period. The results were inconclusive although a slightly higher yield was observed in the less-crowded aquaria (Figure 17).

In order to firmly establish an acceptable reptile model for innate or adaptive immune functions, the effects of crowding may need to be further assessed. The mechanism for the effects, such as increased cortisol, should also be established if possible.

Another characteristic of many reptiles is that of seasonal hibernation. To assess the immuno-competence of the RPEC from lizards revived from hibernation, cellular yield (typical RPEC harvest) and phagocytosis was quantified. Assays were performed on the day of recovery (day 0), day 7, day 16 and day 28.

The mean cellular yields are presented in Figure 18. No clear linear correlation was available, however, it seemed evident that after 28 days post-hibernation, the cellular yield was rising, but still below that reported in Table 1.

Phagocytosis was performed as in the earlier procedure utilizing the GFP expressing bacteria. At day 0, no phagocytosis was measured due to high levels of RBC contamination and clumping thereof. Days 7 and 16 displayed levels of phagocytosis relatively comparable to that of the control cells in Figure 13 (no agonists were used with the hibernation recovery), with day 28 appearing to decrease in phagocytic capacity (Figure 19).

As with the crowding data, further work is warranted concerning any possible effects resulting from the physiological state of the reptiles upon recovery from hibernation. It should be noted that the subjects are typically poorly hydrated for several

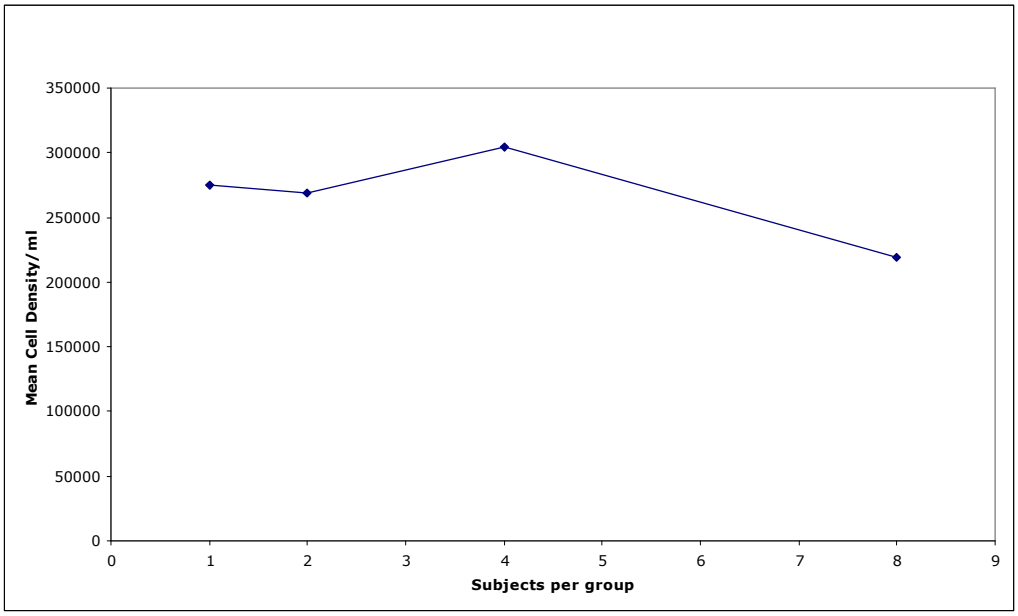

Figure 17. Mean RPEC harvest yields from reptile crowding subjects. Data represent the mean ± the standard error of the mean for a single experimental trial. Offered as preliminary data only.

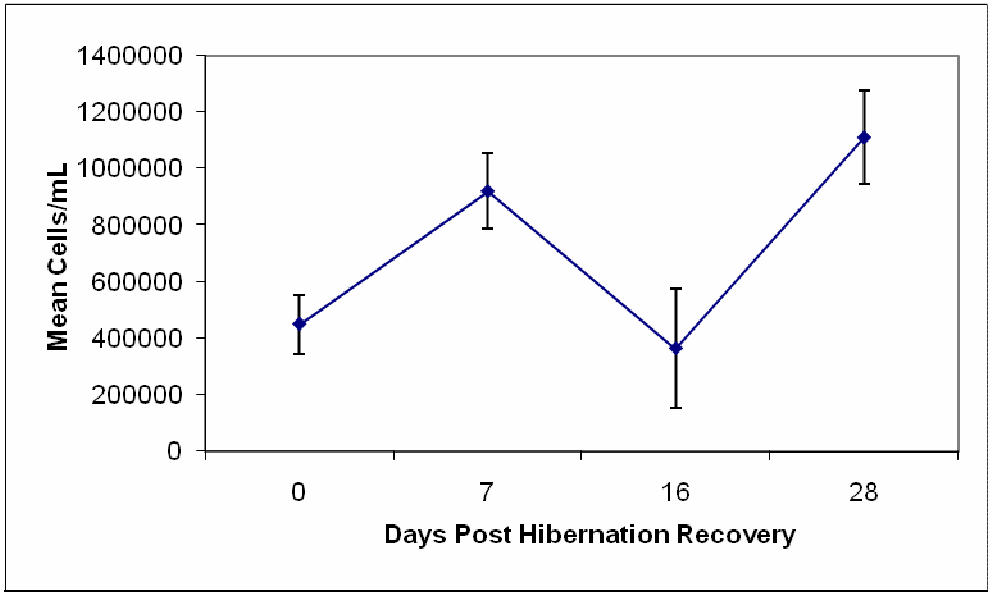

Figure 18. Mean RPEC harvest yield upon recovery from hibernation. The data represent the mean ± the standard error of the mean for a single experimental trial. Offered as preliminary data only.

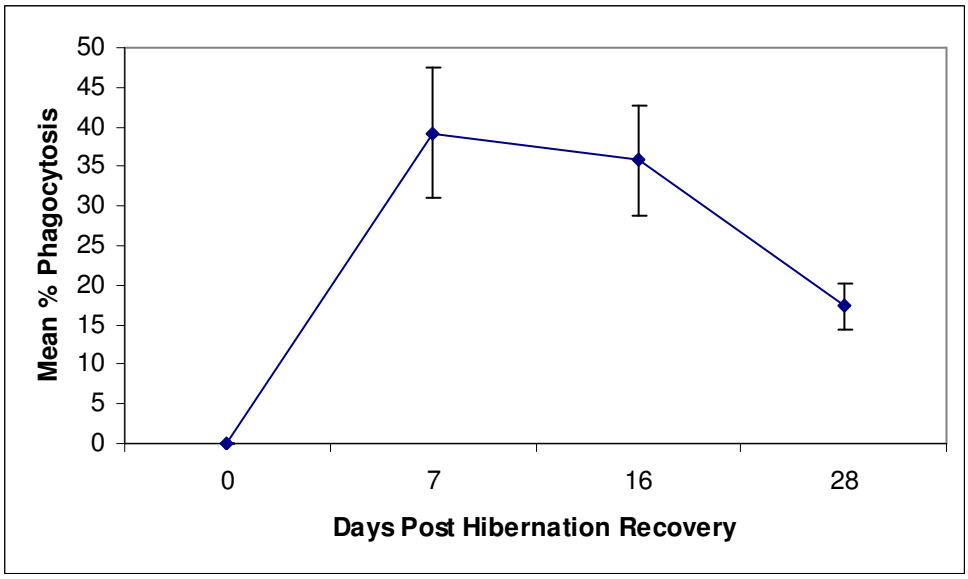

Figure 19. Mean % phagocytosis of GFP bacteria by RPEC during recovery from hibernation. The data represent the mean % phagocytosis ± the standard error of the mean from a single experimental trial. Offered as preliminary data only.

days upon revival from hibernation. This fluid imbalance might play an important role in itself without consideration to other aspects as yet unknown.

CHAPTER V

CONCLUSION

The primary purposes of this study were to explore the extent of innate immune capacities in a potential reptile macrophage model, compare those capacities with those of an established mammalian model and ultimately to add to the breadth of understanding and knowledge of macrophages and the innate immune response in general.

The initial stance taken concerning reptile immunity was that there was a substantial possibility of enhanced innate immune function as compared to mammalian responses. This was based partly on the knowledge that reptile adaptive immunity has been shown to be suppressed under many circumstances such as seasonal, temperature or endocrine changes (Masri, et. al., 1995; Saad, 1989; Saad, et. al., 1986; Saad et. al., 1984). Given the historical age of reptiles and their obvious survival success, it could be inferred that they possess an adequate immune system to ensure such longevity.

With few publications dedicated to reptile immunity and with an experimental animal representing a taxon outside the vast majority of macrophage literature, it was expected that any existing experimental procedures related to the chosen parameters might require modification(s) to exploit the differences in subject physiology. The first and most obvious adaptation was the incubation temperature. Available literature on ectotherms suggested an optimal temperature somewhere between 22 ^{o}C and 30 ^{o}C (Plytycz and Jozkowicz, 1994). Through a series of assays and by microscopic

examination to determine the "healthy" appearance of the reptile cells in culture, it was determined that for our purposes 27 ^{o}C provided the best conditions for optimal immune responses in those aspects explored. The only other broad change that was required was in the duration of some incubation steps.

Verification/identification of the adherent fraction of the mixed cell population obtained at harvest proved to be challenging but ultimately attainable. The results of neutral red uptake and non-specific esterase activity were slightly disappointing as these functions would have provided even stronger evidence for a true macrophage population. However, with the literature supporting the phenomena of spreading and attachment (Nathan and Root, 1977; Stewart, et. al., 1975) and the success of the assays for phagocytic activity, it is unlikely that the presumptive identification was incorrect. Certainly, the more evidence the better for any research project but especially so when attempting to establish a new model system for such diverse and critical functions as macrophages perform.

With the variety of reactive oxygen species and a prominently published subject as nitric oxide, it was thought that respiratory burst would yield vast quantities of data concerning reptile macrophages. However, as noted previously facility limitations relegated the focus of respiratory burst to superoxide production and nitric oxide production. Based on the available literature, these two reactive species are the most prominently studied parameters of macrophage respiratory burst.

Inconclusive early NO data from the reptile macrophages suggested that modification of culture conditions may be necessary and numerous adjustments were made, including temperature, incubation time, % serum supplementing the media and cell

density. Up to this time, no definitive decision had been made concerning a control cell for comparison.

Upon acquiring the RAW 264.7 murine macrophage cell-line, the cells were grown to allow for a sufficient stock to complete this project. When an acceptable reserve had been stored, assays for nitric oxide production were initiated with the cell-line. NO production was seen in response to numerous stimuli, and it was decided to attempt the use of the reptile L-15 complete medium and the reptile-preferred incubation temperature of 27 ^{o}C with the RAW control cells. While they did not perform as well in the RPMI and at 37 ^{o}C, there was enough NO production to rule out media or temperature effects alone or in concert as cause for the lack of detectable NO from the RPEC.

As mentioned earlier, it is highly unlikely that RPEC do not synthesize nitric oxide, but it is more likely that the appropriate conditions and co-factors have yet to be determined. To derive an LPS binding protein specific to the reptile species in an active state or effective concentrations may require more than just pooled serum. Even the effects of cell density have been shown to contribute to NO output in murine macrophages (Jacobs and Ignarro, 2003). Perhaps even a more sensitive assay may be required, however, and the Griess assay has been used successfully for decades and is still the assay of choice for many investigators today.

It should be noted that many of the parameters of respiratory burst have differential expression based on both the tissue origin and the state of activation of the macrophages themselves. In fact, nitric oxide has been obtained and quantified from lizard macrophages harvested from the spleen (Mondal and Rai, 2001; Mondal and Rai,

2002) as well as from peritoneal exudate cells from the turtle, *Trachemys scripta scripta,* (Pasmans, et. al., 2002). These findings support the existence of reptile macrophage production of nitric oxide, but do not discount the probability of tissue- and species-specific variations in total NO production.

Some notable differences exist between the Mondal and Rai studies as compared to this project. The lizard utilized was *Hemidactylus flaviviridis,* the wall lizard. For the purposes of their research, only female lizards were used. Our project involved only male lizards to eliminate the possibility of hormonal fluctuations as an influence on macrophage function. Their research also addressed the issue of temperature variation as a factor and for their subjects 25 ^{o}C was determined to be the optimal temperature. The size of the wall lizards used was approximately one third the size of the *Sceloporus occidentalis* used for our study. The final major difference between the existing and current studies was the tissue of origin for the macrophages. Splenic macrophages were harvested directly from the tissue of the whole spleens and required no elicitation unlike harvest from a serous cavity such as the peritoneum. The cells from the spleens were also pooled which might contribute a mitogenic effect on the function of the macrophages. A pooled population (from two or more *Sceloporus occidentalis)* of RPEC was never explored for NO production. However, from an *in vivo* standpoint, a pooled cell suspension would lend little information as to the function of the macrophages in the individual lizard.

Also, even though human iNOS has been identified, the levels of NO production are far below the observed levels among rodent species. Given these factors, a different

species of reptile may prove to be a good source of RPEC that express prolific production of nitric oxide.

Certainly, with widespread evidence of the multiple functions for nitric oxide in higher organisms and the equally numerous studies that have identified the production of the inducible form by various immune cell-types and species, it is our contention that RPEC from *Sceloporus occidentalis* will eventually prove capable of NO production. With a thorough series of manipulations of the culture and incubation conditions and/or the determination of a binding protein co-factor, or quite possibly an assay that is more sensitive or specific, it is likely that this production will eventually by shown.

The superoxide assay yielded reasonable results with little manipulation of the existing protocols. There was a measurable response to each of the stimuli used at both temperatures explored, although a better response was seen in the lower temperature. It should be noted that the high levels of superoxide displayed by the control (untreated) cells are most likely due to the pre-activated (by elicitation) state of the cells. Production of the reactive species over the range of temperatures as compared to the relative lack thereof from the RAW cells serves as an indicator of our earlier presumption that the innate immune response would be enhanced in at least some regards to offset any suppression of the adaptive immunity.

Much like the superoxide study, lysosomal enzyme production as characterized by the production of acid phosphatase demonstrated good responses at the low temperature extreme and fairly high levels at the higher extreme. It should again be noted that no literature supporting the use of the RAW cells as a control for acid

phosphates was found and that the high activity level seen in the reptile cells is most likely due to their elicited state of activation.

As might be inferred by the common reference “mononuclear phagocyte”, phagocytosis is a prominent feature of macrophage function, and while not unique to macrophages it is distinctive enough to be commonly used to identify macrophage populations (van Furth, 1981; Taffet and Russell, 1981). As mentioned previously, phagocytic capacity is one of the criteria used to make a formal identification of the RPEC used in this study as macrophages.

Phagocytosis was originally posed as an immune function by Elie Metchnikoff in the late 19th century in such primitive and notably non-vertebrate systems as starfish larvae and the water flea *Daphnia* (Hirsch, 1982). In similarly primitive organisms such as the sea sponge, phagocytosis is a means of nutrient uptake (Moen and Svensen, 2004). Likewise, the social soil amoeba *Dictyostelium discoideum* feeds on bacteria in much the same manner (Chen, et. al., 2007).

In higher organisms, phagocytosis is now a common focus of study. Numerous protocols have been developed to determine and/or quantify particulate or whole-cell uptake as a means of maintaining homeostasis or the control and clearance of invading microbes. These methods range from differential interference microscopy of co-cultures (Lopes, et. al., 2006; Bos and de Souza, 2000) to light microscopy of latex beads (Polonio, et. al., 2000) and flow cytometry of fluorescently labeled yeast (Ortega, et. al., 2001).

Due to the complex, multiple aspects required to facilitate phagocytosis in vertebrates, this function, and in fact the macrophage itself, has become an attractive

model for studies of cell biology (Vaux and Gordon, 1981). The stepwise manner in which phagocytosis occurs involves chemotaxis, the cellular migration toward the target. This involves cytoskeletal rearrangement to "crawl" along a signal gradient (Stephens, et. al., 2002). Following chemotaxis is the process of attachment, which requires a cell-surface receptor on the phagocyte. The specificity of this binding ranges from general as in scavenger receptor interactions with host cellular debris and mannose receptor interactions that involve mannose residues that occur on a variety of bacteria to the highly specific binding of Fc receptor to opsonized microbes (Aderem and Underhill, 1999; Underhill and Ozinsky, 2002). The final step of phagocytosis involves the cytoskeletal rearrangement that results in the engulfment of the attached particle/cell (Greenburg, 1999; Aderem and Underhill, 1999; Underhill and Ozinsky, 2002).

Upon completion of phagocytosis, other mechanisms are rapidly activated that aim to control the proliferation of the ingested microbe and/or digest the microbe. Some such mechanisms involve respiratory burst, the production of lysosomal degradative enzymes, and the modulation of intra-lysosomal pH (Underhill and Ozinsky, 2002).

The endocytic studies, while encompassing the poorly demonstrated neutral red uptake, were quite encouraging due to the early success of phagocytosis. Considered by many to be the critical function of an activated macrophage, phagocytosis proved to be relatively easy to achieve. Further refinement of the techniques used in this study might be effective in obtaining a phagocytic index, or a more conventional method such as confocal microscopy might allow for an estimation of such a parameter.

It was quite interesting to observe the phagocytic response to the chosen bacterial species. It seemed obvious to challenge the macrophages with both a Gram (+) and Gram

(-) species given the differences in composition and function. It was not expected that the resulting activity would be so quantitatively similar. Since LPS, a product of Gram (-) bacteria was used as a stimulus in the early phagocytosis work and a fairly robust response was seen, it was expected that the Gram (-) bacteria would elicit a reasonably strong response. No such Gram (+) derived stimulus, such as peptidoglycan, was utilized and it was uncertain how well the macrophages would respond to a Gram (+) species. With the nearly equal response to both species of bacteria, it would be interesting to conduct a more widespread assay with a variety of bacteria and slight modulations to the culture conditions. Such a comprehensive challenge of the reptile macrophages might lend credence to the aforementioned theory that RPEC may respond in an even less specific (innate) manner than previously recognized and established models of macrophage function. Again, this would provide further evidence as to the nature of the long-term survival of reptiles in general.

The failure to perform a successful chemotaxis assay was tempered with what is hoped to be the logical explanation for said failure. If macrophages can migrate to only a single signal, then a different tissue source that does not require elicitation or a more abundant source of resident macrophages is needed. Possibly but not necessarily, a slightly larger reptile species or strain could provide an adequate number of resident macrophages. However, if one considers the mean harvests reported for resident vs. elicited macrophages, there is an obvious migration in response to the TGM injections resulting in the notable increase.

The preliminary data presented revealed further areas to be considered to formally establish a reptile macrophage model. The hibernation studies, while not conclusive by

any means, were presented in poster format at the 2005 American Association of Immunologists meeting in San Diego, CA. For many species of reptile, a complete study of the effects of hibernation recovery would be essential.

In much the same manner, a more thorough crowding study seems necessary based on the early data gathered. In any study involving animal subjects, the husbandry must be taken into consideration when finalizing and/or validating experimental data. There seems to be an obvious effect on humoral immunity (the hemagglutination results) and on cellular/innate immunity (the cell harvest yields) for lizards housed more than two per aquaria.

To begin to address the many observations and even the failures, it seems that an obvious solution to such problems as chemotaxis would be to find a larger experimental subject. While this might provide a larger number of resident macrophages, it would also require animal housing adjustments to avoid the crowding conditions, and this in turn might impede the number of reptiles that a given facility could house at one time. Often, to obtain statistically relevant data, a large sample size is required, or more treatment variables must be considered, both requiring adequate numbers of subjects of the same age and/or sex. Also there is no guarantee that a larger animal would provide sufficient resident cells or that the culture conditions established would be optimal for the new reptile. Even breeding and raising a small sample population to test these measures could be time-consuming and costly.

In regards to the lack of production (or lack of detection) of nitric oxide, NO has been implicated in the formation of neoplasias in mammals. It is also suspected of contributing to numerous pathological conditions, such as Type I Diabetes. To date,

there is a striking absence of published literature dealing with cancers/tumors in reptiles. Given their longevity and the prolific superoxide response, there may be other features of the reptile immune system that preclude the need for NO production, and this "lost" function may provide an explanation for the lack of documented neoplasias in reptiles.

The temperature effects while not surprising when one considers the physiology of ectotherms, raise some questions. Respiratory burst is considered an inflammatory response and yet, superoxide production and acid phosphatase activity was seen at higher levels at lower incubation temperatures. This might indicate a species-specific, temperature-dependent enzyme. However, since reptiles have been shown to bask for thermoregulation when infected (but also for digestive and other functions), one might think that they were raising their body temperature to ward off the infection or prevent microbial colony formation, much as humans experience fever. It seems that if respiratory burst and basking occurred simultaneously, the effect on an invader would be additive.

To verify any of these ideas, however, would require a number of facts to be obtained. It would be critical to first establish the mean non-basking temperature of a healthy reptile as well as the optimal basking temperature of the same species, size and age when experiencing an infection. Further, determination of the common pathogenic microbes and their optimal functioning temperature would be necessary.

To completely characterize and evaluate our assumptions as to reptile innate immune capacities would likely require many more months of research and would likely constitute another complete project by which to normalize the adaptive immune functions in the reptile as compared to mammalian capacities. To effectively establish that a more

robust innate response compensates for seasonally (or otherwise affected) decreases in the adaptive response, it would certainly be necessary to determine the extent of the adaptive responses under different conditions.

At the very least, it is obvious that reptile macrophages exhibit many common parameters of macrophage function as recognized in established species and models. Additionally, it can be seen that some of these reptile functions are equal to or greater than the murine cells used for comparison, and that they are able to function over a much broader range of temperatures. As ectotherms, reptiles are more susceptible to fluctuations in their environmental temperature, therefore a less temperature-dependent immune response would benefit the organism greatly. This ability to function over a range of temperatures adds much evidence for a theory of enhanced innate immunity.

It is also important to recognize the variety of stimuli that the RPEC responded to in an acceptable fashion. While not exhaustive, our approach in selecting effective stimuli was made partially based on economy and availability, as well as "suggested" agonists from the relevant literature. Gram (+) and Gram (-) bacteria as well as LPS, PMA and ConA were all measurably effective in evoking an immune response from the RPEC (except in the case of ConA stimulation of phagocytosis). This lends strength to the thought of a less specific level of immune function which would likely indicate an overall enhanced response.

Taken together, the temperature-independent activity and the seemingly less specific response to agonists are strong indicators of a more active innate immune system, however, a final mention should be made that from an autoimmune standpoint (another complete research project) the lack of specificity could lead to a rise in host-

targeted immune effects. Much like the scope of this project, filling in the small gaps while raising further questions, can only enhance the overall knowledge of the discipline.

REFERENCES

Adams, D.O. and Hamilton, T.A. 1984. The cell biology of macrophage activation. *Annual Reviews of Immunology.* 2: 283-318.

Aderem, A. and Underhill, D.M. 1999. Mechanisms of phagocytosis in macrophages. *Annual Reviews of Immunology.* 17: 593-623.

Akira, S. 2003. Mammalian toll-like receptors. *Current Opinion in Immunology.* 15: 5-11.

Allen, J.M. and Cook, G.M.W. 1970. A study of attachment phase of phagocytosis by murine macrophages. *Experimental Cell Research. 59: 105-116.*

Allison, A.C. and Young, M.R. 1964. Uptake of dyes and drugs by living cells in culture. *Life Sciences.* 3: 1407-1414.

Anderson, C.L. 1989. Human IgG Fc receptors[1]. 1989. *Clinical Immunology and Immunopathology.* 53: S63-S71.

Anderson, K.V. 2000. Toll signaling pathways in the innate immune response. *Current Opinion in Immunology.* 12: 13-19.

Artursson, P., Johansson, D. and Sj□holm, I. 1988. Receptor-mediated uptake of starch and mannan microparticles by macrophages: relative contribution of receptors for complement, immunoglobulins and carbohydrates. *Biomaterials.* 9: 241-246.

Azuma, Y., Shimohara, M., Wang, P.L. and Ohura, K. 2001. Quinolones alter defense reactions mediated by macrophages. *International Immunopharmacology.* 1: 179-187.

Azuma, Y., Shimohara, M., Wang, P.L., Hidaka, A. and Ohura, K. 2001. Histamine inhibits chemotaxis, phagocytosis, superoxide anion production, and the production of TNFα and IL-12 by macrophages via H_2-receptors. *International Immunopharmacology.* 1: 1867-1875.

Babior, B.M. 2000. Phagocytes and oxidative stress. *The American Journal of Medicine.* 109: 33-44.

Badwey, J.A. and Karnovsky, M.L. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annual Review of Biochemistry.* 49: 695-726.

Bae, I.K., Min, H.Y., Han, A.R., Seo, E.K. and Lee, S.K. 2005. Suppression of lipopolysaccharide-induced expression of inducible nitric oxide synthase by brazilin in RAW 264.7 macrophage cells. *European Journal of Pharmacology.* 513: 237-242.

Baier-Anderson, C. and Anderson, R.S. 2000. Suppression of superoxide production by Chlorothalonil in striped bass (*Morone saxatilus*) macrophages: the role of Cellular sulfhydryls and oxidative stress. *Aquatic Toxicology.* 50: 85-96.

Bellows, C.F., Adler, A., Wludyka, P. and Jaffe, B.M. 2006. Modulation of macrophage nitric oxide production by prostaglandin D_2. *Journal of Surgical Research.* 132: 92-97.

Bibel, Debra Jan. Milestones in Immunology: A Historical Exploration. Madison, Wisconsin. Science Tech Publishers, 1988.

Bos, H. and De Souza, W. 2000. Phagocytosis of yeast: a method for concurrent quantification of binding and internalization using differential interference contrast microscopy. *Journal of Immunological Methods.* 238: 29-43.

Brennan, R.E., Russell, K., Zhang, G., and Samuel, J.E. 2004. Both inducible nitric oxide synthase and NADPH oxidase contribute to the control of virulent phase I *Coxiella burnetii* infections. *Infection and Immunity.* Nov. 6666 – 6675.

Brubacher, J.L. and Bols, N.C. 2001. Chemically de-acetylated 2',7'-dichloridihydrofluorescein diacetate as a probe of respiratory burst activity in mononuclear phagocytes. *Journal of Immunological Methods.* 251: 81-91.

Burnham, D.K., Lackey, A., Manering, M., Jaensson, E., Pearson, J., Tyler, D.O., Melson, D. and Talent, L.G. 2003. Effects of 17α-ethinylestradiol on immune Parameters in the lizard *Sceloporus occidentalis*. *Environmental Toxicology.* 18: 211-218.

Burnham, D.K., Keall, S.N., Nelson, N.J. and Daugherty, C.H. 2005. T cell function In tuatara (*Sphenodon punctatus*). *Comparative Immunology, Microbiology & Infectious Diseases.* 28: 213-222.

Byun, J.A., Ryu, M.H. and Lee, J.K. 2005. The immunomodulatory effects of 3-monochloro-1,2-propanediol on murine splenocyte and peritoneal macrophage function *in vitro*. *Toxicology in vitro*. June: 1-7.

Capo, C., Moynault, A., Collette, Y., Olive, D., Brown, E.J., Raoult, D. and Mege, J.L. 2003. Coxiella burnetti avoids macrophage phagocytosis by interfering with spatial distribution of complement receptor 3[1]. *The Journal of Immunology.* 170: 4217-4225.

Carlos, I.Z., Monnazzi, L.G.S., Falcão, D.P. and Machado de Medeiros, B.M. 2004. TNF-α, H_2O_2 and NO response of peritoneal macrophages to *Yersinia enterocolitica* O:3 derivatives. *Microbes and Infection.* 6: 207-212.

Chang, Y., Chen, T.L., Sheu, J.R. and Chen, R.M. 2005. Suppressive effects of ketamine on macrophage functions. *Toxicology and Applied Pharmacology.* 204: 27-35.

Chang, Y.C., Li, P.C., Chen, B.C., Chang, M.S., Wang, J.L., Chiu, W.T. and Lin, C.H. 2006. Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 Macrophages is mediated by cyclooxygenase-2, prostaglandin E_2, protein kinase A, p38 MAPK, and nuclear factor-κ pathways. *Cellular Signalling.* 18: 1235-1243.

Chen, G., Zhuchenko, O. and Kuspa, A. 2007. Immune-like phagocyte activity in the social amoeba. *Science.* 317: 678-681.

Chi, D.S., Qui, M., Krishnaswamy, G., Li, C. and Stone, W. 2003. Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. *Nitric Oxide.* 8: 127-132.

Coffey, R.G., Yamamoto, Y., Snella, E. and Pross, S. 1996. Tetrahydrocannabinol inhibition of macrophage nitric oxide production. *Biochemical Pharmacology.* 52: 743-751.

Condeelis, J. and Pollard J.W. 2006. Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. 124: 263-266.

Coussens, L.M. and Werb, Z. 2002. Inflammation and cancer. *Nature.* 420: 860-867.

Crippen, T.L. 2006. The selective inhibition of nitric oxide production in the avian macrophage cell line HD11. *Veterinary Immunology and Immunopathology.* 109: 127-137.

Crippen, T.L., Sheffield, C.L., He, H., Lowry, V.K. and Kogut, M.H. 2003. Differential nitric oxide production by chicken immune cells. *Developmental and Comparative Immunology.* 27: 603-610.

Cuchens, M.A. and Clem, L.W. 1979. Phylogeny of lymphocyte heterogeneity IV: Evidence for T-like and B-like cells in reptiles. *Developmental and Comparative Immunology.* 3: 465-475.

De Oliveira, C.C., De Oliveira, S.M., Godoy, L.M.F., Gabardo, J. and De F. Buchi, D. 2006. Canova, a Brazilian medical formulation, alters oxidative metabolism of mice macrophages. *Journal of Infection.* 52: 420-432.

Djaldetti, M., Salman, H., Bergman, M., Djaldetti, R. and Bessler, H. 2002. Phagocytosis- the mighty weapon of the silent warriors. *Microscopy Research and Technique.* 57: 421-431.

Djaldetti, M., Salman, H., Bergman, M. and Bessler, H. 2006. Effect of pravastatin, simvastatin and atorvastatin on the phagocytic activity of mouse peritoneal macrophages. *Experimental and Molecular Pathology.* 80: 160-164.

Drath, D.B. and Karnovsky, M.L. 1975. Superoxide production by phagocytic leukocytes. *The Journal of Experimental Medicine.* 141: 257-261.

Drevets, D.A. and Campbell, P.A. 1991. Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. *Journal of Immunological Methods.* 142: 31-38.

Edelson, P.J., Zwiebel, R. and Cohn, Z.A. 1975. The pinocytic rate of activated macrophages. *The Journal of Experimental Medicine.* 142: 1150-1164.

Edelson, P.J. and Cohn, Z.A. 1976. 5'-Nucleotidase activity of mouse peritoneal macrophages: II. Cellular distribution and effects of endocytosis. *The Journal of Experimental Medicine.* 144: 1596-1608.

El Deeb, S.O. and Saad, A.H.M. 1990. Ontogenic maturation of the immune system in reptiles. *Developmental and Comparative Immunology.* 14: 151-159.

El Masri, M., Saad, A.H., Mansour, M.H. and Badir, N. 1995. Seasonal distribution and hormonal modulation of reptilian T cells. *Immunobiology.* Jun 193(1): 15-41.

El Ridi, R., Zada, S., Afifi, A., El Deeb, S., El Rouby, S., Farag, S. and Saad, A.H. 1988. Cyclic changes in the differentiation of lymphoid cells in reptiles. *Cell differentiation.* 24: 1-8.

Espey, M.G. 2006. Tumor macrophage redox and effector mechanisms associated with hypoxia. *Free Radical Biology and Medicine.*

Fang, F.C. 2004. Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nature Reviews: Microbiology.* 2: 820-832.

Firtel, R.A. and Chung, C.Y. 2000. The molecular genetics of chemotaxis: Sensing and responding to chemoattractant gradients. *Bioessays.* 22: 603-615.

Flajnik, M.F. and Du Pasquier, L. 2004. Evolution of innate and adaptive immunity: can we draw the line? *TRENDS in Immunology.* 25: 640-644.

Forman, H.J. and Torres, M. 2001. Redox signaling in macrophages. *Molecular Aspects of Medicine.* 22: 189-216.

Greenberg, S. 1995. Signal transduction of phagocytosis. *Trends in Cell Biology.* 5: 93-99.

Harding, C.V. 1995. Phagocytic processing of antigens for presentation by MHC molecules. *Trends in Cell Biology.* 5: 105-109.

Hatcher, G.E. and Lambrecht, R.S. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. *Journal of Dairy Science.* 76: 2485-2492.

Hirsch, James G. "Host Resistance to Infectious Diseases: A Centennial". *Phagocytic Cells.* Ed. John I. Gallin and Anthony S. Fauci. New York, New York. Raven Press, 1982. 1-12.

Holstad, M., Jansson, L. and Sandler, S. 1997. Inhibition of nitric oxide formation by Aminoguanadine: An attempt to prevent insulin-dependent diabetes mellitus. *General Pharmacology.* 29: 697-700.

Hultmark, D. 2003. Drosophila immunity: paths and patterns. *Current Opinion in Immunology.* 15: 12-19.

Hussein, M.F., Badir, N., El Ridi, R. and El Deeb, S. 1979. Effect of seasonal variation on immune system of the lizard, *Scincus scincus*. *Journal of Experimental Zoology.* 209: 91-96.

Israf, D.A., Khaizurin, T.A., Syahida, A., Lajis, N.H. and Khozirah, S. 2007. Cardamonin inhibits COX and iNOS expression via inhibition of p65NF-$_k$B nuclear translocation and I_k-B phosphorylation in RAW 264.7 macrophage cells. *Molecular Immunology.* 44: 673-679.

Jacobs, A.T. and Ignarro, L.J. 2003. Cell density-enhanced expression of inducible nitric oxide synthase in murine macrophages mediated by interferon-β. *Nitric Oxide.* 8: 222-230.

Jacobson, E.R. and Origgi, F. 2002. Use of serology in reptile medicine. *Seminars in Avian and Exotic Pet Medicine.* 11: 33-45.

Johnston, R.B. Jr., Chadwick, D.A. and Cohn, Z.A. 1981. Priming of macrophages for enhanced oxidative metabolism by exposure to proteolytic enzymes. *Journal of Experimental Medicine.* 153: 1678-1683.

Johnston, R.B. Jr., Godzik, C.A. and Cohn, Z.A. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *Journal of Experimental Medicine.* 115-127.

Jungi, T.W., Lerch, P.G., Cachelin, A.B. and Morell, A. Monomeric and dimeric IgG1 as probes for assessing high-affinity and low-affinity receptors for IgG on human monocyte-derived macrophages and on activated macrophages. *Molecular Immunology.* 25: 719-729.

Kabelitz, D. and Medzhitov, R. 2007. Innate immunity – cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Current Opinion in Immunology.* 19: 1-3.

Kang, J.H., Sung, M.K., Kawanda, T., Yoo, H., Kim, Y.K., Kim, J.S. and Yu, R. 2005. Soybean saponins suppress the release of proinflammatory mediators by LPS-stimulated peritoneal macrophages. *Cancer Letters.* 230: 219-227.

Karnovsky, Manfred L. and Bolis, Liana. Phagocytosis: Past and Future. New York, New York. Academic Press. 1982.

Keil, D.E., Warren, D.A., Jenny, M.J., EuDaly, J.G., Smythe, J. and Peden-Adams, M.M. 2003. Immunological function in mice exposed to JP-8 jet fuel in utero. *Toxicological Sciences.* 76: 347-356.

Kersherwani, V. and Sodhi, A. 2007. Differential activation of macrophages *in vitro* by lectin Concanavalin A, phytohemagglutinin and wheat germ agglutinin: Production and regulation of nitric oxide. *Nitric Oxide.* 16: 294-305.

Kim, S.J., Choi, E.Y., Cho, Y.J., Lee, J.Y., Choi, J.I. and Choi, I.S. 2005. Surface-associated material from *Porphyromonas gingivalis* stimulates the release of nitric oxide by inducing expression of inducible nitric oxide synthase. *Microbes and Infection.* July: 1-8.

Komutarin, T., Azadi, S., Butterworth, L., Keil, D., Chitsomboon, B., Suttajit, M. and Meade, B.J. 2004. Extract of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages *in vitro* and *in vivo*. *Food and Chemical Toxicology.* 42: 649-658.

Kopp, E.B. and Medzhitov, R. 1999. The toll-receptor family and control of innate immunity. *Current Opinion in Immunology.* 11: 13-18.

Kopp, E.B. and Medzhitov, R. 2000. Recognition of microbial infection by toll-like receptors. *Current Opinion in Immunology.* 15: 396-401.

Koshland, D.E. Jr. 1992. The molecule of the year. *Science.* 18: 1861

Krutzik, A.R., Sieling, P.A. and Modin, R.L. 2001. The role of toll-like receptors in host defense against microbial infection. *Current Opinion in Immunology.* 13: 104-108.

Kurtz, J. 2004. Memory in the innate and adaptive immune system. *Microbes and Infection.* 6: 1410-1417.

Kwon, O.D., Yim, C.Y., Jeong, K.S., Jung, K.Y., McGregor, J.R., Bastian, N.R. and Samlowski, W.E. 2004. Suppression of cytokine-inducible nitric oxide synthesis during intraperitoneal Meth A tumor growth. *Journal of Veterinary Medical Science.* 66(4): 357-365.

Lane, R.S. and Quistad, G.B. 1998. Borreliacidal factor in the blood of the Western fence lizard (*Sceloporus occidentalis*). *Journal of Parasitology.* 84(1): 29-34.

Lechner, M., Lirk, P. and Rieder, J. 2005. Inducible nitric oxide synthase (iNOS) in tumor biology: The two sides of the same coin. *Seminars in Cancer Biology.* 15: 277-289.

Lee, J.K., Ryu, M.H. and Byum, J.A. 2005. Immunotoxic effect of β-chlorolactic acid on murine splenocyte and peritoneal macrophage function *in vitro*. *Toxicology.* 210: 175-187.

Leiro, J., Iglesias, R., Paramá, A., Sanmartin, M.L. and Ubeira, F.M. 2001. Respiratory burst responses of rat macrophages to microsporidian spores. *Experimental Parasitology.* 98: 1-9.

Leu, T.H., Charoenfuprasert, S., Yen, C.K., Fan, C.W. and Maa, M.C. 2006. Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNFα secretion in macrophages. *Molecular Immunology.* 43: 308-316.

Lewis, C.E. and Pollard, J.W. 2006. Distinct role of macrophages in different tumor microenvironments. *Cancer Research.* 66: 605-612.

Long, F., Wang, Y.Z., Liu, L., Zhou, J., Cui, R.Y. and Jiang, C.L. 2005. Rapid nongenomic inhibitory effects of glucocorticoids on phagocytosis and superoxide anion production by macrophages. *Steroids.* 70: 55-61.

Lopes, L., Godoy, L.M.F., De Oliveira, C.C., Gabardo, J., Schadeck, R.J.G. and De Freitas Buchi, D. 2006. Phagocytosis, endosomal/lysosomal system and other Cellular aspects of macrophage activation by canova medication. *Micron.* 37: 277-287.

Lunardi, F., Lima, J.H.C. and Assreuy, J. 2006. Comparative study of respiratory burst induced by phorbol ester and zymosan in human granulocytes. *Clinical Biochemistry.* 39: 78-85.

Maeng, O., Kim, Y.C., Shin, H.J., Lee, J.O., Huh, T.L., Kang, K., Kim, Y.S., Paik, S.G. and Lee, H. 2004. Cytosolic $NADP^+$-dependent isocitrate dehydrogenase protects macrophages from LPS-induced nitric oxide and reactive oxygen species. *Biochemical and Biophysical Research Communications.* 317: 558-564.

Magor, B.G. and Magor, K.E. 2001. Evolution of effectors and receptors of innate immunity. *Developmental and Comparative Immunology.* 25: 651-682.

McGettrick, A.F. and O'Neill, L.A.J. 2004. The expanding family of MyD88-like adaptors in toll-like receptor signal transduction. *Molecular Immunology.* 41: 577-582.

Medzhitov, R. and Biron, C.A. 2003. Innate immunity. *Current Opinion in Immunology.* 15: 2-4.

Medzhitov, R. and Janeway, C.A., Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell.* 91: 295-298.

Medzhitov, R. and Janeway, C.A., Jr. 1997. Innate immunity: impact on the adaptive immune response. *Current Opinion in Immunology.* 9: 4-9.

Medzhitov, R. and Janeway, C.A., Jr. 1998. An ancient system of host defense. *Current Opinion in Immunology.* 10: 12-15.

Medzhitov, R. and Janeway, C.A., Jr. 2000. The toll receptor family and microbial recognition. *TRENDS in Microbiology.* 8: 452-456.

Mellman, I. 1988. Relationships between structure and function in the Fc receptor family. *Current Opinion in Immunology.* 1: 16-25.

Meng, Z., Shao, J. and Xiang, L. 2003. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. *Developmental and Comparative Immunology.* 27: 313-321.

Merchant, M.E., Roche, C., Elsey, R.M. and Prudhomme, J. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippienis). Comparative Biochemistry and Physiology Part B.* 136: 505-513.

Merchant, M.E., Roche, C., Elsey, R.M. and Prudhomme, J. 2005. Antiviral activity of serum from the American alligator (*Alligator mississippienis*). *Antiviral Research.* 66: 35-38.

Michl, J., Ohlbaum, D.J. and Silverstein, S.C. 1976. 2-Deoxyglucose selectively inhibits Fc and compliment receptor-mediated phagocytosis in mouse peritoneal macrophages. II. Dissociation of the inhibitory effects of 2-deoxyglucose on phagocytosis and ATP generation. *The Journal of Experimental Medicine.* 144: 1484-1493.

Michl, J., Ohlbaum, D.J. and Silverstein, S.C. 1976. 2-Deoxyglucose selectively inhibits Fc and compliment receptor-mediated phagocytosis in mouse peritoneal macrophages. I. Description of the inhibitory effect. *The Journal of Experimental Medicine.* 144: 1465-1483.

Miliukkiene, V., Biziuleviciene, G., Pilinkiene, A. 2003. Quantitative Evaluation of Macrophage Phagocytosing Capacity by a Fluorometric assay. *Acta Biologica Hungarica.* 54: 347-355

Miller, R.A. and Britigan, B.E. 1995. The formation and biological significance of phagocyte-derived oxidants. *Journal of Investigative Medicine.* 43: 39-49.

Miller, Glenn A., and Morahan, Page S. Use of nonspecific esterase stain. *Methods for Studying Mononuclear Phagocytes.* Dolph O. Adams, Paul J. Edelson and Hillel Koren. New York, N.Y., Academic Press Inc., 1981. 367-374.

Mitchell, M.A. and Shane, S.M. 2001. *Salmonella* in reptiles. *Seminars in Avian and Exotic Pet Medicine.* 10: 25-35.

Mitchell, M.A. and Shane S.M. 2000. Preliminary findings of Salmonella spp. in captive green iguanas (*Iguana iguana*) and their environment. *Preventive Veterinary Medicine.* 45: 297-304.

Moen, Frank Emil, and Svensen, Erling. Marine fish &invertebrates of Northern Europe. Norway. KOM, 2004. 46.

Moeslinger, T., Friedl, R. and Spieckermann, P.G. 2006. Inhibition of inducible nitric oxide synthesis by azathioprine in a macrophage cell line. *Life Sciences.* 79: 374-381.

Mondal, S. and Rai, U. 2001. *In vitro* effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, *Hemidactylus flaviviridis*. *Comparative Biochemistry and Physiology Part A.* 129: 391-398.

Mondal, S. and Rai, U. 2002. Dose and time-related *in vitro* effects of glucocorticoid on phagocytosis and nitrite release by splenic macrophages of wall lizard, *Hemidactylus flaviviridis*. *Comparative Biochemistry and Physiology Part C.* 132: 461-470.

Mosser, D.M. 2003. The many faces of macrophage activation. *Journal of Leukocyte Biology.* 73: 209-212.

Muñoz, F.J. and De la Fuente, M. 2003. *In vitro* proliferation of blood, spleen and thymus leukocytes from the turtle *Mauremys caspica*. *Comparative Biochemistry and Physiology Part C.* 134: 303-309.

Murakami, A., Nakashima, M., Koshiba, T., Maoka, T., Nishino, H., Yano, M., Sumida, T., Kim, O.K., Koshimizu, K. and Ohigashi, H. 2000. Modifying effects of carotenoids on superoxide and nitric oxide generation from stimulated leukocytes. *Cancer Letters.* 149: 115-123.

Nagy, G., Clark, J.M., Buzás, E.I., Gorman, C.L. and Cope, A.P. 2007. Nitric oxide, chronic inflammation and autoimmunity. *Immunology Letters.* April: 1-5.

Nathan, C., Nogueira, N., Juangbhanich, C., Ellis, J. and Cohn, Z. 1979. Activation of macrophages *in vivo* and *in vitro*: Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. *Journal of Experimental Medicine.* 149: 1056-1068.

Nathan, C.F. and Root, R.K. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. *The Journal of Experimental Medicine.* 146: 1648-1662.

Nathan, C. and Shiloh, M.U. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Science.* 97: 8841-8848.

National Human Genome Research Institute. 13, March, 2007. National Human Genome Research Institute. 13, March, 2007. http://www.genome.gov/17015353.

Nunoshiba, T., DeRojas-Walker, T., Wishnok, J.S., Tannenbaum, S.R. and Demple, B. 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proceedings of the National Academy of Science.* 90: 9993-9997.

Olivier, V., Rivière, C., Hindié, M., Duval, J.-L., Bomila-Koradjim, G. and Nagel, M.-D. 2003. Uptake of polystyrene beads bearing functional groups by macrophages and fibroblasts. *Colloids and Surfaces B: Biointerfaces.* August. 1-12.

O'Neill, Luke A.J. 2006. How toll-like receptors signal: What we know and what we don't know. *Current Opinion in Immunology.* 18: 3-9.

Ortega, E., Algarra, I., Serrano, M.J., Alvarez de Cienfuegos, G. and Gaforio, J.J. 2001. The use of 7-amino-aetinomycin D in the analysis of *Candida albicans* phagocytosis and opsonization. *Journal of Immunological Methods.* 253: 189-193

Pabst, M.J. and Johnston, R.B. Jr. 1980. Increased production of superoxide anion by macrophages exposed *in vitro* to muramyl dipeptide or lipopolysaccharide. *Journal of Experimental Medicine.* 151: 101-114.

Pasmans, F., De Herdt, P. and Haesebrouck, F. 2001. Induction of the respiratory burst in turtle peritoneal macrophages by *Salmonella muenchen*. *Developmental and Comparative Immunology.* 25: 159-168.

Pasmans, F., De Herdt, P. and Haesebrouck, F. 2002. Interactions of *Salmonella enterica* serovar Muenchen with macrophages of the turtle *Trachemys scripta scripta*. *Developmental and Comparative Immunology.* 26: 295-304.

Pasmans, F., Martel, A., Boyen, F., Vandekerchove, D., Wybo, I., Immerseel, F.V., Heyndrickx, M., Collard, J.M., Ducatelle, R. and Haesebrouck, F. 2005. Characterization of *Salmonella* isolates from captive lizards. *Veterinary Microbiology.* 110: 285-291.

Paul, William E. Fundamental Immunology. New York, New York. Raven Press, 1984.

Płytycz, B. and J□zkowicz A. 1994. Differential effects of temperature on macrophages of ectothermic vertebrates. *Journal of Leukocyte Biology.* 56: 729-731.

Płytycz, B., R□żanowska, M., and Seljelid, R. 1992. Quantification of Neutral Red Pinocytosis by Small Numbers of Adherent Cells: Comparative Studies. *Folia Biologica (Krak□w).* 40:1-2

Płytycz, B. and Seljelid, R. 1996. Evolution of phagocyte-and lymphocyte-mediated immunity. *Central-European Journal of immunology.* 21:3-11

Polonio, R.E., Wolke, R.E., MacLean, S.A. and Sperry, J.F. 2000. An *in vitro* assay to measure phagocytosis in striped bass hybrids (*Morone saxatilis* x *Morone chrysops*). *Fish & Shellfish Immunology.* 10: 405-418.

Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Experimental Cell Research.* 46: 19-28.

Rabinovitch, M. 1995. Professional and non-professional phagocytes: an introduction. *Trends in Cell Biology.* 5: 85-87.

Rappolee, D.A. and Werb, Z. 1989. Macrophage secretions: A functional perspective. *Bulletin de L'Institut Pasteur.* 87: 361-394.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E. and Aderem, A. 2005. The evolution of vertebrate toll-like receptors. *Proceedings of the National Academy of Science.* 102: 9577-9582.

Roberts, Vernon. The Macrophage. Great Britain. Cambridge University Press, 1972.

Rossi, F., Blanca, V.D. and De Togni, P. 1985. Mechanisms and functions of the oxygen radicals producing respiration of phagocytes. *Comparative Immunology, Microbiology and Infectious Diseases.* 8: 187-204.

Rouzer, C.A., Scott, W.A., Griffith, O.W., Hamill, A.L. and Cohn, Z.A. 1982. Glutathione metabolism in resting and phagocytizing peritoneal macrophages. *The Journal of Biological Chemistry.* 257: 2002-2008.

Saad, A.H. 1989. Sex-associated differences in the mitogenic responsiveness of snake blood lymphocytes. *Developmental and Comparative Immunology.* 13: 225-229.

Saad, A.H. and Plytycz, B. 1994. Hormonal and nervous regulation of amphibian and reptilian immunity. *Folia Biologica.* 42: 3-4.

Saad, A.H. and El Ridi, R. 1988. Endogenous corticosteroids mediate seasonal cyclic changes in immunity of lizards. *Immunobiology.* Sep. 177(4-5): 390-403.

Saad, A.H., and El Ridi, R. 1988. Primary *in vitro* stimulation of antibody production by lizard splenocytes. *Veterinary Immunology and Immunopathology.* 18: 369-377.

Saad, A.H., El Ridi, R., El Deeb, S. and Soliman, M.A. 1986. Effect of hydrocortisone on immune system of the lizard, *Chalcides ocellatus*. III. Effect on cellular and humoral immune responses. *Developmental and Comparative Immunobiology.* Spring 10(2): 235-45.

Saad, A.E.H., El Ridi, R., Zada, S. and Badir, N. 1983. Differential effect of hydrocortisone on T- and B cells of the lizard, *Chalcides ocellatus*. *Developmental and Comparative Immunology.* 7: 769-770.

Saad, A.H., El Ridi, R., Zada, S. and Badir, N. 1984. Effect of hydrocortisone on immune system of the lizard, *Chalcides ocellatus*. II Response of lymphoid tissue and cells to *in vivo* and *in vitro* hydrocortisone. *Developmental and Comparative Immunology.* Vol. 8: 121-130.

Salman, H., Bergman, M., Bessler, H., Alexandrova, S. and Djaldetti, M. 2000. Ultrastructure and phagocytic activity of rat peritoneal macrophages exposed to low temperatures *in vitro*. *Cryobiology.* 41: 66-71.

Sasada, M. and Johnston, R.B., Jr. 1980. Macrophage microbicidal activity: Correlation between phaygocytosis-associated oxidative metabolism and the killing of candida by macrophages. *Journal of Experimental Medicine.* 152: 85-98.

Sasada, M., Pabst, M.J. and Johnston, R.B., Jr. 1983. Activation of mouse peritoneal macrophages by lipopolysaccharide alters the kinetic parameters of the superoxide-producing NADPH oxidase. *The Journal of Biological Chemistry.* 258: 9631-9635.

Shepard, V.L. 1986. The role of the respiratory burst of phagocytes in host defense. *Seminars in Respiratory Infections.* 1: 99-106.

Shepherd, V.L., Lee, Y.C., Schlesinger, P.H. and Stahl, P.D. 1981. L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages. *Proceedings of the National Academy of Science.* 78: 1019-1022.

Silverstein, A.M. 1979. Cellular versus humoral immunity: determinants and Consequences of an Epic 19th century battle. *Cellular Immunology.* 48: 208-221.

Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. 1977. Endocytosis. *Annual Reviews of Biochemestry.* 46: 669-722.

Silverstein, S.C. 1995. Phagocytosis of microbes: insights and prospects. *Trends in Cell Biology.* 5: 141-142.

Sompayrac, Lauren. How the Immune System Works. Malden, Massachusetts. Blackwell Science, Inc. 1999.

Son, C.G., Shin, J.W., Cho, J.H., Cho, C.K., Yun, C.H., Chung, W. and Han, S.H. 2006. Macrophage activation and nitric oxide production by water soluble components of *Hericium erinaceum. International Immunopharmacology.* 6: 1363-1369.

Stix, G. 2001. Saying yes to NO. *Scientific American.* Nov. 34.

Stewart, C.C., Lin, H.S. and Adles, C. 1975. Proliferation and colony-forming ability peritoneal exudates cells in liquid culture. *The Journal of Experimental Medicine.* 141: 1114-1132.

Sung, S.S.J., Nelson, R.S. and Silverstein, S.C. 1983. The role of the mannose/N-acetylglucosamine receptor in the pinocytosis of horseradish peroxidase by mouse peritoneal macrophages. *Journal of Cellular Physiology.* 116: 21-25.

Sveinbjørnsson, B., Olsen R., Seternes, O.M. and Seljelid, R. 1996. Macrophage cytotoxicity against murine Meth A Sarcoma involves nitric oxide-mediated apoptosis. *Biochemical and Biophysical Research Communications.* 223: 643-649

Swanson, J.A. and Baer, S.C. 1995. Phagocytosis by zippers and triggers. *Trends in Cell Biology.* 5: 89-93.

Talent, L.G., Dumont, J.N., Bantle, J.A., Janz, D.M., and Talent, S.G. 2002. Evaluation of western fence lizards (S*celoporus occidentalis*) and eastern fence lizards (*Sceloporus undulatus*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 21: 899-905.

Underhill, D.M. and Ozinsky, A. 2002. Phagocytosis of microbes: Complexity in action. *Annual Reviews of Immunology.* 20: 825-852.

Van Furth, R. Mononuclear Phagocytes: Characteristics, Physiology and Function. Boston, Massachusetts. Martinus Nijhoff Publishers, 1985.

Van Furth, R. 1985. Monocyte production during inflammation. *Comparative Immunology Microbiology, and Infectious Diseases.* 8: 205-211.

Vasquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. and Fang, F.C. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. *Journal of Experimental Medicine.* 192: 227-236.

Walenkamp, A.M.E., Scharringa, J., Schramel, F.M.N.H., Coenjaerts, F.E.J. and Hoepelman, I.M. 2000. Quantitative analysis of phagocytosis of *Cryptococcus neoformans* by adherent phagocytic cells by fluorescence multi-well plate reader. *Journal of Microbiological Methods.* 40: 39-45.

Wan, C.P., Myung, E. and Lau, B.H.S. 1993. An automated micro-fluorometric assay for monitoring oxidative burst activity of phagocytes. *Journal of Immunological Methods.* 159: 131-138.

Wan, C.P., Park, C.S. and Lau, B.H.S. 1993. A rapid and simple microfluorometric phagocytosis assay. *Journal of Immunological Methods.* 162: 1-7.

Wang, Yang and Marsden, Philip A. "Nitric Oxide Synthases: Gene Structure and Regulation." *Nitric Oxide: Biochemistry, Molecular Biology, And Theraputic Implications.* Louis Ignarro and Ferid Murad. San Diego, California. Academic Press, 1995. 71-90.

Watanabe, N., Miura, S., Zeki, S. and Ishii, H. 2001. Hepatocellular oxidative DNA injury induced by macrophage-derived nitric oxide. *Free Radical Biology & Medicine.* 30: 1019-1028.

Weeks, B.A., Keisler, A.S., Myrvik, Q.N. and Warinner, J.E. 1987. Differential uptake of neutral red by macrophages from three species of estuarine fish. D*evelopmental and Comparative Immunology.* 11: 117-124.

Wilkinson, P.C. "Cellular and Molecular Aspects of Chemotaxis of Macrophages and Monocytes." *Immunobiology of the Macrophage.* Ed. David S. Nelson. New York, New York. Academic Press, 1976. 349-365.

Wilson, C.B., Tsai, V. and Remington, J.S. 1980. Failure to trigger the oxidative metabolic burst by normal macrophages: Possible mechanism for survival of intracellular pathogens. *Journal of Experimental Medicine.* 151: 328-346.

Young, M.R. and Newby, M. 1986. Enhancement of Lewis Lung Carcinoma cell migration by Prostaglandin E_2 produced by macrophages. *Cancer Research.* 46: 160-164.

Yun, C.H., Jung, U., Son, C.G., Ju, H.R. and Han, S.H. 2006. 3-Amino-1,4-dimethyl -5H-pyrido[4,3-b] indole (Trp-P-1), a food-born carcinogenic heterocyclic amine, promotes nitric oxide production in murine macrophages. *Toxicology Letters.* 161: 18-26.

Zapata, A.G., Varas, A. and Torroba, M. 1992. Seasonal variations in the immune system of lower vertebrates. *Immunology Today.* 13: 142-147.

Zhang, X. and Morrison, D.C. 1993. Lipopolysaccharide-induced selective priming effects on tumor necrosis factor α and nitric oxide production in mouse peritoneal Macrophages. *Journal of Experimental Medicine.* 177: 511-516.

VITA

DAVID CARL MELSON

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHARACTERIZATION OF THE *IN VITRO IMMUNE* FUNCTION OF REPTILE MACROPHAGES FROM *SCELOPORUS OCCIDENTALIS*

Major Field: Microbiology / Cell and Molecular Biology

Biographical:

Personal Data: Born in Roanoke, Virginia on May 11, 1965, to Isaac and Mary Melson

Education: Graduated from Broken Bow High School in Broken Bow, Oklahoma, in May, 1983; received Associate of Arts Degree in General Education as an honor graduate from Eastern Oklahoma State College in May 1998; received Bachelor of Science Degree in Microbiology from Oklahoma State University in May, 2002; completed the requirements for the Doctor of Philosophy in Microbiology, Cell and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma, in July, 2008.

Experience: Adjunct Faculty Instructor, Department of Biology, Northeastern Oklahoma A & M, June 2008 - July 2008; Instructor, College of Arts and Sciences, Oklahoma State University, August 2007 - December 2007; Graduate Teaching Associate, Department of Microbiology and Molecular Genetics, Oklahoma State University, August 2002 - December 2005, August 2006 - December 2006.

Name: David Carl Melson Date of Degree: July, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHARACTERIZATION OF THE *IN VITRO* IMMUNE FUNCTION OF REPTILE MACROPHAGES FROM *SCELOPORUS OCCIDENTALIS*

Pages in Study: 106 Candidate for the Degree of Doctor of Philosophy

Major Field: Microbiology

Scope and Method of Study: *In vitro* study of cellular immune function

Findings and Conclusions: The project yielded important data for the establishment of a reptile model of study for innate immune functions. The areas of respiratory burst, endocytosis and chemotaxis were explored, as well as model-specific investigations of hibernation recovery and crowding. Phagocytic activity, superoxide production and acid phosphatase production were all quantifiable and exceeded preliminary expectations. Assays yielding poorer results have been addressed and explained to a reasonable level. It is considered that a larger model might provide a better yield for the *in vitro* work described, but is also noted that this would lead to other problems (housing and expense). The tissue origin and the activation state of the macrophage cells might have negatively impacted some assays and this would also likely be alleviated by a larger reptile model.

ADVISER'S APPROVAL: D. Kim Burnham