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Mode of action and characterization of a novel biological response modifier isolated from fractionated caprine serum

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MODE OF ACTION AND CHARACTERIZATION OF A NOVEL BIOLOGICAL RESPONSE MODIFIER ISOLATED FROM FRACTIONATED CAPRINE SERUM

By

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MODE OF ACTION AND CHARACTERIZATION OF A NOVEL BIOLOGICAL RESPONSE MODIFIER ISOLATED FROM FRACTIONATED CAPRINE SERUM

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Immune Cell Potentiating Factor (ICPF) represents a class of biological response modifiers initially only found within active caprine serum fractions. Controlled studies have since demonstrated active ICPF derived from several non-caprine mammalian sources; including equine and human. ICPF is able to increase survivability in murine gram negative induced sepsis (60%) as well as secondary infection and subsequent sepsis in canines infected within canine parvo virus 2 (36%) despite showing no innate antiviral properties. ICPF is able to initiate systemic proteomic changes within several organ systems; including serum, liver, brain, lung, and spleen. ICPF initiated an early acute phase response, specifically through the increased expression of serum amyloid A, with systemic serum levels increasing from 1.5 μg/mL to 403.0 μg/mL within 24 hours and increased to 3,400 μg/mL within 48 hours following ICPF administration. Evaluation of cytokine expression following ICPF treatment revealed the up-regulation of IL-6, INF-γ, and the chemokine CXCL1\KC *in vivo* as well as the expression of IL-6 and IFN-γ *in vitro* within 3 hours of treatment. Development of an *in vitro* bioassay through the expression of IL-6 and IFN-γ within whole blood and peripheral blood mononuclear cells

will allow for further elucidation and testing of ICPF outside of an animal host. The early expression of proinflammatory cytokines and chemokines, an acute phase response including serum amyloid A, and ICPF's inability to alleviate mortality in a lipopolysaccharide animal mortality model strongly indicates an active role for ICPF as an immune regulatory peptide capable of promoting an early inflammatory response to *Salmonella enterica* serovar *Typhimurium* thereby reducing the risk and mortality associated with sepsis.

DEDICATION

This manuscript is dedicated to my mother, Laraine Matyi. From birth to this day, you strive to teach me the importance of hard word, dedication, and tenacity. It was you I called when I was frustrated and discouraged, and it was your who told me to hang on, and that I could do it. You are the embodiment of strength and perseverance, and are both a friend and a mother. Thank you for all you do, not all the lessons stuck, but I got lucky with this one.

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CHAPTER I

LITERATURE REVIEW

Biological Response Modifiers (BRMs) represent a relatively new and untapped class of therapeutic compounds which play a fundamental role in the emerging field of immunotherapy. Traditional therapeutic approaches within the pharmaceutical industry and academia have resulted in specific medicines to serve as treatments to many human and veterinary ailments, but now, with an ever increasing understanding of the complexities and power of the mammalian and avian immune systems, we are closer than ever to being able to modulate subtle attributes of the immune system to abate many pathologies.

While the earliest forms of immunotherapy can be traced back over 70 years to the use of immunoglobulins for the treatment of pneumococcal infections (Donta 2002), it was not until recently that our understanding of the immune system was sufficient to begin truly harnessing its power. The main constituents of immunotherapy include cytokines, chemokines, mono/polyclonal antibodies, immune modulating proteins and peptides, immune stimulating endotoxins (lipids and carbohydrates), and synthetic compounds capable of modulating subtle functions and cell types, such as directing T-cell maturation and phenotypes (Bruns, Meinken et al. 2009). With the advent and successful implementation of interleukin and interferon immunotherapies in oncology, attention and support has once again been redirected toward thwarting viral and bacterial pathologies.

Immunotherapies are of particular interest because they proffer the ability to directly harness the immense power of the immune system, as well as the ability to down modulate an immune response, which if persistent can become detrimental - as is the case with autoimmune deficiencies and sepsis. Instead of developing a drug to combat a specific pathogen or symptom, immunotherapy promises to modulate our current immune system so that it may better address and alleviate the pathology, thereby reducing or eliminating a pathogen's ability to circumvent treatment.

The mammalian immune system can be loosely separated into two distinct entities; the innate and adaptive immune systems. The adaptive immune response is critical to allow an organism to adapt and mount faster and more efficient immune responses to pathogens it has encountered in the past and is therefore likely to encounter again. The induction of an adaptive immune response to a previously un-encountered pathogen is a time consuming process which is tightly regulated by both positive and negative regulatory checkpoints to prevent detrimental autoimmunity. To protect an organism from novel pathogens, the innate immune system is responsible for holding pathogens in check, mounting a frontline defense, recruiting additional cell types, processing pathogens, and presenting antigenic (immunogentic) proteins, lipids and carbohydrates to the cells of the adaptive system.

The innate immune system utilizes two distinct strategies for the detection of pathogens; recognition of missing self, and recognition of microbial non-self (Raulet 2006; Kawai and Akira 2009). Recognition of microbial non-self is usually accomplished by a cell's ability to recognize specific molecular patterns which are well conserved and fundamentally important to the pathogen, yet are not produced or used by the host. Dr. Charles Janeway first proposed and defended the theory that germline

encoded receptors (Janeway) were able to recognize and respond to common pathogen motifs in the late 1980's, a theory which has been shown to be accurate and initiated our current understanding of innate immunology. These Pathogen Associated Molecular Patterns (PAMPS) are recognized by Pattern Recognition Receptors (PRR) on cells, primarily of the innate immune system. PRR are broken into three functional categories; receptors which detect and signal the presence of microbial pathogens and initiate an inflammatory response, soluble secreted PRR which assist in the activation of the compliment immune cascade, opsonization, and serve as coreceptors for other membrane bound PRR, and phagocytic PRR which initiate phagocytosis of the PAMP and possible antigen processing (Medzhitov 2009).

Of particular importance in bridging the gap between adaptive and innate immunity are the Toll-Like Receptors (TLR). These PRR are responsible for detection and signaling for the presence of microbial pathogens and initiating a pro-inflammatory response. PRR are able to recognize and respond to a wide variety of PAMPs such as extracellular type 1 transmembrane, intracellular endosomal (Kuroishi, Tanaka et al. 2007), and recently discovered mammalian soluble cytosolic and secreted TLR (Rad, Ballhorn et al. 2009). Cellular TLR location can often be reflective of its immunological function (Table 1).

Toll-like receptors are able to recognize and bind vastly different PAMPS, including nucleic acids, protein, lipo and glycoproteins, fatty acids, and carbohydrates. These ligands are structurally diverse and unrelated, yet via a mechanism that is not yet fully understood, TLR are able to recognize and bind them with remarkable fidelity. TLRs are comprised of three domain regions, consisting of a leucine rich repeat region, a trans-membrane region, and a Toll/IL-1R domain. The Toll/IL-1R domain is exposed

within the cytosol and interacts with adaptor proteins, resulting in the recruitment of IL-1R associated kinases, and ultimately activation NF-κβ and associated proinflammatory cytokines (Lasker and Nair 2006). An important characteristic of the TLR ligand response is the use of accessory molecules such as *myD88* and lipopolysaccharide binding protein (Schnare, Barton et al. 2001) to further enhance and direct the immune response (Lien, Means et al. 2000). Upon detection, PRR allow for a fast and powerful (inflammatory) immune response which may include the activation of acute phase proteins and compliment from hepatocytes, neutrophil recruitment and influx, modification of cytokine and chemokine networks, changes in cellular morphology, surface receptor profiles, localized immune cell populations, and physiological responses such as pyrogenicity and vasodilation.

Although not perfect, the innate immune system wields awesome power, and even short delays in one aspect of the innate immune response can lead to death from an otherwise innocuous pathogenic infection (Yang, Dorner et al. 2002). Many autoimmune diseases such as rheumatoid arthritis (Vander Cruyssen, Peene et al. 2005), multiple sclerosis (Derfuss, Parikh et al. 2009), sepsis, and septic shock (Nasraway 2003) are the result of poor or mis-regulation and control of the innate/inflammatory system. Modulation of the innate immune system, specifically via TLR and the junction to acquired immunity are of renewed interest for pharmaceutical companies in their development of new immunotherapies. At the time of writing, TLR agonists and antagonists are in development at ten major pharmaceutical companies, with intended uses ranging from next generation vaccine adjuvants to modulators of viral, cancer, auto-immunity, and infectious diseases (Cook, Pisetsky et al. 2004; Hoffman, Smith et al. 2005).

Table 1 Human and mouse Toll Like Receptors

Human and Mouse	Cell Subset	Cellular Location	Recognized PAMPS	Source (Lit.)
TLR 1 (TLR 2 co-receptor)	Ubiquitous	Plasma Membrane	(Triacyl) Lipoproteins, Lipoteichoic acid, zymosan	(Medzhitov 2009; Rad, Ballhorn et al. 2009; Venugopal, Nutman et al. 2009)
TLR 2	Macrophages, dendritic cells, B-cells, epithelial	Plasma Membrane	Peptidoglycans, glycoproteins, Lipoproteins, Lipoteichoic acid, zymosan	(Medzhitov 2009; Rad, Ballhorn et al. 2009; Venugopal, Nutman et al. 2009)
TLR 3	Macrophages, dendritic cells, epithelial	Intracellular	dsRNA, poly (I:C)	(Alexopoulou, Holt et al. 2001; Venugopal, Nutman et al. 2009)
TLR 4	Macrophages, dendritic cells, epithelial, neutrophils, mastocytes, B-cells, endothelial, fibroblasts, muscle	Plasma Membrane	Lipopolysaccarides	(Rad, Ballhorn et al. 2009)
TLR 5	Macrophages, dendritic cells, epithelial	Intracellular	Flagellin	(Uematsu, Myoung Ho et al. 2006; Rad, Ballhorn et al. 2009)
TLR 6 (TLR 2 co-receptor)	Ubiquitous	Plasma Membrane	(Diacyl) Lipoproteins, Lipoteichoic acid, zymosan	(Medzhitov 2009; Rad, Ballhorn et al. 2009)
TLR 7	Plasmacytoid dendritic cells, B-cells	Intracellular	ssRNA	(Lund, Alexopoulou et al. 2004; Venugopal, Nutman et al. 2009)
TLR 8	Myeloid dendritic cells	Intracellular	G-Rich Oligonucleotides	(Gorden, Gorski et al. 2005; Venugopal, Nutman et al. 2009)
TLR 9	Human = Plasmacytoid dendritic cells, B-cells : Mouse = Macrophages, dendritic cells	Endosome/Lysosome	Unmethylated CpG DNA	(Venugopal, Nutman et al. 2009)
TLR 10 (mouse pseudogene)	ND*	ND	ND	(Medzhitov 2009)
TLR 11 (human pseudogene)	ND	ND	Uropathgenic bacteria, Profilin-like molecule	(Lauw, Caffrey et al. 2005; Yarovinsky, Hieny et al. 2008; Rad, Ballhorn et al. 2009)
TLR 12 (human pseudogene)	ND	ND	ND	(Medzhitov 2009)
TLR 13 (human pseudogene)	ND	ND	ND	(Medzhitov 2009)

Principle cellular subset, location, and ligands.
* Not determined

Regulation and communication among various mechanisms of the innate immune system is primarily accomplished through cytokine and chemokine signaling. Cytokines are proteins or peptides secreted from cells which operate in either an autocrine or paracrine fashion. The semantic distinction between cytokine, chemokine, hormone, and growth factor is imprecise; but generally cytokines and chemokines operate in a localized manner, have a short half life, and act locally on targeted leukocytes (Flajnik and Pasquier 2009), however temporal, proximal, and cellular exceptions do exist. Cytokine and chemokine nomenclature has also been problematic, with definitions and names which change as more functional and structural information become available. Cytokines and chemokines can be broadly broken into four families; type 1 and type II cytokines and interferons, interleukin-1, TNF-type cytokines, and chemokines. Cytokines can have a very broad range of effects on cells of the immune system; promoting cellular differentiation of hematopoietic stem cells (Dao and Nolta 2007), chemotaxis (Horuk 2001), apoptosis (Ware 2003), immune cell (de)activation, and systemic proteomic shifts, as seen in the acute phase response (Tissieres, Dunn-Siegrist et al. 2008). While many cytokines are referred to as interleukins, stemming from the initial belief that they were secreted by and targeted leukocytes, there are many *interleukins* which are released by and target somatic cells outside of the traditional immune system.

Type 1 cytokines are a family which shares a conserved structural form consisting of four α helixes in an 'up-up-down-down' topology and similar receptor homology. Knowledge that a cytokine is a member of the type 1 family of cytokines does not indicate function, but does present structural and mechanistic signal transduction information (Leonard 2009). Type II cytokines are primarily composed of interferons (IFNs) and the interleukin-10 family of cytokines which include IL-19, IL-20, IL-22, IL-

24, IL-26, IL-28, and IL-29. IFNs are further separated into type I IFNs consisting of IFN-α, β, ε, κ, ω, δ, τ all encoded from genes clustered on human chromosome 9, while the type II IFN is IFN-γ, encoded from human chromosome 12 (Honda, Takaoka et al. 2006). While IFNs are sometimes referenced of as a separate group from IL-10, they both bind to type II cytokine receptors, show strong structural homology (Zdanov 2004) and are therefore also referred to as type II cytokines.

The interleukin-1 family consists of 11 cytokines, of which four are well characterized, IL-1α, IL-1β, IL-1 receptor agonist, and IL-18. Members of the IL-1 family primarily affect the inflammatory response and are highly pleiotropic, initiating both pro and anti inflammatory cascades. The IL-1β cytokine has been shown to promote gene expression and synthesis of type 2 phospholipase A (Ashraf, Murakami et al. 1996), COX-2 (Duggan, Lindstrom et al. 2007; Ohama, Hori et al. 2007), and nitric oxide synthase (Ohama, Hori et al. 2007). Regulation of the IL-1 family via receptor agonists and monoclonal antibodies has been shown to be effective in modulating inflammatory processes associated with inflammatory based autoimmune diseases such as rheumatoid arthritis (Horai, Saijo et al. 2000; Economides, Carpenter et al. 2003), and type 2 diabetes (Larsen, Faulenbach et al. 2007).

The Tissue Necrosis Factor (TNF) family of cytokines is by far one of the largest, most diverse and intricate cytokine networks. Many functions of TNF lay outside the purview of immunology, these cytokines are nevertheless important for a host of immune responses. The main function of TNF on many immune cells is the activation of transcription factors NF-κB and/or AP-1. NF-κB is of particular importance as it functions to regulate nuclear transcription in nearly all cells undergoing an immunological response from pathogens, formation of secondary lymphoid organ

structures (Siebenlist, Brown et al. 2005) and has more recently been associated with B-cell isotype switching, T and B cell development, and cytokine production (Li and Verma 2002). The ability of the TNF family of cytokines to regulate and control so many diverse functions within the immune system is accomplished, in part, by an evolutionarily ancient and intricate network of associated proteins, soluble and membrane bound cognate receptor molecules, and downstream cell signaling checkpoints and modifications (Li and Verma 2002; Siebenlist, Brown et al. 2005). The large assortment of cross utilization of TNF and associated receptors was initially believed to be a built in redundancy (Kuprash, Alimzhanov et al. 2002), but are now viewed as complete systems with associated functions (Pfeffer 2003). Sub-pathways associated with TNF such as the NF-κβ response, can be further manipulated by the type of activation (receptor form), the cellular state and maturity during activation, and subunit diversification of the transcriptional complex, thus allowing for the modified expression of hundreds of genes, allowing for the alteration of cellular differentiation pathways and survival fates (Ware 2003).

Chemokines are a distinct set of cytokines with specific structural and functional characteristics and all share a level of sequence and morphological homology. Chemokines are defined and divided by structural characteristics and are named based on variations on the number and sequence of cysteine residues, with most having between two and four. The conserved sequence and frequency of cysteine residues results in a highly conserved tertiary structure and non-specific binding of chemokine and receptors within the same family (Horuk 2001). Chemokines are well known for their role in neutrophil recruitment and assistance in initiation of a localized inflammatory response. In addition, chemokines can also function to promote normal and autoimmune

inflammatory events (Cravens and Lipsky 2002), immune cell development (Ohl, Henning et al. 2003), lymphocyte recruitment and activation (Martinez, Gordon et al. 2006), circulatory and nervous system development (Horuk 2001), and as angiogenic and angiostatic agents (Belperio, Keane et al. 2000).

Several pathogens such as the herpes-simplex virus, poxvirus, and murine cytomegalovirus have evolved or acquired specific strategies to neutralize cytokine mediated responses, either through downstream modulation of the immune pathway or direct competitive inhibition of the chemokine to hinder the development of a pro-inflammatory response or TNF induced apoptosis (Benedict and Ware 2001). While the TNF family of cytokines seems to be a common target for pathogen evasion; interferon and other chemokine functions are also repressed (Rahman and McFadden 2006). In addition to utilizing cytokine and chemokine antagonists to evade host defense as many intracellular bacteria, some pathogens such as the Human Immune deficiency Virus (HIV) and the protozoa *Plasmodium* have exploited and incorporated these mechanisms to serve as virulence factors for their own pathogenesis (Catani, Corasaniti et al. 2000; Lusso 2006). HIV utilizes a remarkable method of chemokine exploitation, whereby it expresses its cellular receptors in a two stage method incorporating a trimeric glycoprotein and cellular co-receptors involving CD4 and the chemokine receptor CCR5 or CXCR4. This convoluted strategy allows for the 'expression' of the highly conserved co-receptor binding epitope, while preventing the host from producing neutralizing antibodies (Labrijn, Poignard et al. 2003), thereby allowing HIV entry into a cell, and masking conserved non-self regions from antigen presenting cells.

Pathogens such as HIV, poxvirus, *Plasmodium*, and *Salmonella typhimurium* have evolved strategies that utilize our cytokine and chemokine system both against us to

evade defenses and to their direct advantage in assisting in targeted cellular infection and invasion. Another associated ailment surrounding bacterial (particularly gram negative) pathogenesis is the risk of sepsis or the more severe form, septic shock. Sepsis and septic shock, collectively referred to as Systemic Inflammatory Response Syndrome (SIRS) is responsible for over 100,000 human deaths annually and a mortality rate ranging from 25 – 40% within the United States (Blackwell and Christman 1996). Some nonspecific antigen responses are due to gram positive pathogens, specifically triggered by exotoxins (Schutte, Mayer et al. 1998) such as Lipoteichoic Acid (LTA) or staphylococcal enterotoxin B binding to Major Histocompatibility Complexes (MHC) resulting in a proinflammatory cytokine cascade initiated by T_h cells (Weiser and Nahm 2009). The majority of SIRS induced mortality is due to an autoinflammatory response initiated from a gram negative bacterial infection, usually initiated by the release and binding of lipopolysaccarides (LPS) to TLR-4, resulting in macrophage activation and proinflammatory cytokine release. TNF and IL-1 are two such cytokines implicated in SIRS, and systemic release, even controlled in response to an infection or endotoxin, can result in a type of positive feedback loop, resulting in acute inflammation throughout the body and lethal physiological changes in blood flow and circulation. Endotoxins can be released in great quantity and concentrations following a successful immune response to a pathogen resulting in sepsis or septic shock. Treatment options for sepsis and septic shock are limited; antibiotics and/or antimycotics are used to help curb the underlying infection, while supportive care is typically administered to stave off death. Considering the root causes and regulatory components of sepsis, immunotherapy would appear to be an excellent therapeutic remedy for this autoimmune pathology, however most attempts at utilizing BRMs have resulted in poor therapeutic outcomes.

Most attempts at alleviating sepsis or septic shock via immunotherapy have been centered on inhibiting various forms of TNF or IL-1, usually via monoclonal antibodies, soluble receptors, or as in the case with IL-1, a naturally occurring receptor antagonist which act as competitive inhibitors to both natural forms of IL-1, α and β (Dinarello 1994). Other attempts at immunotherapy have included antagonists to platelet activating factor, a potent mediator of leukocyte activation and platelet aggregation, bradykinin antagonists, and cyclooxygenase inhibitors (Remick 2003). While many of these BRMs have shown promise in pre-clinical trials, little success has been reported in phase II or III clinical trials, including the much anticipated TNF and IL-1 antagonists. Problems utilizing TNF and IL-1 theraputically include ambiguity surrounding dosage and concentrations, proper timing of administration to interfere with the autoimmune process, improper models and definitions of septic patients within the study, and a general lack of understanding as to the precise cause and role the immune system plays within sepsis and septic shock. One notable exception to the overall failure of BRMs and immunotherapy in the treatment of sepsis and septic shock is the administration of the serine protease Activated Protein C, a vitamin K dependant anticoagulant (Warren, Suffredini et al. 2002), however, like other immunotherapies, more work is needed to optimize timing, dosage, and its use for the treatment of sepsis.

Activated Protein C's role in inflammation, sepsis, and cytokine mediated inflammation is a prime example as to the difficulties of modulating the intricate system of cytokine pathways. In the classical route of sepsis, activated macrophages (either via classical or alternative activation (Gordon 2009)) release TNF, IL-6, IL-8, platelet activating factor and other proinflammatory mediators; contributing to the sepsis cascade (Parrillo 2005). IL-1, IL-6, and TNF are also involved in the increased activation and

release of Thromboplastin Tissue Factor (TTF) which is responsible for activation of the extrinsic and intrinsic coagulation pathway and increases thrombin concentrations. Thrombin's role in inflammation includes promoting further expression of proinflammatory cytokines, increased chemokine expression and neutrophil recruitment, and mast cell degranulation, all contributing to sepsis induced multi-organ failure, a leading cause of sepsis mortality in humans. Activated Protein C is believed to protect individuals in late stage sepsis and septic shock by indirectly decreasing thrombin production via inactivation of coagulation factors, down regulating TTF, and promotion of Tissue Factor Pathway Inhibitor (Ely, Kleinpell et al. 2003; Short 2004). With proper dose and temporal considerations it should be possible to treat sepsis with BRMs at an earlier stage to prevent multi-organ failure and death.

A well studied and characterized model organism for an intracellular bacterial infection, human typhoid fever, and gram negative induced sepsis via intraperitoneal injection, is *Salmonella enterica*. *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*) is a common enteropathogenic (Stecher, Robbiani et al. 2007) strain which is extremely virulent in murine models (Bjorkman, Hughes et al. 1998) and is sometimes responsible for Salmonellosis in humans. Salmonellosis infects ~1.5 million Americans annually, 15,000 of which develop severe infections requiring hospitalization. Transmission can occur from zoonotic transmission, contaminated food is the typical vector. Salmonellosis is generally easily treated with antibiotics such as fluoroquinolones and cephalosporins however, increased use of antimicrobials in food animals, livestock, and companion animals are believed to have increased the natural rate and development of specific drug resistant strains (Angulo, Johnson et al. 2000; Wright, Tengelsen et al. 2005). *S. typhimurium*, like many intracellular pathogens, express virulence factors

which enable them to circumvent or inactivate host defense mechanisms, allowing them to remain either impervious or unknown to the host organism. *S. typhimurium* normally enters the body through ingestion and therefore must transverse epithelial cells of the intestinal wall before systemic infection can proceed (Jones, Ghori et al. 1994). This is accomplished by infecting microfold cells which function as a type of APC of the intestinal lumen, transporting ingested antigens across the follicle-associated epithelium to an underlying T or B-lymphocyte, macrophage, or dendritic cell (Vazquez-Torres and Fang 2000). Upon epithelial contact, *S. typhimurium* causes the cell membrane to 'wrinkle' via an actin polymerization reaction which in conjunction with a multi-component virulence complex known as Type III secretion system, the bacteria is able to gain entry into the epithelial cell. *S. typhimurium* ultimately resides and replicates from within the vacuoles of macrophages (Pamer 2009).

Innate immune defense against *S. typhimurium* include PRR's such as TLR's which can recognize a wide range of specific antigens associated with the bacteria and are located on the cellular surface membrane and within macrophage vacuole compartments where the bacteria resides. PAMP's associated with *S. typhimurium* include LPS, flagellin, non-methylated CpG DNA motifs by TLR-4, 5, and 9, respectively. However, the role of TLR-5 in the pathogenesis of *S. typhimurium* is still debated, as TLR-5 deficient mice seem to offer increased resistance to the pathogen, presumably from reduced migration from the lamina propria (Uematsu, Myoung Ho et al. 2006; Pamer 2009). Further innate recognition of *S. typhimurium* occurs within the cytoplasm and is accomplished with the aid of Nod family of proteins, specifically Nod2. The Nod family of proteins represents an intracellular pathogen sensing system which operate independently of TLR's and appear to operate similarly to R-Proteins, found in

plants. Both Nod1 and Nod2 belong to a family of proteins known as the nucleotide binding domain-leucine rich repeats, which are able to recognize, and bind to small peptidoglycan fragments found within the cytosol. In contrast to Nod1, Nod2 is able to detect both gram positive and negative intracellular bacteria due to a smaller peptidoglycan binding motif which does not differentiate between the two. Upon recognition and binding, Nod2 can induce NFκβ signaling through a serine/threonine kinase known as RIP2 (Inohara, Koseki et al. 2000; Philpott and Girardin 2004).

Cytokines, specifically IFN-γ play an essential role in an organism's defense against intracellular bacterial pathogens, as individuals with mutations effecting IFN-γ, IFN-γ receptors, IL-12p40, a subunit shared by IL-12 and IL-23, and STAT1, which are all part of the IFN-γ signaling cascade, all share increased susceptibility or mortality to many intracellular bacterial infections, like *S. typhimurium.* IFN-γ seems to be instrumental in promoting survivability by inducing expression of nitric oxide synthase, the enzyme responsible for the production of nitric oxide (Courtney and Margo 2004). IL-12p30, a subunit common in IL-12 but not IL-23, revealed IL-12's role in Th1 CD T-cell mediated immunity (Courtney and Margo 2004; Pamer 2009) in *S. typhimurium* infection.

Control of systemic *S. typhimurium* is aided by the acute phase inflammatory response. The primary role of the acute phase response is the restoration of homeostasis during or after a challenge by tissue injury, infection, or trauma. The acute phase response can include, but is not limited to; fever, neutrophilia, hypoferraemia, increased gluconeogenesis and muscle protein catabolism, hormonal changes, and induction of acute phase proteins (Han 1997). Acute Phase Proteins (APP) are typically hepatoproteins, synthesized in the liver, and can increase serum protein concentrations by

several orders of magnitude. Cytokines serve as mediators of the acute phase response, specifically in the stimulation and regulation of acute phase proteins. APP are divided into two classes; class I and class II acute phase proteins. Class I APP are induced by the interleukin-1 family of cytokines, in conjunction with the IL-6 family of cytokines. Class I APP include Serum Amyloid A (SAA), C-Reactive Protein (CRP), Compliment C3 (C3) and murine haptoglobin. Class II APP are induced by IL-6 like cytokines only, and include fibrinogen, human (non-murine) haptoglobin, and α1-antichymotrypsin. In addition to being stimulated by IL-6 cytokines, some type II APP such as fibrinogen may also be inhibited by the presence of some IL-1 cytokines, and studies have correlated the expression of IL-1 receptor antagonist and IL-6 in some acute phase reactions, however, similar studies have shown that IL-1 receptor antagonist does not inhibit the production of some type I APP *in vivo*, such as CRP or SAA (Raynes, Eagling et al. 1991; Gabay, Genin et al. 1995; Han 1997).

APP are essential to an organisms response to microbial infections, and have been implicated in several discrete functions. APP such as lipopolysaccharide binding protein (LBP) and soluble CD14 can serve as cofactors for the activation of immune cells through membrane bound receptors, such as TLR. SAA, LBP, compliment C3, myeloid differentiation factor-2 and mannose binding lectins can act as direct bacterial opsonins (Jack, Klein et al. 2001; Shah, Hari-Dass et al. 2006; Tissieres, Dunn-Siegrist et al. 2008) and other APP such as bactericidal permeability increasing protein (BPI) and compliment C3 can directly attack and destroy many pathogens. The systemic increase in soluble APP is also believed to play a role in sepsis, as soluble CD14 and high density lipoproteins like SAA may act as a sink for endotoxins such as LPS (Viriyakosol, Tobias et al. 2001) during sepsis.

Immune Cell Potentiating Factor (ICPF) is a naturally occurring tri-glycosylated peptide first isolated by equilibrium dialysis with molecular porous membrane tubing with a molecular weight cut off range of 6-8 kDa from caprine serum (Parker, Willeford et al. 2005). The preliminary structure of ICPF, a 9 amino acid core consisting of RSVSLSYRF with 3 conjugated fatty acid side chains of stearate, arachidate, arachidonate was determined after first being purified to ≥98% from caprine serum using combined dialysis, ultra-filtration, and Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC). Structural characteristics (Figure 1) were determined using combined Edman degradation, standard protease digestions, anion exchange/reverse phase HPLC, matrix assisted laser desporption/ionization-mass spectrometry and electrospray ionization-mass spectrometry (Ansley and Willeford 2000; Thacker, Fuhrer et al. 2004).

Clinical and experimental observations with ICPF isolated from caprine serum show that it contains one or more components that have an immune modulating effect on various mammalian and avian organisms. This therapeutic BRM reduced the mortality evident in murine and avian typhoid, sepsis and cholera models by upwards of 80% when the subjects were challenged with an otherwise terminal dose of the gram negative bacteria (*Salmonella enterica* (serovar *typhimurium*) and *Pasteurella multocida*) (Willeford, Parker et al. 2000; Parker, Willeford et al. 2001; Willeford, Parker et al. 2001; Parker, Willeford et al. 2005). In association with an increased time to death and survivability of test subjects, a significant reduction in the splenic bacterial load in treated mice was observed. Fractions containing the desired component(s) in question have shown a wide range of attributes which prompted further study. In addition to the reduced mortality in avian and murine subjects against *Salmonella typhimurium,* a

reduction of tumorogenesis in a murine melanoma model (Parker, Willeford et al. 2005) was also documented.

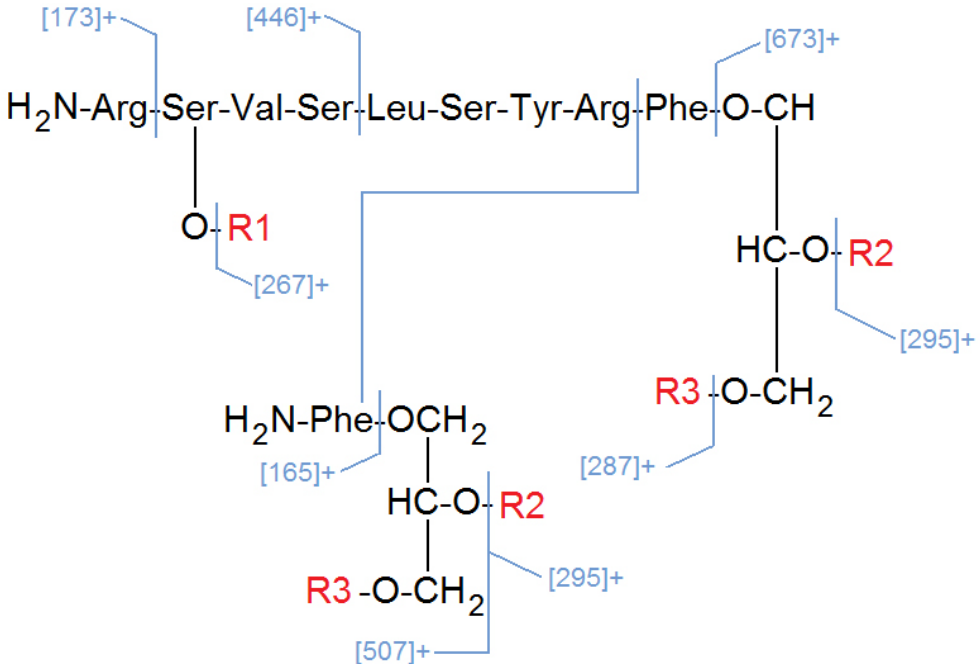


Figure 1 Chemical and mass spectral fragmentation of ICPF

Key ion fragments in the MALDI-MS electro spray MS are indicated where R1 is stearate, R2 is arachidate, and R3 is arachidonate. Figure created with aid of ChemOffice 2000 (Cambridge Soft) (Ansley and Willeford 2000; Thacker, Fuhrer et al. 2004)

CHAPTER II

PROPHYLACTIC EFFECTS OF IMMUNE CELL POTENTIATING FACTOR DERIVED FROM NON-CAPRINE MAMMALIAN SPECIES AND EVALUATION OF BACTERIAL AND VIRAL PATHOGENESIS THROUGH ANIMAL MORTALITY MODELS

Introduction

The prophylactic properties of caprine-serum derived ICPF have been studied in detail over the last several years within our laboratory (Willeford, Parker et al. 2000; Willeford, Parker et al. 2001; Parker, Willeford et al. 2005); and questions concerning efficacy, temporal and dosage requirements, and stability and safety considerations have been addressed and whenever possible, resolved. However, several key questions stemming from this work have remained unanswered. ICPF had only been isolated and validated from caprine serum samples, yet caprine derived biologicals pose a potential zoonotic biohazard, as bovine spongiform encephalopathies and other prion related neurological diseases are believed to have originated from caprine and ovine sources (Agrimi, Conte et al. 2003; Valdez, Rock et al. 2003). To circumvent these potential biohazard concerns as well as possible complications associated with allergic or immune-complex (type III) hypersensitivity reactions (Sarah, Lisa et al. 2004) we attempted to isolate and validate ICPF from additional mammalian hosts; specifically equine, bovine, porcine, and human. Previous animal and immunological studies have been centered on minimal terminal dosage experiments and mainly focused on alleviation of mortality to *S.*

typhimurium. In an effort to address specific questions pertaining to ICPF's mode of action we tested the drug's efficacy against *S. typhimurium's* predominant endotoxin, lipopolysaccharide (LPS). LPS, while not the only *S. typhimurium* derived agent known to illicit an immune response, is by far the predominant endotoxin responsible for bacteremia induced systemic inflammatory response syndrome; toxic shock syndrome. Gram-negative bacteria, in particular via endotoxin release upon lyses, are responsible for septic shock and toxic shock syndrome, resulting in multi-organ failure and ultimately death (Nicoletti, Di Marco et al. 2001; Lynn, Rossignol et al. 2003). ICPF, while known to reduce mortality as well as splenic bacteria counts, has not been evaluated or shown to protect against LPS induced endotoxemia and its associated organ failure. Evaluation of ICPF effectiveness against an endotoxin such as LPS, if successful in alleviation of mortality, would simplify experimental bioassay design and shed new light in a possible mode of action of this immunomodulatory peptide. To address both these questions, previously validated ICPF was prophylactically administered to Specific Pathogen Free (SPF) Swiss Webster Mice challenged with an otherwise lethal dose of inter-peritoneal administered LPS as well as cultured *S. typhimurium* at 3 fold the standard challenge dose of 5×10^3 CFU.

ICPF was further evaluated for effectiveness as an immunotherapy in the treatment of canine patients afflicted with Type-2 Canine Parvovirus (CPV-2). CPV-2 is a small, non-enveloped virus responsible for potentially fatal gastroenteritis in canines, as well as felines in the form of the closely related and potential origin of CPV-2, feline panleukopenia virus (Decaro, Martella et al. 2006; Spibey, Greenwood et al. 2008). There has been little direct evidence that ICPF would be beneficial against additional pathogenic agents such as gram-positive bacteria or viral challenges; however the

presumed non-pathogen specific mode of action and ICPF's apparent ability to potentiate overall immune function presented substantial circumstantial evidence towards possible efficacy for supportive treatment of viral associated diseases. We chose to test ICPF in clinical trials against naturally infected parvovirus type-2 canines in hopes of alleviating secondary post-infections associated with opportunistic pathogens in immune compromised individuals, possible antiviral properties stemming from a modulated immune response, and increasing survival by assisting in the supportive care. In addition to the clinical analysis of the immunotherapuric properties of ICPF, the antiviral and virucidal properties of ICPF where determined by hemagglutination assays of A-72 canine fibroblast cells.

Materials and Methods

Murine Animal Care and Maintenance

Specific Pathogen Free (SPF) female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were allowed to acclimate for 2 weeks upon arrival, during which time they were fed a standard maintenance diet (Laboratory Rodent Diet 5001; PMI Nutrition, Richmond, IN) and watered *ad libitum*. Mice were group-housed (5 mice per cage) in plastic boxes bedded with wood shavings in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Mice were then transferred to an isolation room immediately prior to inoculation with *S. typhimurium* or *S. typhimurium* derived lipopolysaccharides. Experiments were carried out individually so as non-infected/challenged individuals never came in contact or exposed to challenged mice. The isolation room was maintained at 20° C in a controlled negative pressure

environment on a 12-h lighting cycle. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Mississippi State University protocol number 05-051.

Bacterial Culture

S. typhimurium (ATCC 14028) was used as a challenge organism after passage through a murine host no less than three times with subsequent isolation and storage in Dulbecco PBS (dPBS) with 10% glycerol at –80° C. This isolate was supplied from a stock culture from the Department of Biological Sciences (Mississippi State University), where it is maintained as a reference organism. Bacterial counts and CFU's were determined by culturing 20 µl of *S. typhimurium* (ATCC 14028) in 10 mL of Brain Heart Infusion Broth at 37° C for 24 hours on an orbital shaker, prior to serial dilution with sterile Dulbecco phosphate buffered saline (dPBS). *Salmonella typhimurium* growth curves were determined by inoculation of 50 mL Brain Heart Infusion broth (prepared to manufactures specification) by 20 µl stock *S. typhimurium*. At 30 minute intervals 100 µl was removed and diluted with 900 µl of ddH_2O (1:10 dilution) and the optical density measured (referenced against a 1:10 dilution of BHI), plot was fitted with a 5-parameter sigmoidal function (SyStat 2008) (Figure 2). Live bacteria counts were determined by counting Colony Forming Units (CFU) generated by inoculation of 20 µl *S. typhimurium* taken from the cultures stationary phase and cultured on 1.5% agar BHI plate following seven 1:10 serial dilutions. In all cases (unless stated otherwise), bacterial cultures were diluted to 2.5×10^4 CFU per mL, allowing for 5×10^3 CFU per 0.1 mL ip injection and actual inoculations determined by plates following administration.

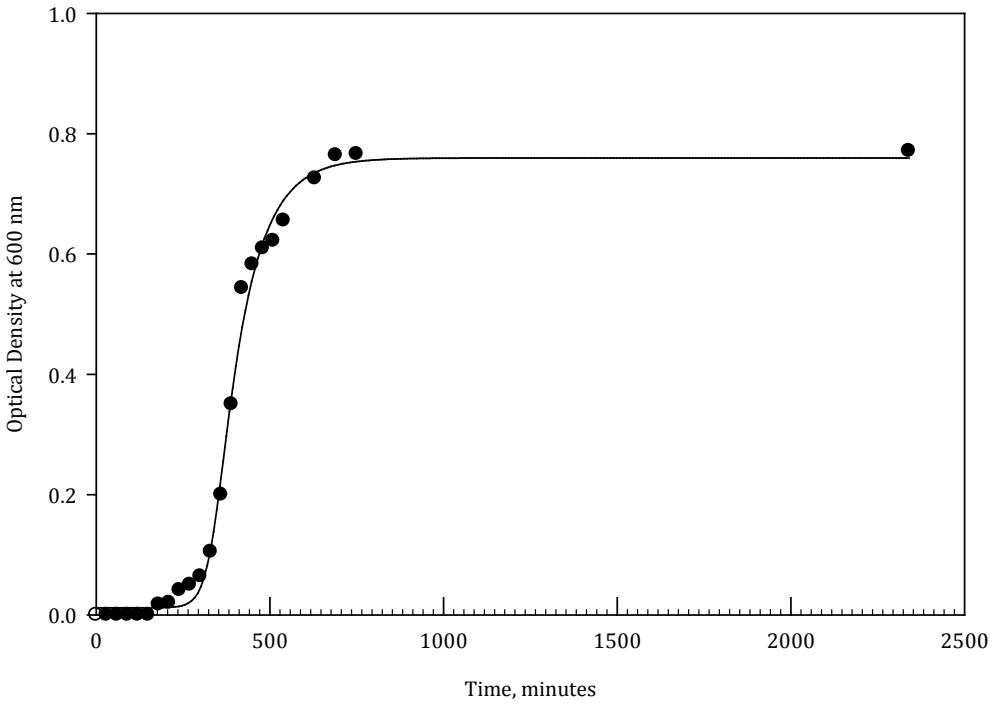


Figure 2 *Salmonella typhimurium* growth curve.

Determined by inoculation of 50 mL Brain Heart Infusion broth (prepared to manufactures specification) with 20 µl stock *S. typhimurium*. At 30 minute intervals 100 µl was removed and diluted with 900 µl of ddH_2O (1:10 dilution) and the optical density measured (referenced against a 1:10 dilution of BHI), plot was fitted with a 5-parameter sigmoidal function (SyStat 2008) resulting in an R^2 value of 0.099 and a standard error of estimate of 0.026.

Lipopolysaccharide Mortality Model

S. typhimurium derived lipopolysaccharide was purchased from Biogenesis, Inc. (Kingston, NH) To induce lethal endotoxemia, LPS was diluted with dPBS to 500 µg/mL and 50 µg LPS (2.5 mg/kg) was administered via a 0.1 mL ip injection. This dosage of LPS was determined by previous experiments and defined by its capability to induce mortality in 80 – 100 % of mice within 3-4 days (Muchamuel, Menon et al. 1997).

ICPF Preparation

ICPF was prepared by equilibrium dialysis utilizing a semipermeable 6-8K molecular weight cut off dialysis membrane (*Spectrum Laboratories, Rancho Dominguez, Ca*). Serum taken from caprine, bovine, equine, porcine, and human subjects underwent dialysis for 24 hours at 4° C at a 1:10 ratio against sterile double distilled de-ionized water. Following dialysis, effluent was lyophilized and stored at -80° C. Lyophilized material was determined to be +95% protein by weight as per the Bradford (*BioRad™, Hercules, Ca.*) and Micro Bicinchoninic Acid (*Pierce; Rockford, IL*) protein determination assays.

Virucidal and Cytotoxic Experimental Design

The virucidal and antiviral interactions between ICPF and CPV-2 were assessed by ViroMed Biosafety Laboratories (*St. Paul, MN*). In the virucidal assay, CPV-2 (obtained from ATCC: VR-2017) was incubated with 4 log dilutions of ICPF (0.1 to 100 μg/ml) for four hours and then added to wells containing canine fibroblast A-72 cells (test wells). Wells were also established to represent a cell control (cells which were neither exposed to CPV or ICPF) and virus control (cells which were exposed to CPV following incubation with culture medium). Prior to inoculation, cells were microscopically determined to be 30 – 50% confluent, and in optimal condition for CPV infection. Following incubation of the cells for 12 days, the supernatants from the cell control, virus control and ICPF treated wells were assayed for virucidal activity using a hemagglutination assay. To determine whether ICPF was directly cytotoxic to the canine fibroblasts, cells were plated and exposed to ICPF. After incubating for 12 days, the wells were assessed for cellular proliferation using a Microculture Tetrazolium Assay (Delhaes *et al.*, 1999). For the antiviral assay, canine fibroblast A-72 cells were exposed

to CPV-2 for two hours and then either ICPF (up to 100 μg/ml) or culture medium was added to establish the treated and virus control samples. Wells plated with cells that had not been exposed to CPV or ICPF were used to establish a cell control well. Cells were incubated for 10 days and their supernatants assayed for viral infectivity using a hemagglutination assay. Tissue Culture Infectious Dose per mL ($TCID_{50}$/mL) was calculated for each titration using the Spearman-Karber method (Svensson, Hjalmarsson et al. 1999). To test ICPF for erythrocyte hemolysis, canine erythrocytes were collected and separated via centrifugation for four minutes at 2000 RCF (relative centrifugal force), the top two layers decanted, and the remaining erythrocytes washed three times with dPBS. dPBS was then added to the cells to make a 10% (v/v) suspension, ICPF was prepared in dPBS and added to concentrations ranging from 0.001 - 1.0 mg/mL (Table 2) and incubated for 30 minutes. A positive control consisting of erythrocytes completely hemolyzed by the addition of 0.2% (v/v) Triton X-100 (Chow, Hedley et al. 2005) was compared to erythrocytes in dPBS and measured spectrophotometrically at 415 nm.

Murine ICPF Experimental Design

Mice comprising the (positive) control and treated populations were injected ip with 0.25 ml of *S. typhimurium* (~5 x 10^3 CFU/mouse) or 0.1 ml (50 μg) *S. typhimurium* derived LPS on day 0. Unless stated otherwise, treated mice were given a 0.2 ml ip injection of ICPF (25 mg protein/ml) at the time designated by the experimental protocol, while control mice received a placebo of physiological saline. Negative control mice were sham-handled in a similar manner to the control and treated populations to evaluate the influence of non-experimental parameters on mortality. Mice were housed up to five per cage and a minimum of six cages were used per treatment group in a randomized

block design. Mice were monitored three times daily and mortality recorded until 80% of the control mice died or for a maximum of 2 weeks.

Canine Virucidal ICPF Experimental Design

Dogs which were diagnosed by both physical examination and ELISA fecal analysis (IDEXX, Westbrook, ME) with parvoviral enteritis where upon admission to the study randomly assigned to either a placebo (n = 25) or ICPF (n = 25) treatment group. Supportive care was initiated and 5 mg of ICPF or dPBS was administered to the respective groups by a 0.5 mL subcutaneous injection. This was a double-blinded study, with neither the researchers or the attending veterinarian and personal staff aware as to the identity of the test articles and groupings until termination of the study. Severity of the disease upon diagnosis was determined by assessing the following parameters: diarrhea (0 – 3) where 0 = none, 1 = mild (soft-formed stool), 2 = moderate (runny stool), and 3 = severe (loose stool with excessive mucous/blood); apathy (0 – 2) where 0 = none, 1 = mild to moderate, and 2 = severe depression/comatose; vomiting (0 = no, 1 = yes), fever (0 = no, 1 = yes), and overall assessment of presenting condition (0 – 3) where 0 = no symptoms, 1 = mild symptoms, 2 = moderate symptoms, and 3 = severe symptoms. All animals were placed in an isolation ward (*Collierville, TN*) and supportive care included; intravenously provided cefazolin (22 mg/kg every 8 hours) to combat bacterial infection, metoclopramide (0.5 mg/kg every 8 hours) to normalize gastrointestinal peristalsis and reduce vomiting, and 5% dextrose lactated Ringers solution with 20 mEq (mmole) KCl/L.

Statistical Analysis

All experiments were arranged in a completely randomized block design. A Kaplan-Meier survival analysis was conducted for data from each survival study utilizing the LIFETEST procedure in SAS for Windows version 9.2 (SAS Institute, Car, NC). When survival plots containing more than 2 curves where required, an adjustment based on Tukey's studentized range test was used. P-values were calculated using Log-Rank or Wilcoxon methods and P-values <0.05 were considered significant.

Results

Lipopolysaccharide Mortality Model

To investigate the potential therapeutic properties of caprine derived ICPF *in vivo* against LPS induced endotoxemia an animal mortality model was developed. LPS acts as a potent endotoxin upon bacterial cell lyses, and is the predominant cause of death in patients systemically infected with several gram negative bacterial pathogens (Klut, Whalen et al. 2001), including food borne Salmonellosis, and *E. coli*. Effectiveness in reducing mortality in *S. typhimurium* challenged mice was previously established in our laboratory (Willeford, Parker et al. 2001), however an early immune response towards the bacteria which curtailed infection and thereby reduced LPS toxemia or a direct mode of action, perhaps relating to an early humoral response has never been determined. In this study however, ICPF was not significantly therapeutic to *S. typhimurium* derived LPS induced endotoxemia, despite its ability to alleviate mortality in a similar gram-negative challenged mouse model (Figure 3). In fact, ICPF treated mice fared worse than their non-treated counterparts, with an 80% increase in time to death and a 7% increase in mortality over the course of the study.

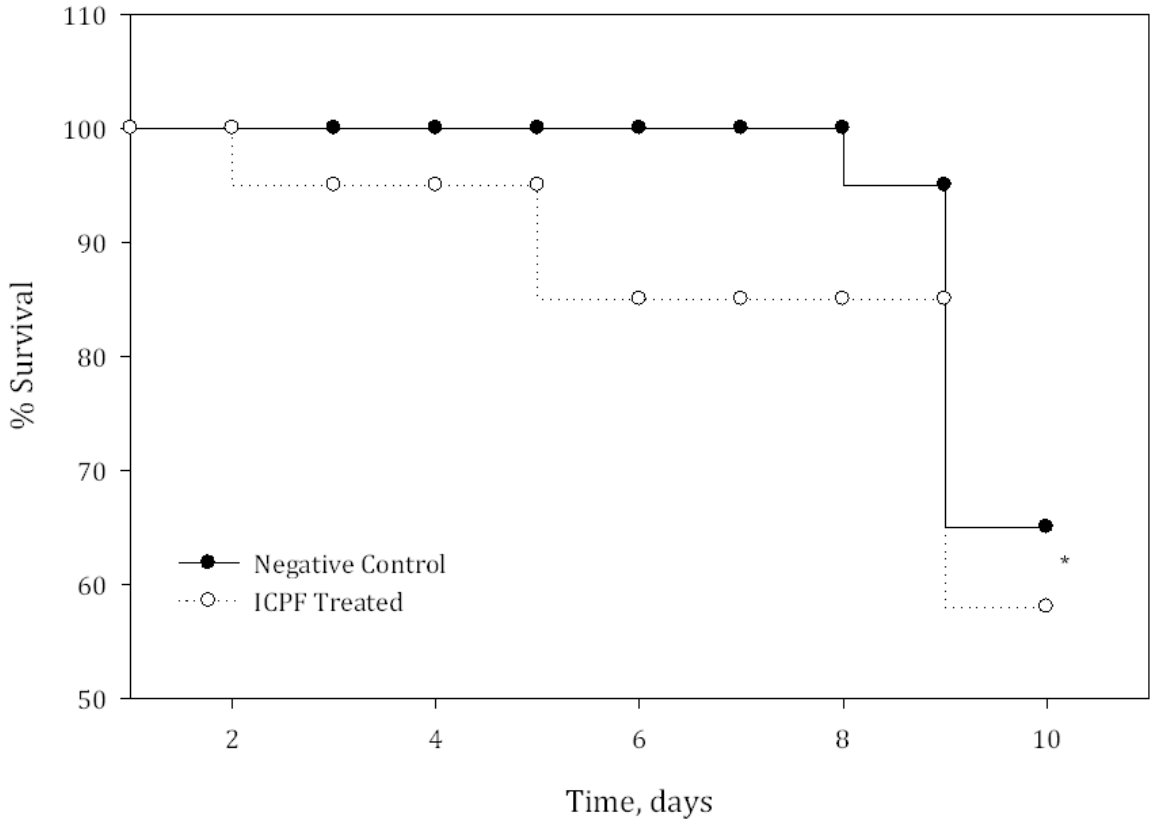


Figure 3 Effect of *S. typhimurium* derived lipopolysaccharide on ICPF treated mice. Control mice –●– were challenged ip with 0.1 ml (50 µg) S. typhimurium derived LPS on day 1, while treated ···○··· mice received both 0.1 ml (50 µg) S. typhimurium derived LPS and a 0.2 mL ip injection of ICPF (5 mg) on day 0. Each data point represents the cumulative percent survival (n = 20), *p > 0.05.

Therapeutic Properties of Bovine, Equine, and Human Derived ICPF on *Salmonella typhimurium* infected Mice

To ascertain any potential therapeutic properties of ICPF derived from non-caprine, mammalian animals, ICPF purified from Bovine, Equine, and Human serum was tested in a *S. typhimurium* animal mortality model (Bovine data not determined in this study, and therefore not included, but results were similar). Serum from the aforementioned species was processed in an identical manner as validated caprine ICPF, as previously described. One day prior to challenge by 5 x 10^3 CFU of *S. typhimurium*, 5 mg of ICPF from Bovine, Equine, and Human serum was administered ip in 0.1 mL

physiological saline. Previously validated caprine ICPF was also administered to serve as a positive control group. A negative control group consisted of challenged mice which were sham handled and injected ip with 0.1 mL physiological saline. Mortality began 6 days post challenge in the negative control and rose to 86% on day 8. As can be seen (Figure 4) mortality of mice treated with caprine derived ICPF also began on day 6, however there was an 80% reduction in mortality, and by day 8, when mortality reached 86% for the negative control, the positive control had reached 13%. Both the human and equine derived ICPF followed this trend, with little deviation. The only divergence being an additional two day delay for time to death for equine derived ICPF, which was not deemed significant ($P > 0.05$). Total reduction of mortality for human and equine ICPF after 8 days was a 73% and 83%, respectively.

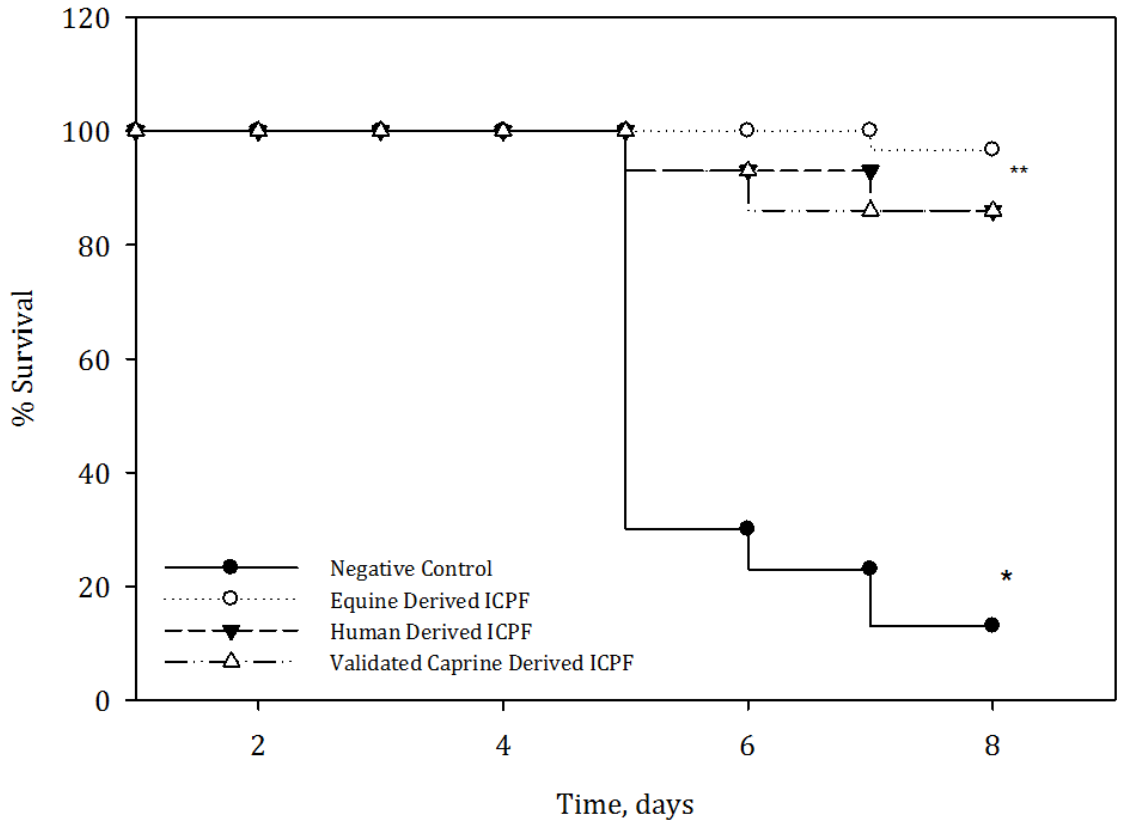


Figure 4 Effect of equine and human derived ICPF on *S. typhimurium* challenged mice

Negative control mice –●– were challenged ip with 0.1 ml (~5 x 10^3 CFU) *S. typhimurium* on day 1, while equine derived, ···○··· human derived, --▼-- ICPF treated mice received both 0.2 mL (~5 x 10^3 CFU) *S. typhimurium* on day 1 and a 0.2 mL ip injection of ICPF (5 mg) on day 0. Previously validated caprine derived ICPF —△— served as a positive control, and received both 0.2 mL (~5 x 10^3 CFU) *S. typhimurium* on day 1 and a 0.2 mL ip injection of ICPF (5 mg) on day 0. Each data point represents the cumulative percent survival (n = 20), *$p < 0.001$ and ** $p > 0.05$.

Increased Bacterial Load (15,000 CFU)

Previous examinations of ICPF centered on the peptide's ability to alleviate, reduce, or postpone mortality in mice challenged with a terminal dose of *S. typhimurium*, specifically 5 x 10^3 CFU which corresponds to an LD_{80} within 8 days post-exposure. ICPF has shown considerable consistency in reducing mortality at this dose, however the immune response necessary to alleviate morbidity or mortality may differ depending on

the dosage received (Hormaeche 1979) and may correlate with ICPF's activity and potency. In this study, two groups of 6 – 8 week old Swiss-Webster mice were inoculated ip with 1.5 x 10^4 CFU of *S. typhimurium*, a 3-fold increase to our standard (5 x 10^3 CFU) LD_{80} over 8 days (Figure 5). The negative control mice were sham handled and received 0.1mL dPBS, while a positive control received 5.0 mg ICPF ip. Mortality initiated five day's post exposure, and reached 95% in seven days within the negative control. We observed a 60% reduction in mortality for treated mice at eight days post exposure.

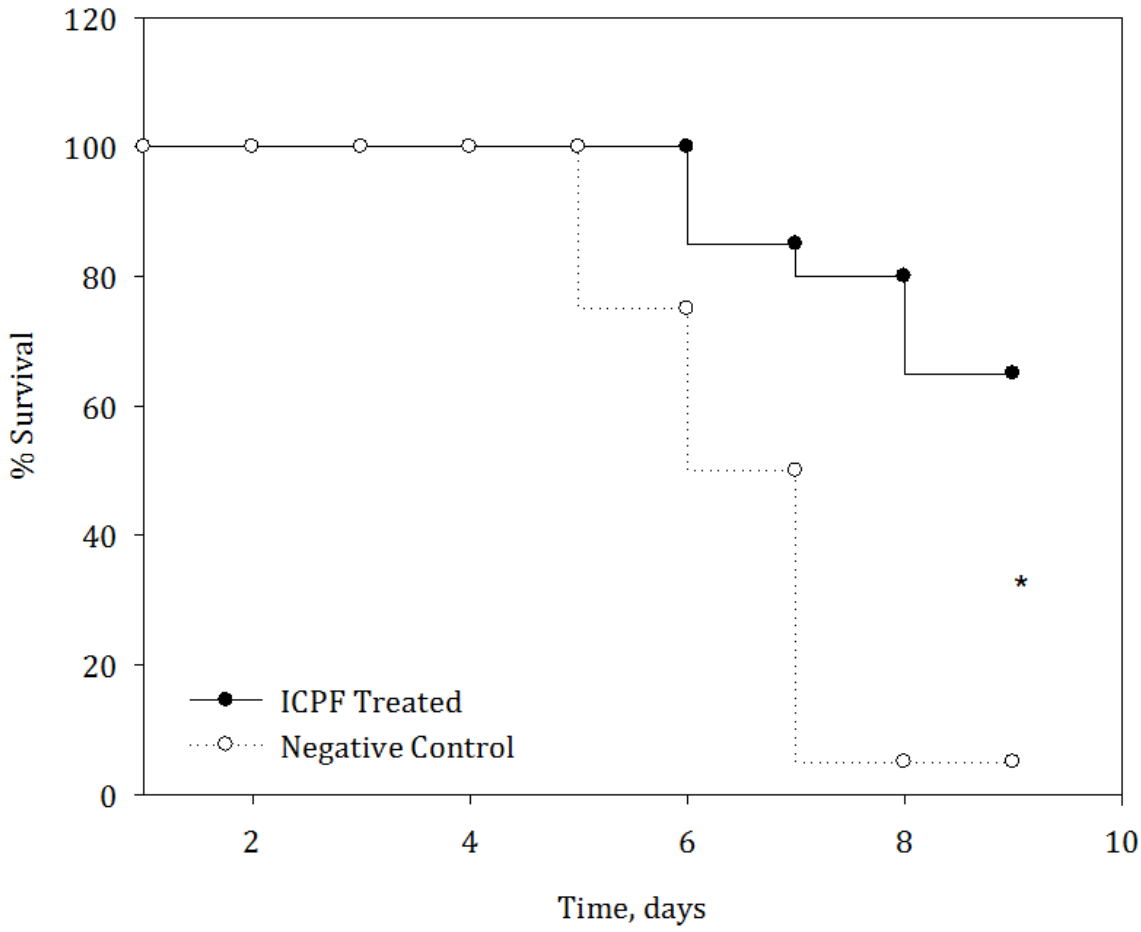


Figure 5 Increased bacterial challenge on ICPF treated mice.

Negative control mice –●– were challenged ip with 0.1 ml (~5 x 10^3 CFU) *S. typhimurium* on day 1, while ICPF treated mice ···○··· received both 0.2 mL (~5 x 10^3 CFU) *S. typhimurium* on day 1 and a 0.2 mL ip injection of ICPF (5 mg) on day 0. Each data point represents the cumulative percent survival (n = 20), *p < *0.001*.

Double Blinded Clinical Canine Parvo-Virus ICPF Analysis

Dogs entering the study demonstrated a reluctance to eat prior to admission and upon presentation. ICPF treated dogs show 68% survival versus 32% for dogs in the placebo group, representing a 2.1 fold increase in survival for animals receiving ICPF augmented treatment. Difference in mortality was significant (P = 0.01) when group comparison statistics were performed. Significance was maintained if animals which

died within a 24 hour time period from either study group were removed from the study population (P = 0.006). There were two such cases within the ICPF treatment group and three within the placebo group, the adjusted measure of survival was 74% and 36%, respectively. Upon admission to the study, 13 dogs were evaluated with severe diarrhea (9 within the placebo group, only one survived). Significance was again sustained (p = 0.015) if the dogs which succumbed to parvo-viral enteritis under these conditions were removed from the study population, with 81% of the animals in the ICPF treatment group surviving versus 47% in the placebo group. If an animal's death ensued after presenting with severe diarrhea upon admission to the study or within 24 hours of admission the survival measure was 50% and 85% for the placebo and ICPF treatment groups, respectively, statistical significance was again noted (p = 0.033) (Figure 6).

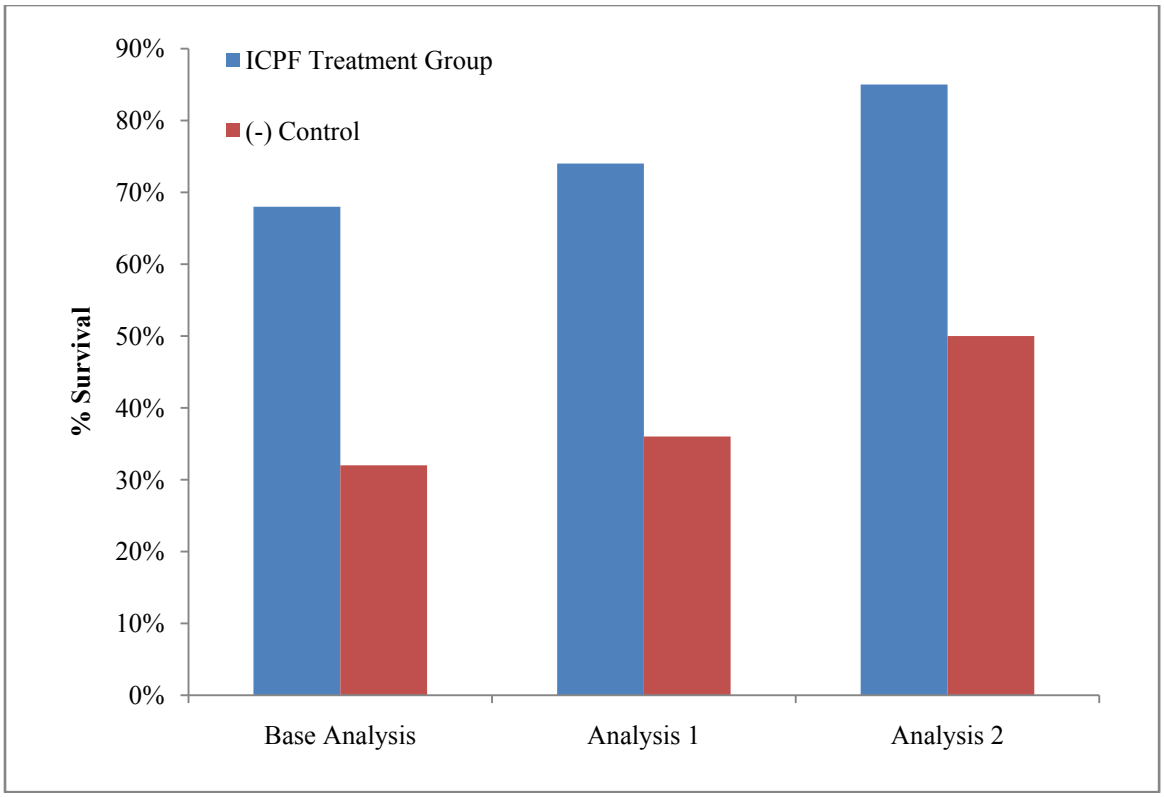


Figure 6 Survival analysis of canines treated with ICPF following diagnosis with parvoviral enteritis

Dogs which received supportive care, denoted ■ consisting of 5 mg ICPF administered sc and ■ denote dogs were given a placebo consisting of 0.5 mL dPBS sc. Base analysis represents survivability of dogs without exclusion of individuals presenting with severe diarrhea or early mortality ($p = 0.010$). Analysis 1 represents total survivability after removal of individuals from study due to mortality within 24 hours post admission ($p = 0.006$). Analysis 2 represents total survivability after removal of individuals from study which presented with severe diarrhea upon administration of the study ($p = 0.015$).

Clinical and Experimental Antiviral/Virucidal Properties of ICPF

Cytotoxicity to ICPF was tested *in situ* by incubating an immortalized line of canine fibroblast cells for 12 days with ICPF concentrations ranging from 0.001 µg/mL to 10.0 mg/ml. These cells showed little adverse effects (as determined by cell viability and proliferation) after exposure to ICPF (Figure 7) with percent proliferation ranging from 75% to 202%. ICPF was not virucidal to CPV at concentrations ranging from 0.001

µg/mL to 1 mg/mL, as infectivity and proliferation of CPV was not affected by prior exposure to ICPF as determined by erythrocyte hemagglutination (Table 2A). Average $TCID_{50}$/mL of CPV virus control wells was determined to be 3.16×10^3. Under the conditions of this assay, a one log reduction in virus production was present in samples incubated with 10 mg/mL ICPF (3.16×10^2 $TCID_{50}$/mL) while no other ICPF concentration resulted in a reduction of CPV titers. Red blood cell hemolysis testing was performed on ICPF to determine the degree to which ICPF may induce hemolysis of erythrocytes, a simple and versatile *in vitro* indicator of drug cytotoxicity. This was performed in partial fulfillment of a drug safety profile for the US Federal Drug Administration. ICPF was shown not to induce erythrocyte hemolysis at concentrations ranging from 1.0 – 0.001 mg/mL (Table 2B*)*.

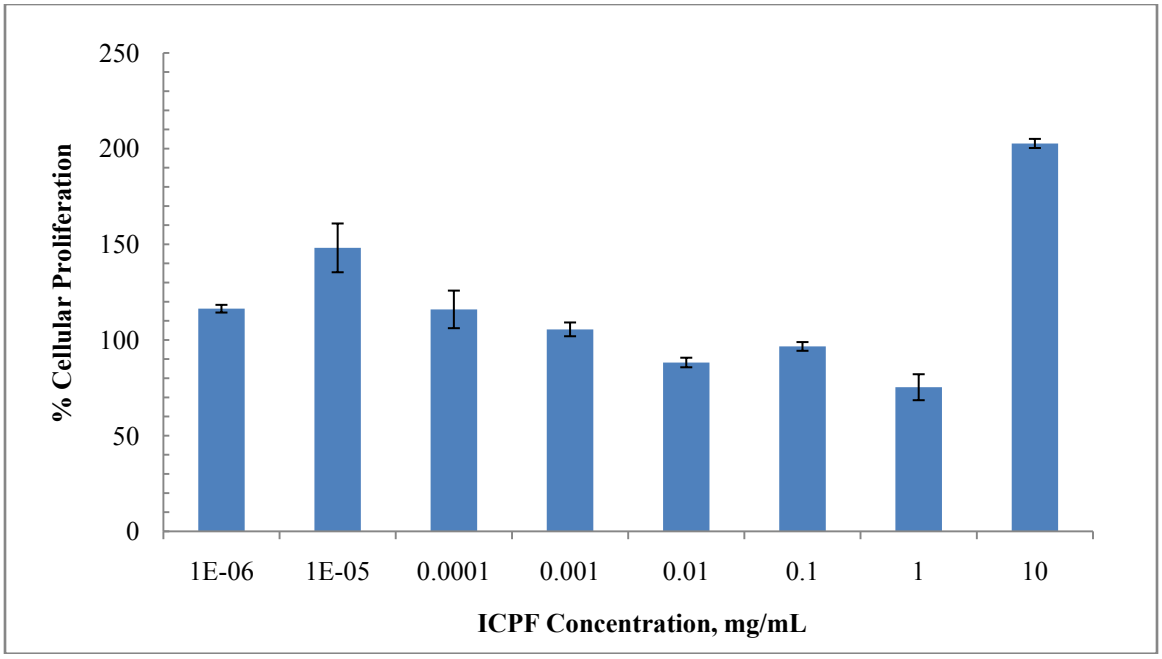


Figure 7 Cytotoxicity of ICPF on canine A-72 fibroblast cells.

ICPF was administered to A-72 Fibroblast cells at concentrations ranging from 1×10^{-6} to 10 mg/mL. Percent cellular proliferation ranged from 75.3% to 202.7%. Cellular

proliferation was measured using a microculture tetrazolium assay, using 2,3bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenyl-amino)-cabonyl-2H-tetrazolum hydroxide (XTT), viable cells metabolize XTT, producing a soluble formazan dye. Percent cellular proliferation was measured as a mean of toxicity/mean of cell control (cells not exposed to ICPF) ± S.E.M of n=3.

Table 2 Effect of ICPF on CPV proliferation and Erythrocyte Hemolysis

A.

ICPF Concentration	**CPV ($TCID_{50}$/mL)**
Virus Controls	3.16×10^3
10 mg/mL	3.16×10^2
1.0 mg/mL	3.16×10^3
0.1 mg/mL	3.16×10^3
0.01 mg/mL	3.16×10^3
0.001 mg/mL	3.16×10^3
0.0001 mg/mL	3.16×10^3
0.00001 mg/mL	3.16×10^3
0.000001 mg/mL	3.16×10^3

B.

Sample	**1**	**2**	**3**	**Avg.**	**% Hemolysis**
Positive Control	0.135	0.131	0.134	0.133	100
Negative Control	0.002	0.003	0.002	0.002	0
0.001 mg/mL	-0.001	-0.001	-0.002	-0.001	0
0.01 mg/mL	-0.001	-0.002	-0.002	-0.002	0
0.1 mg/mL	-0.003	-0.002	-0.002	-0.002	0
1 mg/mL	-0.002	-0.002	-0.003	-0.002	0

A. $TCID_{50}$/mL as determined by erythrocyte hemagglutination. Wells positive for CPV in the presence of erythrocytes are characterized by the formation of a proteinaceous lattice over the bottom of the well. B. Canine erythrocytes incubated in 0.2% Triton X-100 served as a positive control and spectrophotometric reference for erythrocyte lysis while cells incubated in a 10% dPBS solution served as a negative control. Percent hemolysis was calculated using the equation *(Sample Absorbance at 415 nm – Negative Control)/(Positive Control-Negative Control) x 100*. ICPF did not induce erythrocyte lysis at any concentration ranging from 0.001 – 1.0 mg/mL.

Discussion

When considering any new therapeutic agent, especially one which appears to work as a biological response modifier, it is important to consider its broader range of activity, often beyond the compounds intended or anticipated medicinal roles. With this

in mind, we entered into this portion of our investigation with an emphasis on further elucidating relative safety, toxicity, and increased range of activity with respect to pathogenic presentation and cross species interactions. When looking beyond the preconceived roles of any therapeutic, it is often prudent to concurrently address any potential problems. Risk associated with systemic bacterial infections are often two fold; systemic proliferation of the bacteria and endotoxins, such as LPS, released following elimination and lyses by the associated immune response and any antibiotics administered. ICPF appears to act as an immune modulator, with increased immune effectiveness 24 hours prior to exposure of a bacterial challenge. This was reestablished indirectly by ICPF's inability to alleviate LPS associated mortality. It is known that the balance between systemic levels of pro and anti-inflammatory cytokines play a key role in the severity and outcome of *S. typhimurium* associated sepsis (Walley, Lukacs et al. 1996), it is of little surprise that an immunotherapy which is believed to operate by potentiating an early immune response would be of limited use in an LPS induced sepsis model. Previous studies (Tracey, Fong et al. 1987; Block, Berg et al. 1993) have shown reduction of pro-inflammatory cytokines, by monoclonal antibodies or receptor antagonists, or the administration of anti-inflammatory cytokines to offer protection against endotoxemia or septic shock. Current evidence suggests that ICPF acts in a manner to increase early pro-inflammatory cytokine response, which aid in keeping pathogenic bacteria concentrations at a level manageable by the immune system.

ICPF derived from caprine serum has shown both consistency and reliability in activity and function, however caprine derived biologicals are under increasing surveillance and restrictions by the USDA. Additional active mammalian sources of ICPF would be beneficial in further understanding the biological role of ICPF and to

assuage concern with respect to its accepted use. The confirmation of immunological activity in ICPF derived from bovine, equine, canine and human serum suggests a role fundamental to the mammalian immune response. The presence of non-mammalian ICPF has not been reported, however mammalian ICPF has been validated within our laboratories to illicit an immune response and offer protection in avian subjects (Willeford, Parker et al. 2000). The presence of active ICPF across several mammalian species in conjunction with biological activity within avian test subjects suggests a mode of action shared by mammals and birds. Within mammals there exists considerable homology within Toll-Like Receptor structure and function and while differences exist between human and mice (Table 1), these difference are usually the result of gene duplications. The presence of ICPF in several mammalian species, ranging from human to goat, all of which are able to potentiate an immune response in mammalian and avian test subject, strongly suggests an evolutionarily primitive pathway, related to the innate immune response, such as TLR. Direct evidence linking ICPF function and TLR, would require proof of either a direct (ICPF-receptor) mediated response or confirmation of its emanate cytokine cascade.

ICPF has been shown to be non-virucidal against CPV-2 and does not interfere with the virus' ability to infect or proliferate within tested cell types, specifically canine A-72 fibroma cells. In what was the first double blinded study to characterize the therapeutic effects of ICPF in a viral challenge, ICPF reduced canine mortality by 36%, a ($p = 0.010$) significant amount. It is clear from the data provided that ICPF promotes resistance to CPV infection without acting as a directly virucidal agent, the mechanism by which it accomplishes this remains to be elucidated. It is possible that ICPF provokes a PRR mediated response to the viral presence or perhaps rallies the immune system

giving the subject the time it needs to circumvent death while developing permanent immunity to CPV. The results of this study, while providing new and valuable insight to ICPF potential as an antiviral therapeutic, does not address specific questions concerning mode of action or efficacy. What this study has shown however, is a direct correlation between specific immune pathways and clinically relevant results. Mortality associated with CPV-2 infections rarely result from the virus itself in individuals infected prior to 8 weeks of age (Greene 1984). Mortality is usually proceeded by hemorrhagic enteritis, myocarditis, secondary infections, and gram negative sepsis (Greene 1984; Wessels and Gaffin 1986; Evermann, Abbott et al. 2005), followed by disseminated intravascular coagulation and death. Pathogenesis resulting in death in dogs over 8 weeks of age may take one of two routes; CPV infections of lymphoid tissue and marrow or infection within epithelial cells particularly within the crypts of the small intestines (Favrot, Olivry et al. 2000). Dogs with CPV infected lymphoid tissue suffer from associated immunodeficiency resulting in secondary infection and gram-negative sepsis. Intestinal epithelial cell infections usually result in increased intestinal permeability, resulting in a drop of colloidal pressure and fluid loss, and decreased nutritional absorption, ultimately followed by either recovery or secondary infection and sepsis, the later usually resulting in death by disseminated intravascular coagulation. ICPF intercedes in this process by either interfering with viral pathogenesis, moderating the secondary affects resulting from immunodeficiency, or through tempering both these activities.

We have previously shown that ICPF does not protect mice suffering from acute gram-negative derived endotoxemia, (Figure 3) yet is capable of assisting compromised individuals suffering from systemic bacteriameia (Figure 5) when prophylactically treated. This study, being clinical in nature, was not designed or intended to differentiate

between sepsis induced by inflammation of the gastrointestinal tract or immunodeficiency, and we are therefore limited in our ability to attribute a specific systemic mode of action to ICPF in CPV-2 infected dogs. ICPF's ability to alleviate mortality in such a model does further establish its role as an immune potentiator and suggests additional clinical uses in both human and veterinary medicine, possibly as an adjuvant for the treatment of immune stressed/compromised individuals or patients at risk of severe inflammatory reactions as seen in CPV patients. Further work utilizing a different viral model, specific pathogen free animals, and a better understanding of the specific immune response to ICPF may clarify the full extent of ICPF's bioactivity and mode of action.

CHAPTER III

SYSTEMIC *IN VIVO* PROTEOME CHANGES RESULTING FROM ADMINISTRATION OF IMMUNE CELL POTENTIATING FACTOR

Introduction

ICPF mediated prophylaxis to a systemic *S. typhimurium* infection has been shown to display both temporal and dose dependencies (Parker, Willeford et al. 2001). These time and dosage considerations of successful treatments can be indicative of a systemic proteomic shift as well as underlining ICPF's immunological mode of action. A medium time frame of 24 hours pre-infection for peak immunological performance was determined for the administration of ICPF in specific pathogen free (SPF) female Swiss Webster Mice and therefore, the first 24 hours serves as an ideal initial time frame to monitor for such changes. A pre-challenge window of 24 hours makes an adaptive or humoral response within that time frame unlikely, however it may be indicative of an innate mediated inflammatory response (Medzhitov 2009).

Systemic proteomic shifts in response to traditional drugs are somewhat rare and undesirable, nevertheless an immune initiated acute phase response would be possible and fitting based on known prophylactic time frames. In order to determine if ICPF had any systemic effects on various protein concentrations and profiles, several experiments were conducted. To determine the effect of ICPF on Acute Phase Proteins (APP), Serum Amyloid A concentrations were determined following ICPF treatment with and without a

bacterial challenge. APP are an ideal staging point to search for possible systemic proteomic changes related to immunity, as serum levels of APP can increase 1,000 fold within minutes of an inflammatory incident, (Beer, Baltz et al. 1982; Raynes, Eagling et al. 1991). While the chemical trigger initiating APP production (e.g, cytokines) will spike temporally (providing a detection window of perhaps minutes) APP increases can persist for hours or days. These collective features make APP detection straightforward, while if discovered, simultaneously validating the impetus to seek its trigger.

The roles of APP are complex, ranging from initiation of compliment, opsonization, homeostasis, lipid transport, and immune cell activators but are, by themselves, of limited use in further elucidation of ICPF's mode of action (Szalai, VanCott et al. 2000; Shah, Hari-Dass et al. 2006; Tissieres, Dunn-Siegrist et al. 2008). Their diverse functions open the door to more complex and specific proteomic changes within the body. To better understand the homeostatic and physiological changes associated with ICPF, a more inclusive and quantitative examination of differential proteome profiles is required. To facilitate this need, proteomic changes associated with ICPF were mapped out in serum, spleen, lung, liver, and central nervous tissue (brain) using Two-Dimensional Fluorescence Difference in Gel Electrophoresis (2D DIGE). Serum was evaluated for proteomic changes for it serves as a conduit for nearly all endocrine and immune signaling, is essential for swift bacterial dissemination within the body, and on the forefront of the innate immune response. Splenic tissue was evaluated as it represents the largest single organ of the immune system. Respiratory (i.e., lung) tissue was selected for proteomic examination as ICPF showed promise in alleviation of equine lower respiratory disease *in vivo* (Hamm, Willeford et al. 2002) and alveolar macrophage activation *in vitro* (unpublished). The innate immune response is specific to

the lung – wherein this large respiratory surface area is constantly being exposed to inhaled particles and microorganisms. Central nervous tissue was examined to assess physiologic changes related to immune function and homeostasis. Brain and liver (the latter being the primary site of APP production) also serve as a more stable platform to monitor proteomic changes in contrast to the dynamic nature within the circulatory system.

Materials and Methods

Murine Animal Care and Maintenance

Specific Pathogen Free (SPF) female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were allowed to acclimate for 2 weeks upon arrival, during which time they were fed a standard maintenance diet (Laboratory Rodent Diet 5001; PMI Feeds) and watered *ad libitum*. Mice were group-housed (5 mice per cage) in plastic boxes bedded with wood shavings in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Mice were then transferred to an isolation room immediately prior to inoculation with *S. typhimurium* or *S. typhimurium* derived lipopolysaccharides. Experiments were carried out individually so as non-infected/challenged individuals never came in contact or were exposed to challenged mice. The isolation room was maintained at 20° C in a controlled negative pressure environment on a 12-h lighting cycle. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Mississippi State University protocol number 05-051.

Murine Treatments and Sample Collection

Two groups of SPF female Swiss-Webster Mice (n=3 per group) comprising a treated population and (negative) control were injected ip with either 5 mg of previously validated caprine ICPF in 0.2 mL dPBS or 0.2 ml dPBS, respectively. Following administration of treatment or placebo, one individual from each group was sacrificed at 0, 3, and 24 hours by CO_2 asphyxiation and 0.8 - 1.0 mL of blood drawn by cardiac puncture. Whole blood was transferred into a 5 mL sterile serum tube, gently inverted several times, placed upright and allowed to clot at ~25° C for 30 - 45 minutes. Samples were then centrifuged for 10 minutes at 1,500 RCF. 50 - 100 µl serum were collected with a micropippette and stored at -80° C. Cardiac, liver, respiratory, spleen, CNS (e.g., brain), and ocular tissue where then aseptically removed and stored at -80° C for further analysis.

Serum Amyloid A Experimental Design and Analysis

Three groups of SPF female Swiss Webster Mice (n=5 per group) were utilized in this experiment. Group 1 received 5 mg ICPF ip in 0.2 mL dPBS, while a second group received 5 x 10^3 CFU ip of *S. typhimurium* and the final group received 5 mg ICPF ip in 0.2 mL dPBS 24 hours prior to 5 x 10^3 CFU ip of *S. typhimurium*. An additional group consisting of n=5 served as a baseline and negative control for comparison, were sham handled and received 0.2 mL dPBS. At 6, 24, and 48 hours following challenge of *S. typhimurium*, the mice were sacrificed by CO_2 asphyxiation and 0.8 - 1.0 mL of blood was drawn by cardiac puncture. Whole blood was transferred into a 5 mL sterile serum tube, gently inverted several times, placed upright and allowed to clot at ~25° C for 30 - 45 minutes. Samples were then centrifuged for 10 minutes at 1,500 RCF. 50 - 100 µl serum were collected with a micropippette and stored at -80° C. Serum Amyloid A

values were determined using a solid phase sandwich ELISA (Tri-Delta Diagnostics, Rockaway Valley, NJ) and a streptavidin-HRP conjugate in a 96 well microplate format.

2D DIGE Tissue Preparation

Respiratory (lung), liver, central nervous tissue (brain), and splenic tissue were washed with Washing Buffer (10 mM Tris-HCl and 5 mM magnesium acetate, pH 8.0) in order to remove contaminates such as extraneous tissue and blood. 200 µl of 2D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) were mixed with 10 mg of tissue sample and sonicated at 4° C. Samples where then centrifuged for 30 minutes at 10,000 RFC and supernatant collected. Protein concentration was determined by either the Bradford (BioRad™, Hercules, Ca.) or Micro Bicinchoninic Acid (Pierce; Rockford, IL) protein determination assays. Samples were diluted to between 4 - 8 mg/mL.

2D DIGE Proteomic Identification and Analysis

2D DIGE was performed by Applied Biomics (Hayward, CA); briefly: Following protein extraction from tissue samples, proteins were covalently bonded to Cy2, Cy3, or Cy5 dyes (GE Healthcare, Piscataway, NJ) and subjected to isoelectric focusing on a 3 - 10 immobilized pH gradient strip prior to analysis on a 9 - 13% gradient SDS-gel, performed at 15° C until the dye front reached end of gel running plate. Image scans were carried out immediately following the SDS-PAGE using Typhoon TRIO (Amersham BioSciences, Piscataway, NJ). Scanned images were then analyzed by Image Quant software (version 5.0, Amersham BioScience, Piscataway, NJ), and subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.0 (Amersham BioSciences, Piscataway, NJ). Selected spots were picked up by Ettan Spot Picker

(Amersham BioSciences, Piscataway, NJ) following the DeCyder software analysis and spot picking design. The selected protein spots were subjected to in-gel trypsin digestion, peptide extraction, desalting, and then subjected to MALDI-TOF/TOF (Applied Biosystems) analysis to determine protein identity. ProteinID scores >95% were considered significant identifications.

Statistical Analysis

All experiments were arranged in a completely randomized block design and a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Pairwise Multiple Comparison (Student-Newman-Keuls Method) to calculate P-values (SyStat 2008) was used to compare data. Differences with P-values < 0.05 were considered significant.

Results

Effect of ICPF on Serum levels of the Acute Phase Protein Serum Amyloid A

Stimulation of acute phase protein Serum Amyloid A (SAA) by ICPF, alone, and with costimulation with *S. typhimurium* was evaluated in female Swiss Webster mice. Naïve mice served as a negative control and a baseline with normal physiological concentration of SAA of 1.05 ± 0.07μg/mL, correlating with established norms (Tape, Tan et al. 1988; Lindhorst, Young et al. 1997). Within 6 hours of ICPF treatment, serum concentration of SAA increased to 128 ± 51.7 μg/mL reaching 403 ± 80.9 μg/mL at 24 hours, and 3,480 ± 270 μg/mL at 48 hours (Figure 8). To serve as a positive control, mice were injected ip with 1 x 10^3 CFU of *S. typhimurium (Clarissa and Alexander 1999; Hari-Dass, Shah et al. 2005)*. In this positive control, SAA values increased to 423 ± 30.3 μg/mL within 6 hours post exposure, peaked at 3,710 ± 95.8 μg/mL in 24 hours and returned to 55 ± 14.8 μg/mL within 48 hours, slightly elevated, but not indicative of a

typical acute phase response. Finally, we evaluated the response of SAA in animals concurrently treated with ICPF and exposed to *S. typhimurium*. When mice where both treated and challenged, a novel response by SAA was observed. Within 6 hours serum concentration of SAA increased to 561 ± 100.0 μg/mL continued to increase to 1,331 ± 155.1 μg/mL at 24 hours and finally peaked at 1,671 ± 705 μg/mL, 48 hour post treatment. Serum levels of SAA following ICPF stimulation indicate an increased acute phase response when compared to the positive control at 48 hours ($P < 0.05$), as determined by SAA concentration, however, a temporal shift of 24 hours results in a delay for peak SAA concentration within the system when compared to positive control ($P < 0.05$). During costimulation with ICPF and *S. typhimurium*, systemic levels of SAA increased more rapidly than ICPF treated mice at 6 hours ($P < 0.05$) but no significant differences were noted compared to *S. typhimurium* challenged mice ($P > 0.05$). SAA concentrations in mice costimulated with ICPF and *S. typhimurium* were lower than mice treated with ICPF alone ($P < 0.05$).

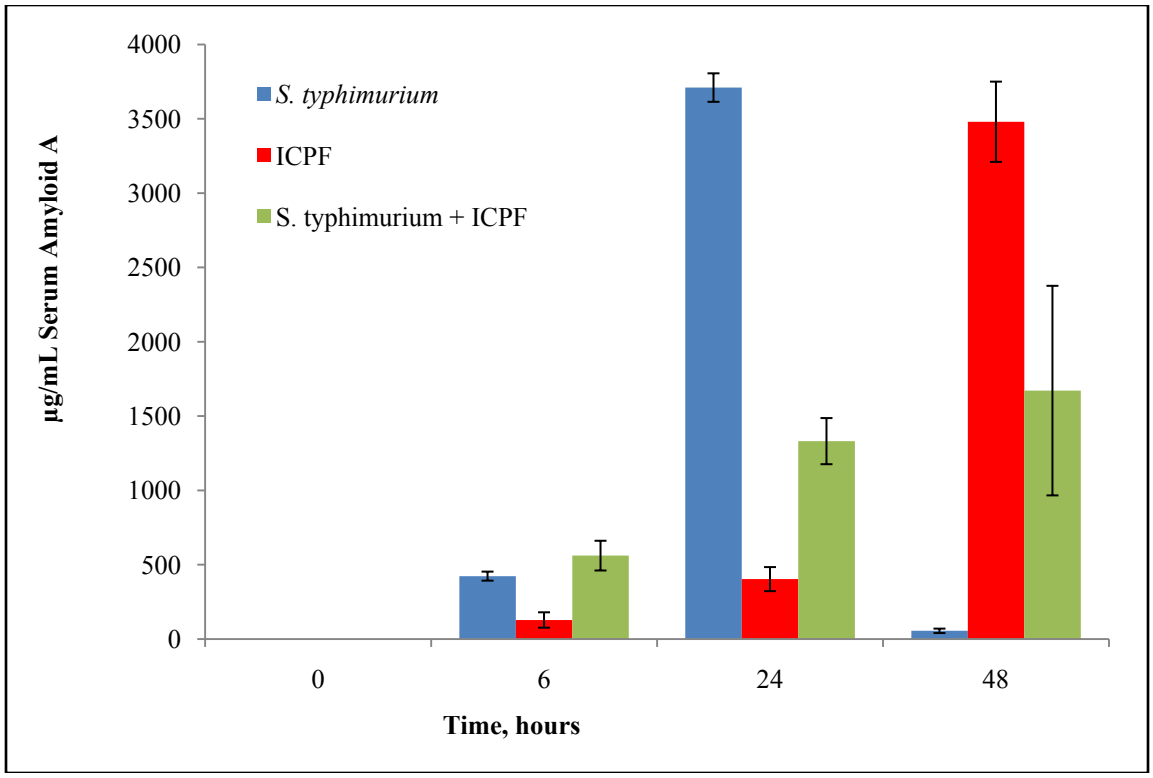


Figure 8 Synergistic effects of ICPF and endotoxin stimulation on serum levels of Serum Amyloid A.

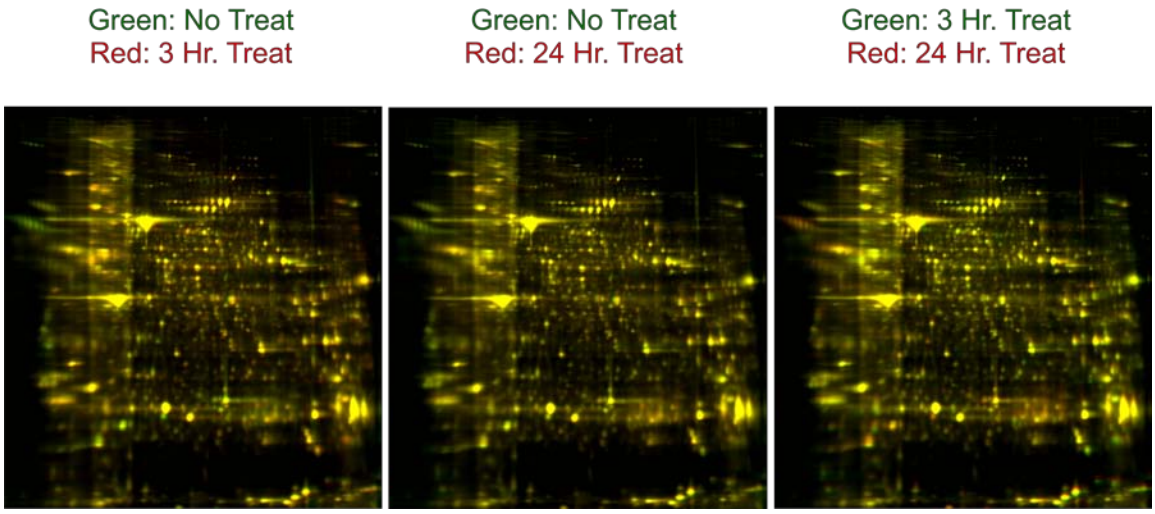
ICPF, *S. typhimurium*, and combined ICPF and *S. typhimurium* on Serum Amyloid A concentration in serum was determined using a sandwich ELISA in mice treated with 0.5 mg ICPF administered ip in 0.2 mL dPBS ■, mice challenged ip with 5 x 10^3 CFU of *S. typhimurium* ■, and both ICPF and *S. typhimurium* ■, ± S.E.M of n=5

2D DIGE Proteomic Identification and Analysis

Using 2D DIGE and DeCyder 6.0 image analysis software we investigated any potential effects ICPF may have on systemic protein concentrations and post translational modification on a systemic level. Following ip administration of 5 mg ICPF as previously described, subjects were sacrificed and tissue samples analyzed by 2D DIGE. For serum, respiratory, splenic, liver, and central nervous tissue, protein expression profiles from a zero hour (control), 3 hour and 24 hour post treatment were determined, a representative gel can be seen on (Figure 9). Several criteria were used to choose spots

for possible MS/MS protein identification. A high degree of up/down regulation, at least a 2 fold increase or decrease in intensity in order to select proteins with a greater likelihood of being selectively modified. Intensity of spots, either weak or strong played a role in identification likelihood, as weak proteins prove difficult to obtain a positive mass spectrometry identification, while a strong spot indicates a higher probability of being a structural protein or housekeeping enzyme by virtue of its high concentration within the gel, so spots with medium intensity were chosen. Finally, whenever possible we avoided known landmark protein locations, such as actin, tubulins, and heat shock proteins, as they are not likely to play a significant role in the immunological reaction.

A.



B. C.

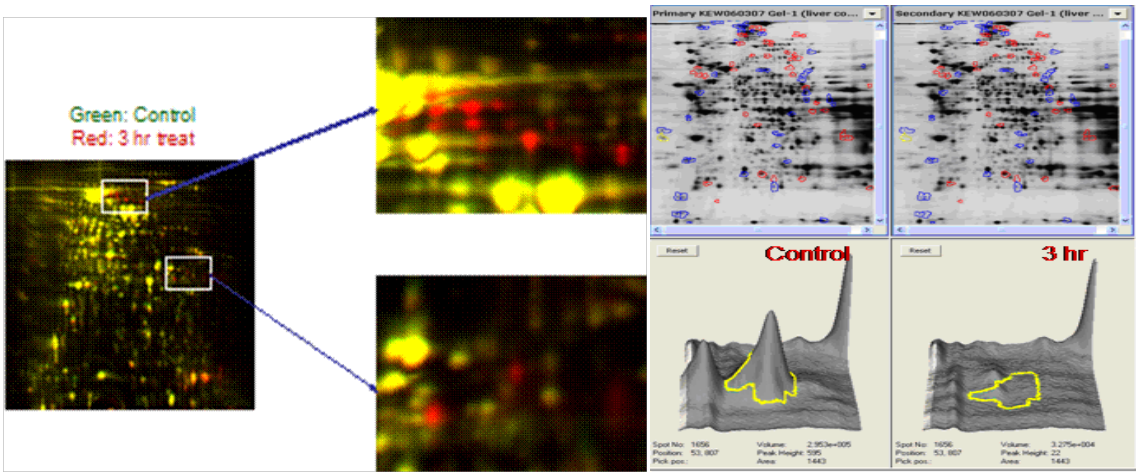


Figure 9 Representative 2D DIGE analysis.

A. 2D DIGE gel image of homogenized respiratory tissue at 0, 3 and 24 hours following 0.2 mL ip administration of 5.0 mg ICPF in SPF female Swiss Webster Mice. Proteins were covalently bonded to Cy2, Cy3, or Cy5 dyes (GE Healthcare, Piscataway, NJ) and subjected to isoelectric focusing on a 3 - 10 immobilized pH gradient strip prior to analysis on a 9 - 13% gradient SDS gel. B. A detailed view of boxed region within 2D DIGE image of homogenized liver tissue. C. 3-D fluorescence intensity profile of a selected spot within the gel.

Within central nervous tissue, 25 spots where chosen for protein identification by MALDI-Mass Spectrometry. Of these 25 spots, 3 yielded possible secondary protein identifications, resulting in 28 potential proteins which were differentially expressed or underwent post translational modification(s) (Table 3). Respiratory tissue, consisting predominantly of lung, yielded ten spots with an additional five secondary protein identifications (Table 4). For serum, 20 spots were selected, based on the above mentioned criteria for protein identification, however seven had weak mass spectrum readings and conclusive protein identification was not possible, of the remaining 13 protein spots analyzed only six different proteins were identifiable (Table 5) with serum albumin constituting the predominate duplicate/contaminating protein. Splenic tissue yielded 15 unique differentially expressed proteins (Table 6) and hepatic (liver) tissue (Table 7) yielded 21, 17 unique, differentially expressed proteins, of which ten have specific enzymatic functions.

Table 3 Differentially expressed central nervous tissue proteins identified by MALDI-TOF/TOF

	3 Hour/Control		24 Hour/Control		24 Hour/3 Hour				
Spot #	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Most Likely Protein ID**	**Accession**	**Functional Type**
1	Increased	2.49	Similar	1.09	Decreased	-2.41	Dnm1 protein [Mus musculus]	21961254	Cytoskeleton
2	Increased	2.06	Similar	1.15	Similar	-1.88	Transferrin [Mus musculus]	18606172	Carrier
3	Increased	2.02	Similar	1.44	Similar	-1.47	Low signal	--	--
4	Similar	-1.87	Similar	-1.63	Similar	1.09	Transferrin [Mus musculus]	18606172	Carrier
5	Increased	2.53	Similar	1.13	Decreased	-2.36	BiP [Mus musculus]	2598562	Chaperone
6	Decreased	-3.56	Decreased	-2.67	Similar	1.27	Neurofilament-L	200038	Cytoskeleton
7	Increased	4.94	Similar	1.05	Decreased	-4.96	Protein disulfide-isomerase precursor	IPI00133522	Enzyme
8	Decreased	-3.05	Decreased	-3.6	Similar	-1.24	Dihydropyrimidinase-like 2 [Mus musculus]	40254595	Enzyme
9	Similar	1.39	Increased	2.16	Similar	1.48	Coronin, actin binding protein 1A [Mus musculus]	31418362	Cytoskeleton
10	Decreased	-2.38	Decreased	-2.31	Similar	-1.02	Pyruvate dehydrogenase E1 alpha 1 [Mus musculus]	13938051	Enzyme
11	Increased	2.53	Similar	-1.09	Decreased	-2.89	Eef2 protein [Mus musculus]	38511951	Elongation Factor
11'	Increased	2.53	Similar	-1.09	Decreased	-2.89	Serpinb1a [Mus musculus]	56206897	Protease Inhibitor
12	Increased	5.09	Similar	-1.2	Decreased	-6.43	Car6 protein [Mus musculus] (Carbonic anhydrase VI)	29612611	Enzyme
13	Increased	3.96	Similar	1.05	Decreased	-3.94	Interferon inducible protein 1 [Mus musculus]	6680351	Chemokine
16	Similar	1.48	Similar	1.58	Similar	1.02	G protein beta subuit like [Mus musculus]	475012	Second Messenger
16'	Similar	1.48	Similar	1.58	Similar	1.02	Vacuolar ATP synthase subunit E (V-ATPase E subunit)	1718091	ATPase
17	Increased	2.68	Similar	-1.02	Decreased	-2.87	G protein beta subuit like [Mus musculus]	475012	Second Messenger
18	Decreased	-3.48	Decreased	-2.98	Similar	1.11	Quininoid dihydropteridine reductase [Mus musculus]	12805283	Enzyme
19	Increased	7.97	Similar	-1.42	Decreased	-11.84	Phosphomannomutase 2 [Mus musculus]	28374408	Enzyme
19'	Increased	7.97	Similar	-1.42	Decreased	-11.84	Internexin neuronal intermediate filament protein, alpha	34328368	Cytoskeleton
20	Decreased	-3.29	Similar	-1.28	Increased	2.45	Peroxiredoxin-6	IPI00555059	Enzyme
21	Similar	1.81	Similar	-1.01	Similar	-1.92	ES1 protein homolog, mitochondrial precursor	IPI00133284	Mitochondrial
21'	Similar	1.81	Similar	-1.01	Similar	-1.92	Peroxiredoxin-6	IPI00555059	Enzyme
22	Increased	3.27	Similar	-1.33	Decreased	-4.55	Superoxide dismutase [Mn], mitochondrial precursor	IPI00109109	Enzyme
23	Increased	9.53	Similar	-1.28	Decreased	-14.34	Glandular kallikrein K6 precursor	IPI00323869	Serine Protease
24	Similar	1.81	Similar	-1.15	Decreased	-2.45	17 kDa protein (Peptidyl-prolyl cis-trans isomerase A)	IPI00551246	Isomerase
24'	Similar	1.81	Similar	-1.15	Decreased	-2.45	Down syndrome cell adhesion molecule-like protein	IPI00408489	Cellular Adhesion
25	Decreased	-3.3	Decreased	-2.37	Similar	1.33	Splice Isoform 2 of Syntaxin-binding protein 1	IPI00415403	Binding Protein

Proteins which were either up or down regulated within central nervous tissue, accession numbers correlates to either NCBI GenBank (Benson, Karsch-Mizrachi et al. 2005) or the International Protein Index (Kersey, Duarte et al. 2004) which is denoted by an 'IPI' preceding the number. Spot numbers denoted with a prime signify situations where more than one likely protein was identified, alternative protein ID listed in the subsequent row.

Table 4 Differentially expressed respiratory tissue proteins identified by MALDI-TOF/TOF

	3 Hour/Control		24 Hour/Control		24 Hour/3 Hour				
Spot #	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Most Likely Protein ID**	**Accession**	**Functional Type**
1	Increased	2.55	Increased	6.03	Increased	2.38	NA (weak mass spectrum)	--	--
2	Decreased	-2.03	Similar	-1.37	Similar	1.49	Albumin 1 [Mus musculus]	29612571	Carrier
3	Similar	1.65	Similar	1.07	Similar	-1.54	Dihydropyrimidinase-like 2 [Mus musculus]	40254595	Enzyme
3'	Similar	1.65	Similar	1.07	Similar	-1.54	EH domain containing protein [Mus musculus]	13938647	Cytoskeleton
4	Similar	1.99	Similar	1.09	Similar	-1.82	Electron transferring flavoprotein, dehydrogenase	15214778	Enzyme
4'	Similar	1.99	Similar	1.09	Similar	-1.82	Lamin A, isoform A [Mus musculus]	15929761	Kinase
5	Similar	-1.75	Similar	-1.17	Similar	1.5	Pzp protein [Mus musculus]	34785996	Protease Inhibitor
6	Similar	1.73	Similar	-1.22	Decreased	-2.11	Calponin 2 [Mus musculus]	14318693	Cytoskeleton
7	Similar	-1.47	Similar	1.01	Similar	1.49	Extracellular superoxide dismutase [Mus musculus]	3320907	Enzyme
7'	Similar	-1.47	Similar	1.01	Similar	1.49	Pzp protein [Mus musculus]	34785996	Protease Inhibitor
8	Similar	1.31	Similar	1.82	Similar	1.39	Carbonic anhydrase III [Mus musculus]	10717134	Enzyme
8'	Similar	1.31	Similar	1.82	Similar	1.39	Aldh1a1 protein [Mus musculus]	28386049	Enzyme
9	Similar	-1.99	Similar	-1.28	Similar	1.57	1-Cys peroxiredoxin protein [Mus musculus]	4139186	Enzyme
9'	Similar	-1.99	Similar	-1.28	Similar	1.57	Peroxiredoxin 6 [Mus musculus]	82892635	Enzyme
10	Similar	-1.98	Similar	-1.41	Similar	1.4	Apolipoprotein A-I precursor [Mus musculus]	109571	Carrier

Proteins which were either up or down regulated within respiratory tissue, accession numbers correlates to either NCBI GenBank (Benson, Karsch-Mizrachi et al. 2005) or the International Protein Index (Kersey, Duarte et al. 2004) which is denoted by an 'IPI' preceding the number. Spot numbers denoted with a prime signify situations where more than one likely protein was identified, alternative protein ID listed in the subsequent row.

Table 5 Differentially expressed serum proteins identified by MALDI-TOF/TOF.

	3 Hour/Control	24 Hour/Control	24 Hour/3 Hour			
Spot No.	**Abundance**	**Abundance**	**Abundance**	**Most Likely Protein ID**	**Accession**	**Functional Type**
1	Increased	Similar	Decreased	Transferrin [Mus musculus]	18606172	Carrier
2	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
3	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
4	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
5	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
6	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
7	Increased	Similar	Decreased	Keratin 18	254540068	Cytoskeleton
8	Increased	Similar	Decreased	NA (weak mass spectrum)	--	--
9	Increased	Increased	Decreased	NA (weak mass spectrum)	--	--
10	Increased	Similar	Decreased	NA (weak mass spectrum)	--	--
11	Increased	Similar	Decreased	NA (weak mass spectrum)	--	--
12	Increased	Increased	Decreased	NA (weak mass spectrum)	--	--
13	Increased	Increased	Similar	Immunoglobulin light chain variable region	5705888	Immunoglobulin
14	Increased	Increased	Decreased	Immunoglobulin light chain variable region	5705888	Immunoglobulin
15	Increased	Similar	Decreased	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
16	Increased	Increased	Similar	Serum Albumin (or its precursors or degradation product)	20330098	Carrier
17	Increased	Increased	Similar	NA (weak mass spectrum)	--	--
18	Increased	Increased	Similar	Beta-2 Globin	17647499	Immunoglobulin
19	Increased	Increased	Similar	Beta-1 Globin	4760590	Immunoglobulin
20	Increased	Similar	Similar	NA (weak mass spectrum)	--	--

Proteins which were either up or down regulated within serum, accession numbers correlates to either NCBI GenBank (Benson, Karsch-Mizrachi et al. 2005) or the International Protein Index (Kersey, Duarte et al. 2004) which is denoted by an 'IPI' preceding the number. Spot numbers denoted with a prime signify situations where more than one likely protein was identified, alternative protein ID listed in the subsequent row.

Table 6 Differentially expressed splenic tissue proteins identified by MALDI-TOF/TOF.

	3 Hour/Control		24 Hour/Control		24 Hour/3 Hour				
Spot No.	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Abundance**	**Volume Ratio**	**Most Likely Protein ID**	**Accession**	**Functional Type**
1	Increased	2.8	Increased	3.76	Similar	1.35	Albumin 1	33859506	Carrier
2	Similar	-1.83	Similar	-1.9	Similar	-1.04	Placental protein 39	76639367	Elongation Factor
3	Similar	1.42	Increased	2.01	Similar	1.42	Methylenetetrahydrofolate dehydrogenase 1	20270275	Enzyme
4	Decreased	-3.76	Decreased	-3.69	Similar	1.02	Protein disulfide isomerase associated 2	20899196	Enzyme
5	Increased	2.63	Similar	-1.11	Decreased	-2.91	Albumin 1	33859506	Carrier
6	Similar	1.79	Similar	1.23	Similar	-1.45	HNRPL	71119320	Ribonuclear
7	Decreased	-10.5	Decreased	-12.75	Similar	-1.21	Hypothetical protein LOC67745	34304054	Unknown
8	Decreased	-3.4	Decreased	-3.45	Similar	-1.01	Zinc finger protein 647	27370220	DNA Binding Protein
9	Similar	1.98	Similar	1.89	Similar	-1.04	Gamma actin	809561	Structural
10	Similar	1.84	Similar	1.67	Similar	-1.1	Gamma actin	809561	Structural
11	Increased	2.58	Similar	1.32	Similar	-1.95	Carbonic anhydrase 3	31982861	Enzyme
12	Increased	2.24	Increased	2.11	Similar	-1.05	Car 1 protein	15029975	Cytoskeletal
13	Decreased	-3.56	Similar	-1.31	Increased	2.74	Peroxiredoxin 6	6671549	Enzyme
14	Similar	-1.92	Similar	-1.37	Similar	1.41	HNRPA 3	23274114	Ribonuclear
15	Decreased	-2.24	Similar	-1.5	Similar	1.5	Glutathione S-transferase, Pi 1	10092608	Enzyme

Proteins which were either up or down regulated within splenic tissue, accession numbers correlates to either NCBI GenBank (Benson, Karsch-Mizrachi et al. 2005) or the International Protein Index (Kersey, Duarte et al. 2004) which is denoted by an 'IPI' preceding the number. Spot numbers denoted with a prime signify situations where more than one likely protein was identified, alternative protein ID listed in the subsequent row.

Table 7 Differentially expressed hepatic tissue proteins identified by MALDI-TOF/TOF.

	3 Hour/Control		24 Hour/Control		24 Hour/3 Hour				
Spot No.	Abundance	Volume Ratio	Abundance	Volume Ratio	Abundance	Volume Ratio	Most Likely Protein ID	Accession	Functional Type
1	Decreased	-9.48	Decreased	-14.31	Similar	-1.49	Myosin heavy polypeptide 2	56206250	Structural
2	Increased	2.53	Increased	5.54	Increased	2.22	Carbamoyl phosphate synthetase 1	82879179	Enzyme
3	Similar	1.96	Increased	2.43	Similar	1.26	Carbamoyl phosphate synthetase 1	82879179	Enzyme
4	Similar	-1.48	Similar	-1.76	Similar	-1.18	Leprecan-like 1 protein	27527202	Unknown
5	Similar	1.14	Increased	2.98	Increased	2.66	Carbamoyl phosphate synthetase 1	82879179	Enzyme
6	Increased	2.12	Increased	2.3	Similar	1.1	Hypothetical protein XP_890111 isoform 1	82942647	Unknown
7	Increased	2.08	Increased	2.53	Similar	1.23	Transferrin	20330802	Carrier
8	Decreased	-2.43	Decreased	-2.81	Similar	-1.14	Phosphoenolpyruvate carboxykinase 1	7110683	Enzyme
9	Increased	2.84	Increased	2.79	Similar	-1	Aldehyde dehydrogenase 1	27532959	Enzyme
10	Similar	1.21	Increased	4.07	Increased	3.4	Heat Shock Protein 90, Grp94	14714615	Chaperone
11	Similar	-1.67	Similar	-1.65	Similar	1.03	Glutamate dehydrogenase 1	6680027	Enzyme
12	Decreased	-2.11	Decreased	-2.33	Similar	-1.09	3-hydroxy-3-methylglutaryl-CoA synthetase 2	31560689	Enzyme
13	Similar	1.07	Increased	3.74	Increased	3.55	Immunoglobulin heavy chain variable region	5705888	Immunoglobulin
14	Decreased	-3.41	Decreased	-3.94	Similar	-1.14	4-hydroxyphenylpyruvate dioxygenase	849053	Enzyme
15	Similar	1.69	Increased	4.08	Increased	2.46	S-adenosyl-L-homocysteine hydrolase	904132	Enzyme
16	Decreased	-2.47	Decreased	-2.14	Similar	1.17	Leprecan-like 1 protein	27527202	Unknown
17	Decreased	-8.54	Decreased	-6.38	Similar	1.36	Calreticulin	13097432	Chaperone
18	Increased	3.53	Increased	7.16	Increased	2.05	Carbonyl reductase	15215242	Enzyme
19	Decreased	-2.46	Similar	-1.07	Increased	2.32	Indolethylamine N-methyltransferase	6678281	Enzyme
20	Similar	1.37	Increased	3.45	Increased	2.55	Carbonyl reductase	15215242	Enzyme
21	Decreased	-2.73	Decreased	-2.81	Similar	-1.02	Major Urinary protein 2	47059037	Enzyme

Proteins which were either up or down regulated within hepatic tissue, accession numbers correlates to either NCBI GenBank (Benson, Karsch-Mizrachi et al. 2005) or the International Protein Index (Kersey, Duarte et al. 2004) which is denoted by an 'IPI' preceding the number. Spot numbers denoted with a prime signify situations where more than one likely protein was identified, alternative protein ID listed in the subsequent row.

Discussion

An immune response capable of reducing levels of a systemically infected bacterium, such as the one initiated by ICPF would also be expected to alter protein expression throughout several of an organism's systems. Utilizing 2D DIGE our aim was to (1) to verify that ICPF instigates a time-related proteomic shift and (2), identify potential key proteins which, through their differential expression (as assessed by relative abundance or post-translational modification) may help elucidate ICPF's mode of action. 2D DIGE combined with MS/MS protein identification serves as a valuable tool for biomarker identification of both diseased states and treatment efficiencies (Kondo 2008; Poschmanna, Siteka et al. 2008).

We chose to investigate such changes within central nervous tissue as ICPF has been implicated (unpublished) as a therapeutic agent for the treatment of symptoms associated with multiple sclerosis, an autoimmune disease which results in neurodegenerative demylenation of nerve cells. Cytokines, which are believed to play a role in ICPF's mode of action are able to alter blood brain barrier characteristics and integrity while triggering the release of additional downstream cytokines, prostaglandins, nitric oxide, and other paracrine factors (Banks, Lynch et al. 2008) within central nervous tissue. We used a subset of the total number of resolved proteins for identification and several of the identified proteins show promise in having specific immune modulating properties; particularly interferon inducible protein 1 (Irgm1) which showed a nearly fourfold increase in expression within three hours of ICPF administration. Irgm1 expression is regulated by a type II interferon, IFN-γ, as well as the endotoxin lipopolysaccharide (MacMicking, Taylor et al. 2003; Feng, Zheng et al. 2008) and has been shown to be required for innate immune defense against several species of

intracellular bacteria, including *S. typhimurium* (Shenoy, Kim et al. 2008). While little is currently known about how Irgm1 is able to provide protection against such infections, murine Irgm1 has been shown to increase autophagy and elimination of intracellular bacteria within macrophages (Singh, Davis et al. 2006), and its presence and rapid up regulation in the absence of lipopolysacharide implicate ICPF as either an inducer of IFN-γ, a pluripotent innate immune cytokine or as a direct inducer if Irgm1. The later is unlikely as there is no reason to suspect that ICPF would be able or does cross the blood brain barrier, and IFN-γ shows little to no structural or sequence homology with ICPF. Cytokines however, are typically transiently expressed (often in a series of wavelike emanations) as low abundant proteins. The restoration of Irgm1 to a near baseline level 24 hours post-administration of ICPF may well mirror such an induced cytokine cascade.

In addition to interferon inducible protein, several proteins without direct or apparent immunological functions have been identified within the brain as being up or down regulated following ICPF administration. Five cytoskeletal related proteins where found to be differentially expressed; dynamin like-1 protein which is believed to function in microtubule transport of cellular vesicles and endocytosis (Leinonen, Nardone et al. 2006) was increased within three hours of treatment. Neurofilament-L and tropomyosin β chain were decreased following treatment while α-internexin was increased before subsequent down regulation at 24 hours. Neurofilament-L is a form of intermediate filament which along with M and H forms, comprise neurofilaments (Shah, Flanagan et al. 2000). Tropomyosin β chain binds actin filaments in association of with the troponin complex (Brown, Kim et al. 2001) and α-internexin serves as an intermediate neural filament which plays a role in cytoskeletal development and neuronal morphogenesis (Liem and Messing 2009). Coronin-1A showed an increase at 24 hours following

treatment and is believed to be a crucial component of the cytoskeleton of mobile cells and is involved in cellular locomotion and phagosome fusion with lysosomes (Mugnier, Nal et al. 2008). Several enzymes were differentially expressed within the brain following ICPF treatment including; disulfide isomerase, carbonic anhydrase VI, phosphomannomutase 2, and superoxide dismutase which were all up-regulated three hours post treatment. Disulfide isomerase catalyzes the formation, breakage and rearrangement of disulfide bonds (Darby, Freedman et al. 1994), carbonic anhydrase is responsible for the reversible hydration of carbon dioxide, phosphomannomutase 2 is involved in the synthesis of the GDP-mannose and dolichol-phosphate-mannose which is required for a number of critical mannosyl transfer reactions (Mizugishi, Yamanaka et al. 1999; Leinonen, Nardone et al. 2006) and superoxide dismutase which destroys radicals produced within the cells that are normally toxic to most biological systems. Enzymes which were down regulated or expressed following ICPF treatment include dihydropyrimidinase-like 2, pyruvate dehydrogenase, quinoid dihydropteridine reductase, and peroxiredoxin-6. Dihydropyrimidinase roles include axon growth and guidance as well as neural cell migration (Deo, Schmidt et al. 2004; Leinonen, Nardone et al. 2006) while pyruvate dehydrogenase E1 α is a member of the pyruvate dehydrogenase complex which catalyzes the conversion of pyruvate to acetyl-CoA and CO_2 (Gopalakrishnan, Rahmatullah et al. 1989; Leinonen, Nardone et al. 2006). Quinoid dihydropteridine reductase produces tetrahydrobiopterin (BH-4) (Tegeder, Costigan et al. 2006), an essential cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases and peroxiredoxin-6, a highly conserved thiol specific antioxidant protein (Eismann, Huber et al. 2009). Additional proteins which showed an increase in expression within three hours of treatment include; transferrin, a carrier protein responsible for iron sequestering and

transport, a 70 kDa heat shock protein and chaperone found within the endoplasmic reticulum, tubulin which is a major component of cellular microtubules, a G protein β subunit which acts as an intracellular receptor to protein kinase C with potential kinase activating properties (Leinonen, Nardone et al. 2006), and kallikrein, which is able to selectively cleave Met-Lys and Arg-Ser bonds in kininogen to release Lys-bradykinin (Marcondes and Antunes 2005). Syntaxin-binding protein 1 expression was decreased three hours following treatment and is thought to play a role in synaptic vesicle endocytosis (Zanner, Gratzl et al. 2004; Leinonen, Nardone et al. 2006). Down syndrome cell adhesion molecule-like protein expression was decreased at 24 hours and plays a role in axon guidance, receptor regulation, targeting, and branching (Li and Guan 2004) within drosophila, however its role in mice and humans is still unknown.

Liver and splenic tissue was evaluated due to ICPF's ability to reduce splenic bacterial load in *S. typhimurium* infections (Willeford, Parker et al. 2001) and the liver's physiologic role in protein metabolism, acute phase protein regulation, and toxin filtration. Within both splenic and hepatic tissue, no proteins of direct immunological importance were identified. However, several metabolic enzymes such as phosphoenolpyruvate carboxykinase, peroxiredoxin-6, methylenetetrahydrofolate dehydrogenase 1, and aldehyde dehydrogenase (Table 7) and (Table 8), did show significant alterations in expression levels, indicating possible modulation of metabolic processes. Proteins which were decreased within the spleen following ip treatment with ICPF include an uncharacterized disulfide isomerase, zinc finger protein 647 which is thought to play a role in transcriptional regulation (Leinonen, Nardone et al. 2006), peroxiredoxin-6 (previously described), and glutathione S-transferase which catalyses the conjugation of reduced glutathione. Both carbonic anhydrase (previously described) and

methylenetetrahydrofolate dehydrogenase were increased following IPCF administration within the spleen. Methylenetetrahydrofolate dehydrogenase roles include the catalysis of the interconversion of tetrahydrofolate derivatives which are required for synthesis of purines, thymidylate, and methionine (Tremblay, Mejia et al. 1992). Two heterogeneous nuclear ribonucleo proteins (hnRNP A3 and hnRNP L) were identified within spleen samples, however no changes in expression where noted. Within hepatic tissue, a myosin associated peptide, phosphoenolpyruvate carboxykinase, 1,3-hydroxy-3-methylglutaryl-CoA synthetase, 4-hydroxyphenylpyruvate dioxygenase, leprecan-like 1 protein, calreticulin, and indolethylamine N-methyltransferase were all down regulated within three hours of ICPF administration. Phosphoenolpyruvate carboxykinase catalyzes the conversation of oxaloacetate to phosphoenolpyruvate for the formation of glucose in gluconeogenesis. 1,3-hydroxy-3-methylglutaryl-CoA synthetase condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA (Chang and Limanek 1980; Leinonen, Nardone et al. 2006) and 4-hydroxyphenylpyruvate dioxygenase catalyzes the catabolism of tyrosine (Leinonen, Nardone et al. 2006). Leprecan-like 1 protein (Prolyl 3-hydroxylase 2) catalyses the post-translational formation of 3-hydroxyproline in collagen types IV and V (Mizuno, Hayashi et al. 2004; Leinonen, Nardone et al. 2006; Vranka, Pokidysheva et al. 2010). Calreticulin is a calcium binding protein with chaperone properties commonly found within the endoplasmic reticulum and indolethylamine N-methyltransferase catalyzes the methylation of tryptamine (Leinonen, Nardone et al. 2006). Protein expression which increased in hepatic tissue following ICPF treatment include carbamoyl phosphate synthetase which is involved in ammonia removal via the urea cycle, 10-formyltetrahydrofolate dehydrogenase which catalyzes the oxidation of 10-formyltetrahydrofolate to CO_2 and tetrahydrofolate (Schirch, Villar et al. 1994), heat

shock protein 90, a stress induced chaperone, carbonyl reductase, an enzyme with a broad substrate specificity able to catalyze the reduction of various carbonyl compounds (Leinonen, Nardone et al. 2006). S-adenosyl-L-homocysteine hydrolase is believed to play a role in the control of intracellular methylation reactions by regulating the intracellular concentration of adenosylhomocysteine, which is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions (Malanovic, Streith et al. 2008).

Our serum profile was unable to detect any low abundant proteins (e.g. cytokines, chemokines), possibly due to masking from several high abundant proteins such as serum albumin, transferrin, and immunoglobulins, all of which were present at high concentrations. Eighty five percent of serum protein composition (Echan, Tang et al. 2005) is composed of only six high abundant proteins, making the detection and identification of differentially expressed low abundant proteins a challenging prospect. Changes in protein concentrations within serum are carefully regulated by the body, and often transient – compounding the experimental challenges in resolving them.

Respiratory tissue was evaluated due to ICPF's ability to alleviate lower respiratory disease within horses (Hamm, Willeford et al. 2002) as well as the lungs immunological role in early detection of inhaled pathogens (Murphy, Davis et al. 2004). We attempted to identify 10 potential proteins which yielded differential expression levels, however only three of the identified proteins, serum albumin, dihydropyrimidinase-like 2, and Calponin 2 were differentially expressed with any level of significance. Serum albumin represents the main protein of plasma and it serves as a transfer protein with good binding capacities for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, bilirubin and drugs and in addition to analyte transport, it functions in the

regulation of colloidal osmotic pressure of blood (Don and Kaysen 2004; Leinonen, Nardone et al. 2006). Calponin 2 is a cytoskeletal protein which is believed to play a role in the regulation and modulation of smooth muscle contraction, likely through its ability to bind actin and calmodulin (Sugenoya, Yoshimura et al. 2002). Dihydropyrimidinase-like 2, an enzyme involved in nucleoside and nucleotide metabolism, was found to be up regulated three hours following ICPF administration. The significance of this find relates to previous work conducted in our lab which shows that ICPF is able to reduce tumorgenesis by nearly 50% in murine respiratory melanoma (Parker, Willeford et al. 2005). While the precise role of dihydropyrimidinase-like 2 in the formation of lung cancer remains questionable, it has been found to be down regulated in murine lung adenocarcinoma models and up regulated in the presence of other immunotherapies which have also been shown to be potent inhibitors of lung tumorigenesis (Bortner, Das et al. 2009) however, another conflicting study, while also validating the down regulation of dihydropyrimidinase-like 2 by carcinogens and in lung cancer, have yielded no evidence of its upregulation in the presence of chemoprotective agents (Kassie, Anderson et al. 2008). Five additional enzymes were identified in lung samples treated with ICPF; however they showed no significant alterations in expression following treatment. Electron transfer flavoprotein-ubiquinone oxidoreductase is a mitochondrial membrane protein which is able to accept electrons from mitochondrial electron transfer flavoprotein dehydrogenases and transport them to ubiquinone (Beckmann and Frerman 1985). Retinol dehydrogenase, a member of the aldehyde dehydrogenase family and its roles include in the oxidation of aldehydes derived from biogenic amines such as epinephrine and norepinephrine, as well as the aldehydes generated via lipid peroxidation, and is often found in the liver, lung, and testis (Zhang, Chen et al. 2001;

Maeda, Maeda et al. 2005; Leinonen, Nardone et al. 2006). Peroxiredoxin-6, carbonic anhydrase, and superoxide dismutase (previously described) were also identified in lung samples. A-2 macroglobulin, a protease inhibitor (Armstrong and Quigley 1999) also referred to as pregnancy zone protein, was identified. Two intracellular proteins, an endosomal eps15-homology (EH) domain (Braun, Pinyol et al. 2005) and the structural protein laminin A which are components of the nuclear lamina (Fong, Ng et al. 2006).

2D DIGE is a powerful tool in the search of differentially expressed proteins which may play a role in pathogenesis or the therapeutic properties of a drug or immune therapy, however it was not particularity well suited for the identification of small peptides or low abundant proteins, especially in complex tissue system such as serum. Removal of select high abundant proteins prior to 2D DIGE by either Cibricon Blue, a chlorotriazine dye with a high affinity towards albumin (Gianazza and Arnaud 1982), as well as immunoglobulin depletion matrixes Protein A and Protein G, where considered but poor protein specificity and retention of low abundant proteins, which are often nonspecifically bound to albumin, prevented its use. 2D DIGE also has limitations in its resolving power, despite its impressive ability to resolve several hundred proteins on a single gel, this number represents a fraction of the estimated 20,000 proteins expressed in the mouse (Nekrutenko 2004).

In a more focused approach, systemic levels of the apolipoprotein Serum Amyloid A was evaluated by ELISA. Acute phase proteins play a central role in innate immunity, and SAA along with C-Reactive Protein are the major acute phase protein within mammals, including man and has been used to screen for infectious diseases, to monitor their response to therapy, and to distinguish between bacterial and viral infections (Ducret, Bruun et al. 1996). SAA exists naturally within serum at low concentrations (1-

5 μg/mL), usually complexed with high density lipoproteins, and during an acute phase response may increase to nearly 1 mg/mL within 24 - 36 hours post stimulation (Clarissa and Alexander 1999). SAA exists as 12kDa monomer, who's structure has not been fully elucidated, in two isoforms, SAA1 and SAA2, however SAA1 is the predominant acute phase form. During our analysis of SAA we were unable to differentiate between SAA1 and SAA2 due to considerable structural and sequence homology, they differ by 5 - 7 amino acids and both have 2 regions of high homology (Ducret, Bruun et al. 1996), therefore we will use SAA to imply both isoforms, SAA1 and SAA2. ICPF's ability to stimulate SAA expression shows for the first time a possible direct mode of action for this peptides ability to alleviate mortality associated with gram negative induced sepsis. SAA is capable of rapidly binding with great affinity to a common protein on the membrane of gram negative bacteria, including *S. typhimurium*, known as Outer Membrane Protein A (Hari-Dass, Shah et al. 2005) and act as an opsonizing agent by assisting in phagocytosis for monocyte derived macrophages and neutrophils (Shah, Hari-Dass et al. 2006). SAA is a type 1 acute phase protein produced largely by hepatocytes initiated synergistically by Il-1 and Il-6 proinflamatory cytokines (Jiang, Lozanski et al. 1995). It is currently unknown if ICPF increase SAA concentration *in vivo* by direct activation of hepatocytes or by triggering the synthesis and release of Il-1 and Il-6 from an as yet unknown mechanism.

The upregulation of both Irgm1 and SAA and to a lesser extent, dihydropyrimidinase-like 2, help advance our understanding of the immunotherapeutic properties of ICPF within our model system. The opsonization and increased phagocytosis induced by SAA in conjunction with possible increased elimination of phagocytized intracellular bacteria from upregulation of the IFN-γ effector molecule,

Irgm1 provides, for the first time, a detailed and working hypothesis for a mode of action associated with ICPF's immune modulating properties for the sequestering and elimination of *S. typhimurium*.

CHAPTER IV

BIOASSAY DESIGN AND ANALYSIS OF CYTOKINE EXPRESSION *IN VIVO* AND *IN VITRO* FOLLOWING ADMINISTRATIONS OF IMMUNE CELL POTENTIATING FACTOR

Introduction

Cytokines and chemokines are key components in immune regulation and activation, often thought of as the "hormones" of the immune system, they are typically endogenous, extracellular immunoregulatory compounds. Cytokines are instrumental in disease progression and diagnosis, as well as immune signaling with potential for both autocrine and paracrine functionality. Both cytokines and chemokines are typically small and proteinaceous, and their roles in the immune system, while often overlapping, may differ considerably. Cytokines help regulate an immune response by signaling for a particular cell type to increase in number (proliferation), differentiation, or maturation, as in the case of lymphocytes and monocytes. The presence or absence of a particular cytokine during an immunological challenge can alter the route of an infection as well as the body's reaction to a pathogen. Chemokines are typically responsible for bringing different cell types together, operating in conjunction with specific receptors on both myeloid and lymphoid cells, they work toward directing chemotaxis and generating a localized immune response, as well as cellular activation, proliferation, and memory (Murphy 2009). Both cytokines and chemokines typically work synergistically to generate a myriad of immune responses, from an inflammatory event surrounding a

wound, to systemic reactions such as; generating a fever, initiating an acute phase response, or the activation of the complement cascade (Andrews, Feldhoff et al. 2003).

In recent years cytokines and chemokines have come to the forefront of immunology as we have further elucidated their roles in disease and diagnosis. Many autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and lupus have been shown to be caused, diagnosed, or successfully controlled (in some cases treated) with cytokine and chemokine intervention therapies, such as etanercept, and adalimumab. Both of which work by suppressing TNF-α (Lovell, Ruperto et al. 2008; Griffiths, Strober et al. 2010) and anakinra, which is believed to act as an IL-1 receptor antagonist (Botsios, Sfriso et al. 2007). Nearly every BRM currently on the market has a role as an agonist or antagonist of cytokines or chemokines, additionally some BRM's are nothing more than recombinant cytokines, as is the case for IFN-α, IFN-γ and IL-2 (H Bönig, H-J Laws et al. 2000; Stefania, Antonella et al. 2001) for the treatment of some cancers.

Our understanding of ICPF's role in the inflammatory response, its reported success in alleviating some of the symptoms of multiple sclerosis, as well as its apparent potency in the murine sepsis model, may all be attributed to the regulatory control of cytokines and chemokines. Cytokines and chemokines never work alone. One cytokine can have several different effects on a system, sometimes starkly opposite effects. Depending on the cell(s) that secreted the cytokine, the cell(s) the cytokine interact with, and the location in the body of the previous dependencies, all play a role in the effect of the immunological response, and ultimately on the organism. It is now also believed that antibodies and other biological chemicals can interact with these cytokines to augment or alter these effects, leading to yet another level of immunological regulation (Bradney, Sempowski et al. 2002; Wassef and Plaeger 2002).

Due to the pleotropic nature of most cytokines, the potential number of cells and systems being affected by ICPF, and the possibility that ICPF may not be the only component in the immune augmentation we have observed; delineating an accurate mode of action for this compound has proven difficult. Through the use of single analyte assays and systemic proteomic profiling by means of ELISA and 2D DIGE, we have shown that ICPF can have a modulating effect on several proteins within the murine model. However, a traditional ELISA is typically only able to measure a single cytokine level within a sample at any given time and 2D DIGE, while capable of screening for hundreds of potential proteins, has been inefficient at measuring specific, low abundant proteins such as cytokines and chemokines in complex tissue samples. While knowing the regulation of one cytokine can point us in a direction, it is imperative that we survey several cytokine levels simultaneously within a single sample, as temporal variations within different individuals, even within the same species, can vary dramatically. Through the use of infrared (IR) multiplex protein arrays we can accurately measure the levels of dozens of cytokines and chemokines at the same time within a single sample from one individual, thereby elucidating complex cytokine pathways and a potential mode of action.

In addition to elucidating ICPF's mode of action, developing a new bioassay to supplement our current animal mortality model was imperative. Further characterization of ICPF as well as its immunological potentials has proven difficult due to the time, cost, and ethical constraints involved in our animal mortality model. It was for these reasons that we set out to develop an *in vitro* assay utilizing murine whole blood. A whole blood assay capable of mirroring some of the *in vivo* biological responses to ICPF would allow for greater flexibility for future testing and analysis of this and any subsequent

derivatives/modifications of the peptide. Developing a new *in vitro* bioassay to study mode of action was complicated due to our lack of understanding of the specific biological changes induced by ICPF *in vivo* and we therefore chose to tackle both issues concurrently by mapping any alterations in cytokine and chemokine expression by way of a multiplex IR based Fluorescent Linked Immuno-Sorbent Assay (IR-FLISA).

Materials and Methods

Murine Animal Care and Maintenance

Specific Pathogen Free (SPF) female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were allowed to acclimate for 2 weeks upon arrival, during which time they were fed a standard maintenance diet (Laboratory Rodent Diet 5001; PMI Feeds) and watered *ad libitum*. Mice were group-housed (5 mice per cage) in plastic boxes bedded with wood shavings in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. The isolation room was maintained at 20° C in a controlled negative pressure environment on a 12-h lighting cycle. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Mississippi State University protocol number 05-051.

Whole Blood Culture

Twenty five mL of EDTA heparinized murine whole blood (Bioreclamation, Liverpool, NY) was diluted 1:5 (v/v) in RPMI 1640 (Thermo Scientific, Waltham, MA) culture medium, distributed into 24-well tissue culture plates (1.5 mL/well) and inoculated with 7.5 µl of 10 mg/mL ICPF (50 µg/mL) and serving as a negative control 7.5 µl sterile dPBS. Plates were incubated at 37° C with 5% CO_2, cells and supernatant

were collected at 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours, centrifuged at 900 RCF for five minutes at 4° C and supernatant collected and frozen at -80° C until needed. Nucleated cellular enumeration was determined utilizing a Nucleocounter™ from New Brunswick Scientific (Edison, New Jersey) as per manufactures specifications.

Peripheral Blood Mononuclear Cell Preparation and Culture

Fifty mL of EDTA heparinized murine whole blood (Bioreclamation, Liverpool NY) was centrifuged at 400 RCF for 10 minutes at 25° C. Buffy coat (thin white layer between erythrocytes and serum) was collected and transferred to 14 mL conical tubes. 1:1 (v/v) of AKC erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA•Na_2) was added to buffy coat extract and gently agitated. Cellular suspension was centrifuged at 400 RCF for 10 minutes at 25° C and supernatant discarded. The process was repeated one additional time if needed. Remaining cells, predominantly leukocytes and platelets were then diluted 1:5 (v/v) in RPMI 1640 culture medium, distributed into 24-well tissue culture plates (1.5 mL/well) and inoculated with 7.5 µl of 10 mg/mL ICPF (50 µg/mL) and serving as a negative control, 7.5 µl sterile dPBS. Plates were incubated at 37° C with 5% CO_2. Cells and supernatant were collected at 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours, centrifuged at 900 RCF for five minutes at 4° C and supernatant collected and frozen at -80° C until needed. Nucleated cellular enumeration was determined utilizing a Nucleocounter™ from New Brunswick Scientific (Edison, New Jersey) as per manufactures specifications.

Murine Serum Preparation and ICPF Administration

Sixty-nine mice were broken into two groups, a negative control and an ICPF treated group. Mice within the treated group received a 0.2 mL ip inoculation of 5.0 mg

ICPF in sterile dPBS. Negative control mice were sham handled and injected ip with 0.2 mL sterile dPBS. At 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 24 hours post treatment, three mice from both groups (except 0 time point which consisted of only one group) were anesthetized by CO_2 asphyxiation and 0.8 – 1.0 mL blood collected by cardiac puncture, mice were then humanely euthanized. Blood was placed in a 2 mL microtube and allowed to clot for two hours at room temperature with gentle agitation. Samples were then centrifuged at 1500 RCF for 10 minutes at 4° C and serum (30 – 40 µl) transferred to a sterile tube and stored at -80° C until needed.

IL-6 IR-FLISA Development

Capture antibodies consisting of rat monoclonal anti-murine IL-6 (Abcam, Cambridge MA) were diluted to 1.5 µg/mL and 100 µl added to each well of an optically clear opaque walled 96-well fluorescence microplate (Thermo Scientific, Waltham, MA). Capture antibodies were allowed to incubate overnight at 4° C with gentle agitation. Non-bound capture antibodies were removed and wells washed 3 – 5 times with dPBS and 0.05% Tween-20 to increase stringency of wash. 300 µl of blocking solution consisting of either sterile 5% bovine serum albumin or 5% non-fat dried skim milk was added to each well, and incubated at room temperature for 2 – 3 hours. Protein standards consisting of recombinant murine IL-6 (Bender Medsystems, San Diego CA) were added at 8.5 – 5,000 pg/mL in an 11 step 1:3 serial dilution, with one well serving as a blank. Samples consisting of murine serum following ICPF administration at 0, 3, and 24 hours (n = 3 per group) were diluted 1:1 in reagent diluent (0.1% BSA, 0.05% Tween-20, 0.02M Tris-Base, and 0.15 NaCl, pH 7.2), to reduce non-specific protein-protein interactions and added to wells for eight hours at 4° C. Samples and standards were

removed and wells washed as previously described. 100 µl rabbit anti-mouse IL-6 polyclonal antibody (Abcam, Cambridge, MA) was added to all wells at 8.5 µg/mL and allowed to incubate for five hours at 4° C. Following an additional wash step, 100 µl of goat anti-rabbit IR-680 antibody (Li-Cor Bioscience, Lincoln, Nebraska) was added. The microtiter plate was scanned with an Odyssey™ Infrared Imaging System (Li-Cor Bioscience, Lincoln, Nebraska) at 700 nm. IL-6 sample concentrations were determined with Softmax Pro 4.6 (Molecular Devices, Silicon Valley, CA) software, using curve fit models (log-log or 4-PL) as suggested by the manufacturer.

Multiplex Cytokine Analysis

The Pierce Endogen SearchLight™ Multiplex Protein Array (Pierce Biotechnology, Rockford, IL), a 96 well plate based assay was utilized for the detection of multiple cytokines and chemokine concentration determination. Calibration curves were prepared in sample diluent, with a range of 5000 pg/mL to 0.4 pg/mL, dependent upon the analyte being analyzed. Assay controls consisted of the sample diluent and the corresponding calibration standard. The Pierce Searchlight Assay was essentially performed as per the manufacturer's instructions, briefly; 50 µl of cytokine/chemokine standards and samples were loaded in duplicate (samples assayed in triplicate) to a 96 well microtiter plate, covered and allowed to incubate at 20 – 25° C for 3 hours on an orbital shaker. Following the incubation period, the contents of the plate were discarded and plate washed four times. 50 µl biotinylated antibody reagent was added to each well, covered, and allowed to incubate for an additional 30 minutes. Contents were again discarded and the plate washed four times. 50 µl of DyLight™800 (Pierce Biotechnology, Rockford, IL) was added to each well and incubated for 30 minutes,

discarded, and washed, as previously described. 300 µl ddH_2O was added to each well, and the plate centrifuged at 300 RFC for five minutes. The plate was then assayed using an Odyssey™ Infrared Imaging System (Li-Cor Bioscience, Lincoln, Nebraska). Instrumentation was set at a resolution of 84 µm, with a focus offset of 4 mm and scanned at 800 nm. Sample cytokine concentrations were determined with Softmax Pro 4.6 (Molecular Devices, Silicon Valley, CA) software, using curve fit models (log-log or 4-PL) as suggested by the manufacturer (Figure 10) and (Table 8).

Statistical Analysis

All experiments were arranged in a completely randomized block design and a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Pairwise Multiple Comparison (Student-Newman-Keuls Method) to calculate P-values (SyStat 2008) was used to compare data. Differences with P-values < 0.05 were considered significant.

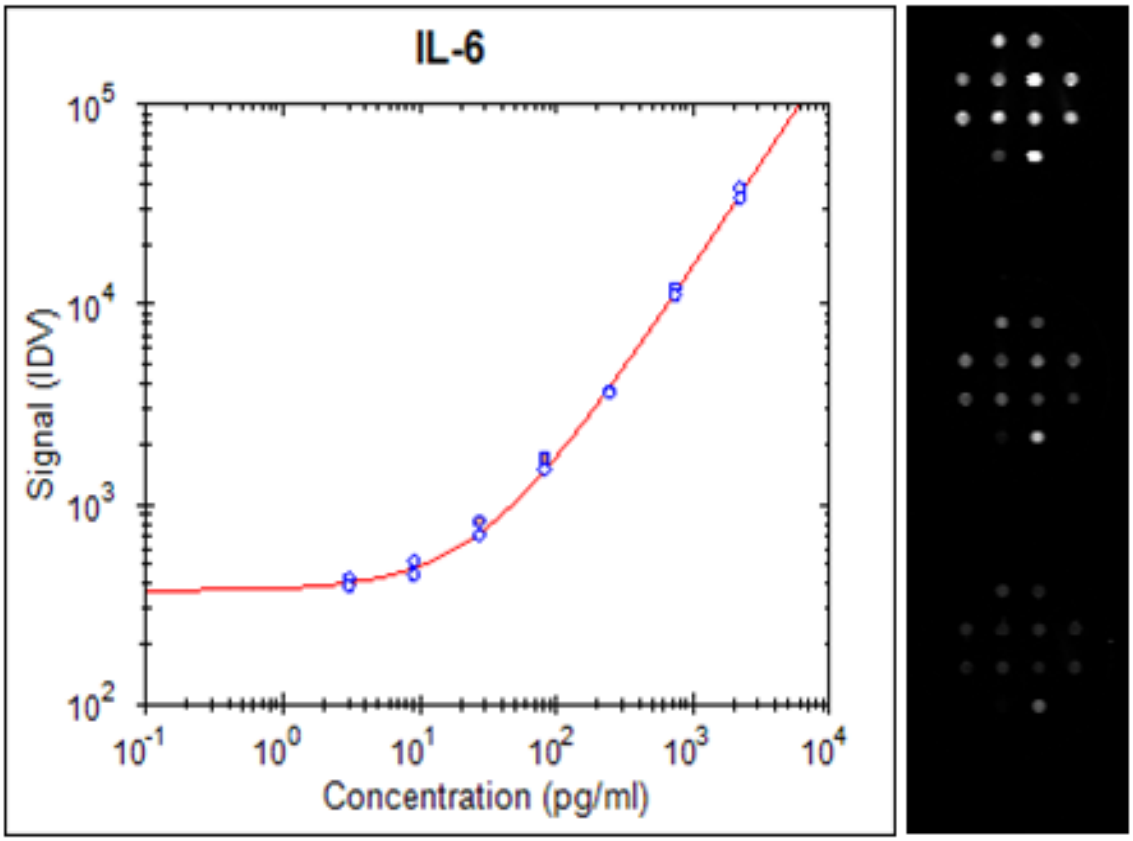


Figure 10 Representative scan and analysis of Searchlight™ Multiplex Protein Array

Four parameter logarithmic standard curve of IL-6 and representative IR-FLISA scan with cytokine and chemokine designation overlay

Table 8 Representative Multiplex FLISA Standard Curve

Rep 1	Rep 2	AVG	%C.V.	S.D.	pg/ml	BACKFIT
38236	34137	36186.4	8	2898.4	2200	2258.7
12017	11150	11583.4	5.3	613.7	733.33	746.1
3643	3610	3626.4	0.6	23.4	244.44	229.4
1509	1715	1611.7	9	145.8	81.48	91.4
702	823	762.9	11.2	85.5	27.16	30.5
519	446	482.4	10.6	51.3	9.05	9.2
426	393	409.5	5.8	23.6	3.02	3.4
382	352	367	5.7	20.9	0	1

a	370.31
b	1.05
c	39541026
d	1.01E+09
R^2	0.9923
LOD (pg/ml)	3.33
LOQ (pg/ml)	10.01

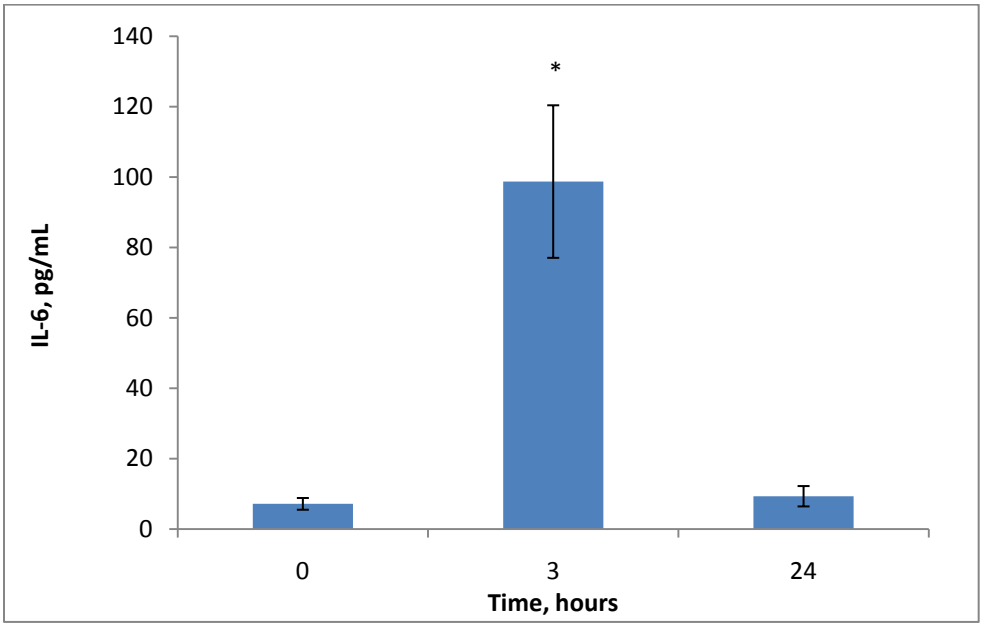
Representative Curve Fit Analysis and coefficient of determination and intra assay coefficient of variance.

Results

IL-6 *in vitro* IR-FLISA Development

A single analyte *in vitro* IR-FLISA was developed to detect IL-6 in both Peripheral Blood Mononuclear Cells (PBMC) as well as whole blood to determine any effect ICPF had over cytokine expression and as a 'proof of concept' before multiplex protein array technology would be considered. Initial IL-6 levels were at or below the assays detectable limits of 5.5 pg/mL. Following administration of 7.5 µl of 10 mg/mL ICPF (50 µg/mL), IL-6 concentration increased to 113 ± 28.1 pg/mL within three hours ($P < 0.05$) and returned to an *in vitro* physiological baseline (≤5.5 pg/mL) ($P < 0.05$) within 24 hours post exposure (Figure 11a). An intra assay coefficient of variance was calculated to be <25%. SDS-PAGE and fluorescent western blot analysis was conducted on samples and standards to determine the presence of any potential non-specific or antibody cross-reactivity which would compromise FLISA results (Figure 11b) and antibody binding appeared limited to IL-6. Cellular enumeration was determined to be 1.83×10^6 live nucleated cells per well for isolated PBMC.

A.



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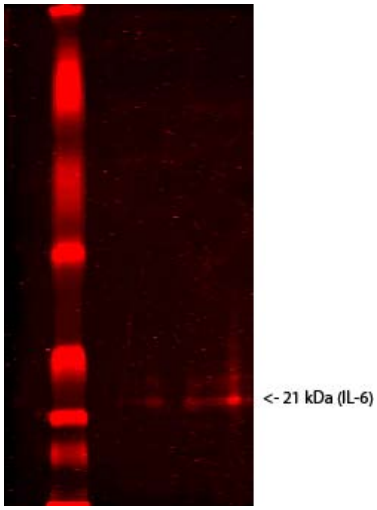


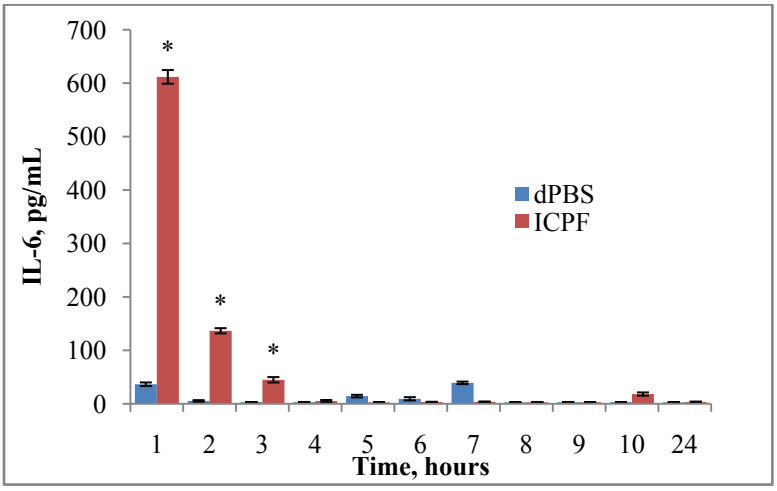
Figure 11 IR-FLISA development

A. Murine PBMC in a 24 well culture plate were treated with 50 µg/mL ICPF. Non-treated cells served as a zero time point and baseline for IL-6 expression. At 3 and 24 hours post ICPF treatment, cells were removed, centrifuged and supernatant assayed for IL-6 expression. IL-6 concentration was determined in pg/mL ± S.E.M of n=3. Significant differences between ICPF treated samples and controls ($P < 0.05$) are noted with an asterisk. B. IR-Fluorescent western blot analysis of IL-6 standards with detection and signal antibodies utilized in IR-FLISA.

In vivo Multiplex Cytokine Analysis

SPF Swiss Webster mice cytokine profiles were evaluated using Pierce Searchlight Multiplex protein array technology. Cytokine expressions evaluated following 5 mg ip ICPF injection included IL-1α, IL-1β, IL-2, Il-4, Il-5, IL-6, KC (CXCL1), IL-10, IL-12p40, IL-13, IFN-γ, and TNF-α with dPBS serving as a negative control. IL-6 concentrations increased to 611±22.1 pg/mL ($P < 0.05$) within one hour of ICPF treatment. Within two to three hours, IL-6 levels began dropping, reaching 136±8.5 ($P < 0.05$) and 44.8±8.7 pg/mL ($P < 0.05$) respectively. Within four hours IL-6 had returned to a concentration not significantly different than physiological baseline ($P > 0.05$) and remained at these levels for the duration of the study (Figure 12a). IFN-γ concentration increased to 197 ±65.0 pg/mL ($P < 0.05$) within one hour post treatment and returned to a concentration not significantly different than physiological baseline ($P > 0.05$) within two hours (Figure 12b). Slight fluctuations within IFN-γ continued for the duration of the study, however they remained low and were determined to not be significantly different from negative control.

A.



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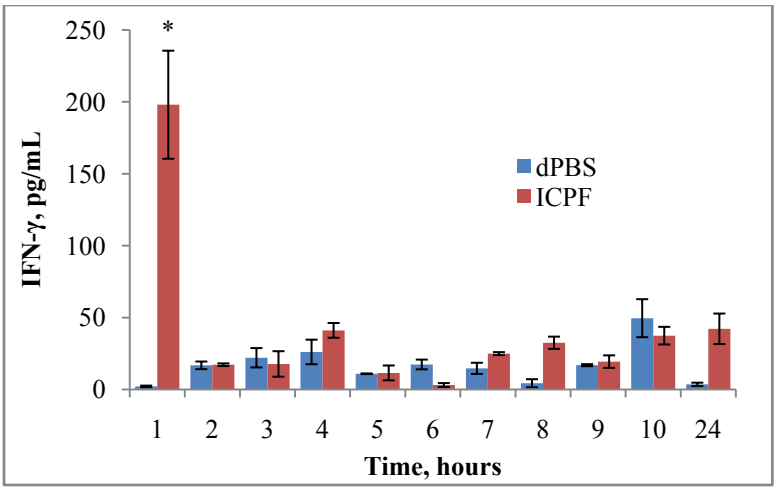


Figure 12 *In vivo* cytokine expression following ICPF treatment

A. IL-6 expression *in vivo* following ip administration of 5 mg ICPF. Three mice from each time point received ip 0.2 mL dPBS serving as a negative control, while three additional mice from each time point were treated ip with 5 mg ICPF. IL-6 concentration was determined in pg/mL ± S.E.M of n=3 with an average intra assay coefficient of variance of 7.0%. Significant differences between ICPF treated mice and controls ($P < 0.05$) are noted with an asterisk. B. Expression of IFN-γ within previously described mice. IFN-γ concentration was determined in pg/mL ± S.E.M of n=3 with an average intra assay coefficient of variance of 7.25%. Significant differences between ICPF treated mice and controls ($P < 0.05$) are noted with an asterisk.

The only murine chemokine to show any significant increase over the negative control following ICPF treatment was KC, also known as CXCL1. KC is a murine chemokine belonging to the CXC family of mouse chemokines. Following ICPF administration, KC increased in concentration to 61.3±3.3 pg/mL ($P < 0.05$) from a physiological baseline of 2.5 pg/mL, KC continued to increase in the blood for an additional hour; peaking at 104.7 ± 6.4 pg/mL ($P < 0.05$) at two hours post injection before beginning to decrease to 40.2 ± 7.6 pg/mL ($P < 0.05$) and beginning at three hours and within four hours KC had returned to physiological base line. At seven hour post treatment, the systemic concentration briefly increased to 40.46±13.0 pg/mL ($P < 0.05$) before once again returning to baseline which was determined to not be significantly different from negative control ($P > 0.05$) (Figure 12).

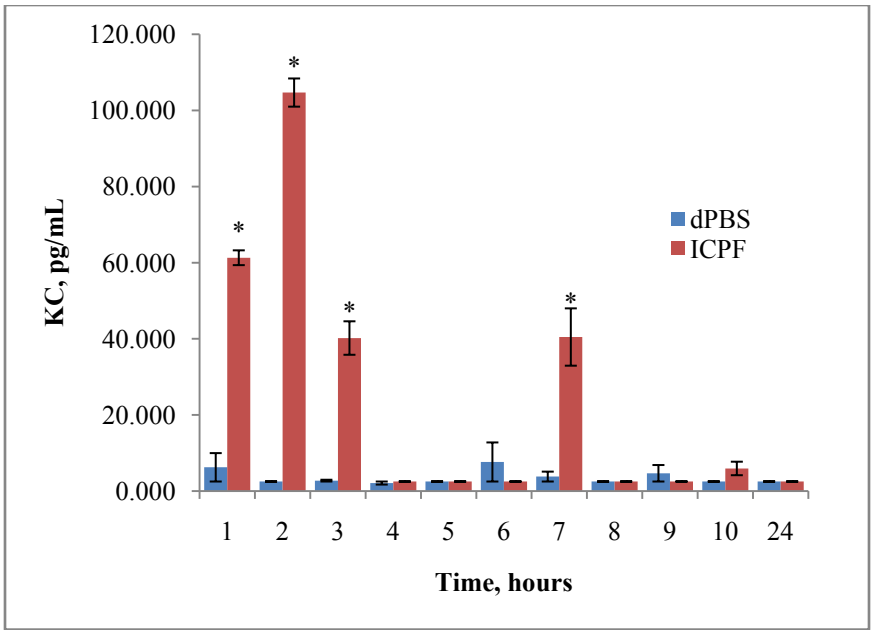


Figure 13 *In vivo* chemokine expression following ICPF treatment

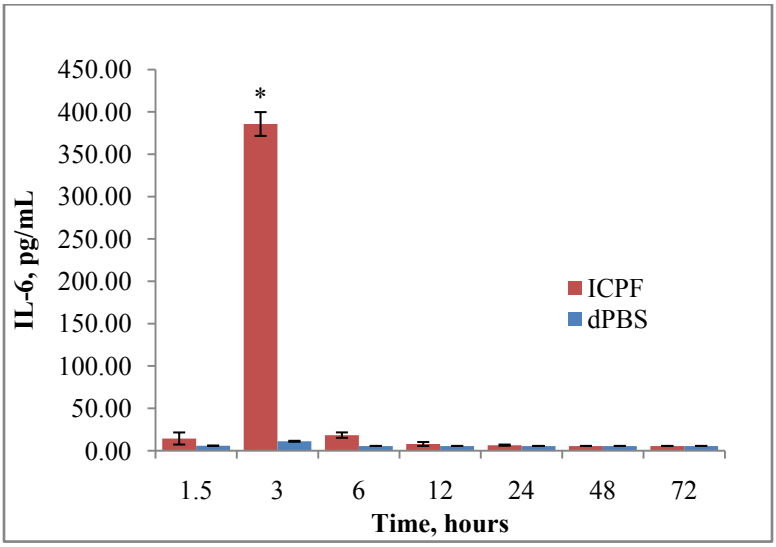
KC expression *in vivo* following ip administration of 5 mg ICPF. Three mice from each time point received ip 0.2 mL dPBS serving as a negative control, while three additional mice from each time point were treated ip with 5 mg ICPF. KC concentration was determined in pg/mL ± S.E.M of n=3 with an average intra assay coefficient of variance of 8.5%. Significant differences between ICPF treated mice and controls ($P < 0.05$) are noted with an asterisk.

In vitro Multiplex Cytokine Analysis

In vitro cytokine analysis was performed on whole blood in order to both confirm results documented within *in vivo* studies as well as aid in future bioassay design. IL-6 and IFN-γ were both expressed in similar patterns as seen *in vivo*, with IL-6 expression peaking at 385±24.3 pg/mL ($P < 0.05$) at three hours before returning to baseline which was determined to not be significantly different from negative control ($P > 0.05$). IFN-γ expression increased slightly earlier to 77.8±7.9, pg/mL ($P < 0.05$) within 1.5 hours of treatment with ICPF and remained elevated at 54.0±4.3, pg/mL ($P < 0.05$) at three hours post treatment (Figure 14). The ratio of IFN-γ to IL-6 expression showed similar trends between *in vivo* and *in vitro* studies, with IFN-γ expression being approximately 30% of

IL-6 in the live mouse and 20% in tissue culture. In addition, the temporal relationship between the two cytokine expression profiles, with IFN-γ preceding IL-6 indicates a potential causal relationship. There were however, no significant changes noted within the CXCL1 chemokine expression *in vitro* ($P > 0.05$). Cellular enumeration was determined to be 5.89×10^6 live nucleated cells per well for this whole blood culture.

A.



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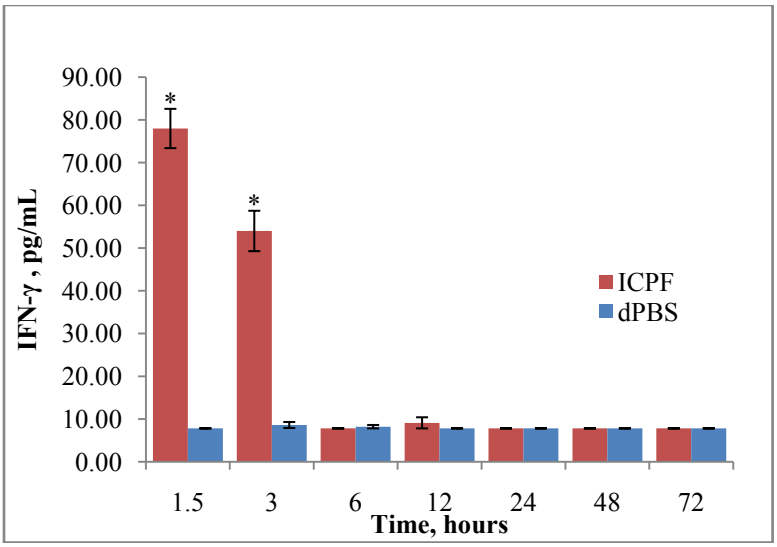

Figure 14 IL-6 and IFN-γ expression *in vitro* following ICPF treatment of murine whole blood

A. IL-6 expression *in vitro* following treatment of 50 µg/mL ICPF in whole blood and B. IFN-γ expression *in vitro* following treatment of 50 µg/mL ICPF in whole blood. IL-6 and IFN-γ concentration was determined in pg/mL ± S.E.M of n=3 with an average intra assay coefficient of variance of <15%. Significant differences between ICPF treated samples and controls ($P < 0.05$) are noted with an asterisk.

Discussion

Septicemia is a highly complex and poorly defined condition that results from a systemic inflammatory response, usually the result of endotoxemia caused by a systemic infection from a gram-negative bacteria and the body's natural attempt at eradicating the pathogen. Following a successful immunological response to such an infection, typically catalyzed by the administration of antibiotics, dead and dying bacteria release large quantities of endotoxin, usually in the form of lipopolysaccharides (LPS). Circulating LPS is quickly bound by LPS-Binding Protein (LBP) which forms an immune complex recognized by TLR-4 and complement receptors of the innate immune system that trigger the release of proinflammatory cytokines (Li and Verma 2002; Li, Holers et al. 2002; Nautiyal, McKellar et al. 2009) such as IL-1, IL-6, TNF-α, and the murine chemokine KC (Osuchowski, Welch et al. 2006; Peyssonnaux, Cejudo-Martin et al. 2007). It is believed that an over expression of these proinflammatory cytokines, possibly compounded with inadequate expression of anti-inflammatory cytokines, all contribute as the primary causative agent of sepsis and septic shock. Due to this mechanistic understanding, many attempts have been made to utilize anti-inflammatory cytokines and antibodies directed at proinflammatory cytokines to preemptively target this underlining cause of sepsis. Despite several successful animal trials (Zeni, Freeman et al. 1997; Nasraway 1999) none, save IFN-γ for the treatment of injury induced sepsis (Polk Jr, Cheadle et al. 1992), have proven successful in human clinical phase II or III trials (Nasraway 2003) and some have concluded with disastrous results, leading to potential cytokine storms and increased mortality (Fisher, Agosti et al. 1996).

While ICPF has been shown to induce the expression of proinflammatory cytokines (Figure 15) while reducing mortality in a bacterial induced sepsis model

(Figure 5) the effect on endotoxemic (LPS) induced sepsis (Figure 3) was dismal. Recently another theory attempting to explain sepsis has been gaining momentum, which challenges the notion that an initial overproduction of proinflammatory cytokines is the primary causative agent in the susceptibility to septic shock (Netea, van der Meer et al. 2003). This theory proposes that an initial deficiency in proinflammatory cytokine expression and production allows for rapid proliferation and systemic spread of the invading bacteria, resulting in higher levels of endotoxin release following the corresponding immune response, resulting in systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS), which can lead to multisystem organ failure, shock, and ultimately death (Netea, van der Meer et al. 2003).

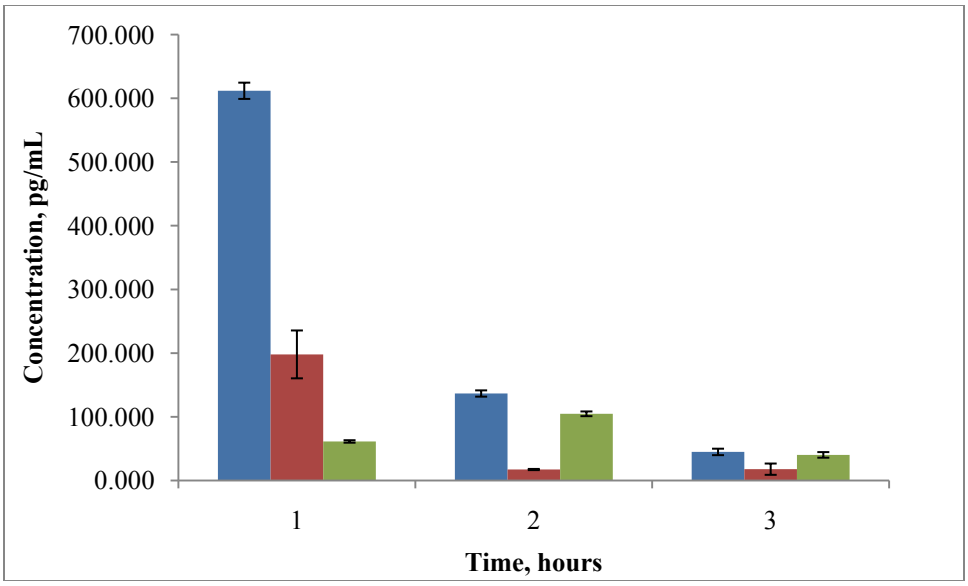

Figure 15 Expression of proinflammatory cytokines following administration of ICPF

■ IL-6, ■ IFN-γ, and ■ KC *in vivo* expression following ip administration of 5 mg ICPF. Three mice from each time point received ip 0.2 mL dPBS serving as a negative control (data not shown), while three additional mice from each time point was treated ip with 5 mg ICPF. Cytokine concentration was determined in pg/mL ± S.E.M of n=3 with an average intra assay coefficient of variance of 7.0%.

The rapid expression of IL-6, IFN-γ, and CXCL1 (KC) by ICPF may be alleviating septicemia induced mortality by priming the innate immune system and allowing for an increased immune response to the invading pathogen, preventing systemic bacterial dissemination, endotoxemia, and ultimately septic shock. While ICPF has been known to reduce systemic levels of *S. typhimurium* within two days of treatment and infection (Willeford, Parker et al. 2001), no mechanism for this activity has previously been proposed.

Critical to further study and analysis of ICPF is the development of a reliable and cost effective bioassay to serve as an alternative to the current murine animal model. In addition to analyzing cytokine expression *in vivo*, cytokine values were determined *in vitro* following ICPF treatment. It was our hope that the cytokine expression profiles

would match that seen within the organism, as we wanted the indicator to be a function of ICPF and its immune effect. While CXCL1 was not identified as an up regulated chemokine *in vitro*, both IFN-γ and IL-6 were up regulated within three hours of treatment, at nearly the same time as *in vivo*. We believe that the combined temporal proximity and relative expression levels of IL-6 and IFN-γ *in vivo* and *in vitro,* in response to administration of ICPF to be an excellent indicator of potential immune function, and further speculate that a whole blood cellular assay which mimics, at least in part, the prophylactic properties induced by ICPF is now in preliminary testing. Before such a bioassay can be validated, it remains to be seen if the same mechanism inducing these early proinflammatory cytokine expressions *in vivo* is taking place *in vitro* which future work hopes to address.

CHAPTER V

CONCLUSION

IL-6, while being a highly pleiotropic cytokine with both pro and anti-inflammatory properties, has been shown to be instrumental for early activation of both B and T lymphocytes (Van Snick 1990) and hepatic induction of type 1 and 2 acute phase proteins, such as C-reactive protein and Serum Amyloid A (SAA), both of which serve a protective role as innate immune opsonins for *S. typhimurium* and other gram negative bacteria (Szalai, VanCott et al. 2000; Hari-Dass, Shah et al. 2005; Shah, Hari-Dass et al. 2006). The increased expression of IL-6 by ICPF is able to explain for the first time the systemic increase in SAA previously observed (Figure 8). IFN-γ plays an essential role in the activation of macrophages and successful clearance of phagocytized intracellular pathogens such as *S. typhimurium*. Upon phagocytosis of certain intracellular bacteria, macrophages release IL-12 which induce IFN-γ production by NK and T-cells (Stark 2007). The IL-12 subunit, IL-12p40 was also shown to be up regulated (data not shown) by ICPF *in vitro* however we were unable to detect its expression *in vivo*, possibly due to its temporal expression and our selection of chosen time points or insufficient systemic levels being expressed. IFN-γ is essential to the activation of interferon inducible protein 1 (Irgm1) which was also found to be up regulated by ICPF. IFN-γ and Irgm1 are both important to a successful immune response towards *S. typhimurium*, as intracellular bacteria have evolved several specific strategies to avoid host defense and clearance once phagocytized by a macrophage, such as preventing IFN-γ mediated macrophage

maturation and possibly Irgm1 induced phagosome clearance (Donné, Pasmans et al. 2005; Henry, Daniell et al. 2007). We contend that induction of IFN-γ by ICPF, either directly or by activation of macrophages, possibly via a TLR, is able to activate Irgm1, thereby allowing for increased intracellular bacterial clearance.

CXCL1/keratinocyte-derived chemokine (KC) is one of only four murine CXC chemokines containing the Glycine-Leucine-Arginine (ELR) motif, despite the identification of seven such chemokines within humans (Lin, Carlson et al. 2007). CXCL1 also serves as a murine homolog of the human oncogene termed GRO (Growth Regulated Oncogene), is mainly induced by LPS in macrophages and vascular cells and is associated with neutrophil recruitment and inflammation (Rubio and Sanz-Rodriguez 2007). $ELR^{+/-}$ chemokines, such as CXCL1 and CCL2 have been shown to precede proinflammatory cytokine and innate inflammatory responses within six hours of infection and impedance of this early chemokine response can result in more aggressive bacterial infection, proliferation, and possibly increased mortality (van der Zee, Dik et al. 2010).

Osuchowski, Welch et al. (2006) provides an excellent analysis of both pro and anti-inflammatory cytokine profiles following experimentally induced sepsis by cecal ligation and puncture in mice. While their observed increase in total serum concentration of these cytokines was considerably higher (1,000 fold) than what we observed following ICPF administration their results are otherwise very similar to our own in respect to proinflammatory cytokines.

Osuchowski, Welch et al. observed a significant increase in IL-6 and CXCL1 in mice which succumbed to sepsis within 1-5 days post challenge yet these proinflammatory mediators were not noted in mice which survived or took longer to

succumb, suggesting a temporal relationship between the correlation of increased IL-6 and CXCL1, and mortality attributed to sepsis. In addition to the increased levels of IL-6 and CXCL1 as compared to ICPF, their earliest reported point of measurement was six hours post challenge, while we observed an increase in IL-6 and CXCL1 within 1-4 hours, and a return to baseline by six hours (Figures 12 and 13). While excessive levels of IL-6 in the range of 10 μg/mL have been shown to jeopardize host defense against a bacterial infection (Soda, Kano et al. 2003), our highest concentration of IL-6 (611±22.1 pg/mL) were well below those shown to increase mortality. Osuchowski's analysis of proinflammatory cytokines prior to sepsis did not include IFN-γ; deficiences in IFN-γ expression have been reported to be associated with increased susceptibility to infections and potential sepsis (Borges, Augustine et al. 2000; Netea, van der Meer et al. 2003; Takeyama, Tanaka et al. 2004) and its administraion has been used successfully to treat sepsis following severe injury (Polk Jr, Cheadle et al. 1992).

The prophylactic properties generated by ICPF in response to *S. typhimurium* induced sepsis are propagated through a rapid stimulation of the innate immune response, via stimulation and expression of proinflammatory mediators. Specifically IL-6, IFN-γ, and CXCL1 and the rapid elimination of the causative agent *S. typhimurium*, partially through activation and increased expression of Irgm1 and SAA before systemic levels of bacteria and endotoxins are able to overwhelm the body's ability to compensate (Figure 16). This theory is further supported by the inability of ICPF to protect mice directly challenged with *S. typhimurium* derived lipopolysaccharides, as the induction of proinflammatory cytokines would be of little help, and possibly detrimental to survivability during such a challenge.

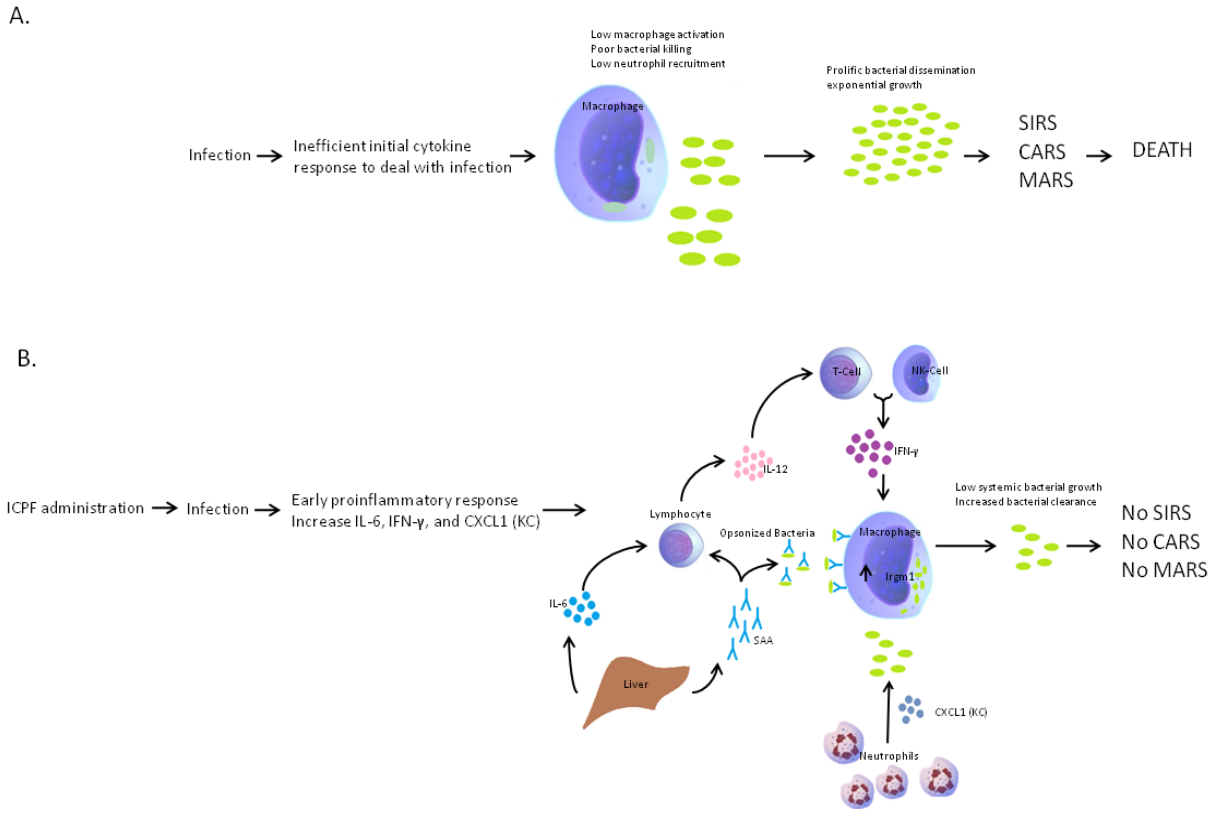

Figure 16 Diagram of proposed mechanism for ICPF prophylactic treatment of *S. typhimurium*

A. Sepsis model where low proinflammatory cytokine expression immediately following an infection results in poor activation of innate immune systems, deficient elimination of bacterial infection, and exponential systemic bacterial growth. When the innate immune system is able to mount an effective response to the infection, the increased bacterial load would result in a massive proinflammatory response resulting in Systemic Inflammatory Response Syndrome (SIRS) followed by death or Compensatory/Mixed Anti-inflammatory Response Syndrome (CARS/MARS), staling of the immune response, possibly resulting in death. B. Proposed mode of action for ICPF following *S. typhimurium* infection. IL-6, IFN-γ, and CXCL1 (KC) expression as

determined by multiplex FLISA analysis, Serum Amyloid A expression as determined by ELISA, and interferon inducible protein 1 determined by 2D DIGE. We propose that an early increase in proinflammatory cytokines and chemokines, specifically IL-6, IFN-γ, and CXCL1, combined with an prolonged acute phase response with Serum Amyloid A (SAA), and augmented macrophage intracellular bacterial clearance from increased expression of interferon inducible protein 1 (Irgm1) stemming from prophylactic administration of ICPF, allows an organism to more fully respond to an initial bacterial challenge, preventing systemic bacterial growth and proliferation, and ultimately preventing sepsis induced death *rium* derived sepsis

Continued research pertaining to ICPF's mode of action should concentrate on the importance of the up-regulated cytokines and chemokines following ICPF administration, either by utilizing cytokine deficient mice (developed through selective breeding programs or via molecular knockout) or by coupling ICPF with neutralizing antibodies directed against select proinflammatory cytokines. Utilizing *in vivo* protein profiles, while ideal, presents several limitations in terms of costs and practicality associated with the use of live animal models. To address these restrictions, single or mixed cell cultures should be evaluated and if possible, developed to further advance our understanding of the cells responsible for these early cytokines. The development of a mono-culture able to respond to ICPF in a manner which mirrors an *in vivo* response will also be essential to determine the pharmacokinetics and specific receptors involved with ICPF.

Discovering agents that potentiate the immune response is a driving force in modern bio-rational drug research (Taylor and Wright 2008) and our ability to analyze immunological, biochemical, and molecular pathways have led to significant discoveries in this field over the last three decades. While there are many potential pathways, both immunological and pathogenic to potentially exploit, the number of successful and safe therapies remain low (Chan 2008). General immune stimulants and cytokine factors such as interferons, interleukins, and inflammatory antagonists currently in use, although potentially providing dramatic therapeutic results, require dosages at concentrations that

often produce toxic side effects which make them intolerable to many patients and have thus not proven good candidates for wide-spread, general use (Kruth 1998; Nasraway 2003). The need to augment our antimicrobial arsenal is driven not just by the desire to increase human welfare, but by the increasing threat of antimicrobial resistant pathogens in both human medicine and food animal production (Liu, Huang et al. 2008; Silbergeld, Graham et al. 2008). Currently, the majority of antibiotics in use today were discovered over 50 years ago and methacillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and multidrug resistant gram negative bacteria, such as *Salmonella enteritis* have become an increasingly serious problem for human welfare (Taylor and Wright 2008).

Despite the considerable questions which remain surrounding ICPF's mode of action, ICPF has proven to safely provide a significant health benefit when administered prophylactically. ICPF can be provided prophylactically or administered therapeutically without interfering with other therapeutic modalities such as those requiring antibiotic or antiviral regimes (Figure 6). The typical "ICPF dose" (0.25 mg/g) has not promoted a recorded harmful side effect. ICPF did not affect cell viability or inhibit protein translation as assessed in canine diploid fibroblasts (Figure 7) or cause the hemolysis of red blood cells when the culture mediums were supplemented with up to 1 mg/ml ICPF (Table 2). Animals previously subjected to ten times the therapeutic dosage did not demonstrate toxicity nor experience a "Delayed Type Hypersensitivity Reaction" when re-exposed (data not shown) and PulmoClear ™, which constitutes a crude formulation of ICPF, is a USDA approved product for equine lower respiratory disease. World-wide, infectious disease and sepsis remain a leading cause of mortality. It seems promising that if further researched and developed, ICPF could augment our therapeutic arsenal.

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