HEPATIC TRANSCRIPTIONAL DYNAMICS FOLLOWING BURN AND SEPSIS: THE PROFILE OF THE SYSTEMIC INFLAMMATORY RESPONSE

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ABSTRACT OF THE THESIS

Hepatic Transcriptional Dynamics Following Burn and Sepsis: The Profile of the Systemic Inflammatory Response

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This thesis aims to understand the dynamic impact of injury on liver metabolism and protein production. Hypermetabolism is a major clinical complication arising from systemic inflammation in the liver. Thus, this thesis hypothesizes that major inflammation driving stressors will be able to induce both metabolic and pro-inflammatory changes to liver gene expression. Animals were induced into an inflammatory state either by Burn, Cecal Ligation and Puncture (CLP), or Sham Cecal Ligation and Puncture (SCLP). CLP and SCLP, characterized previously by their cytokine outputs, were functionally annotated and subjected to pathway analysis following RNA expression measurement. Acute phase differences between CLP and SCLP show a much more severe acute CLP response, including a significant number of anti-bacterial proteins. Long term responses to CLP compared to SCLP show a significant anti-inflammatory surge, along with signs of oxidative stress sensitivity. When primed with burn injury, the CLP acute inflammatory response becomes depressed, but surges back stronger at later time points, suggesting early immune vulnerability followed by deleterious overcompensation. Overall, these studies

reveal an underlying complexity to the sepsis models that suggest multiple avenues towards deleterious inflammation that may be clinically addressed.

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In addition, I wish to acknowledge that the contents of this thesis are primarily derived from published work during my education:

Dynamics of Hepatic Gene Expression Profile in a Rat Cecal Ligation and Puncture Model [1]

Long-term gene expression profile dynamics following cecal ligation and puncture in the rat [2]

Impact of burn priming on immune and metabolic functions of whole Liver in a rat cecal ligation and puncture model [3]

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Introduction

Clinical Sepsis

Severe sepsis is defined as a potentially deadly medical condition which is characterized by a whole-body inflammatory state [4]. Despite progress in the study of inflammation, sepsis still poses a significant challenge in hospitals, and remains a major burden on time and care costs for patients. Although better treatment options have decreased the fatality rates of severe sepsis cases between 1993 and 2003, the incidence of the illness is increasing [5]. Furthermore, the inflammatory response that drives sepsis is the hallmark of other severe injuries, including major burns and traumas. Like sepsis, these injuries are difficult to properly treat, due to the persistence of the patient's inflammatory response following injury. Though treatment options are improving, the lack of fundamental understanding of the pathophysiology of inflammation prevents the discovery of drugs and techniques that can significantly relieve the burdens of treatment. One major difficulty in the treatment of inflammatory pathologies is the fact that it occurs in patients over an extended period of time, and is characterized by a slow wasting of body mass, which causes weakness over an extended period of time. In addition, the inflammatory response is fundamentally a natural response to injury, but it can develop into pathologies such as sepsis when gone awry. Counteracting the detrimental effects of these disorders must be tempered with an appropriate lack of interference with the host's natural defenses to the original insult (burn, infection, or trauma) in order to facilitate recovery. Understanding the mechanisms that drive innate immune, metabolic and signaling changes associated with the host's response to injury is critical towards identifying key points where

the healthy response transforms into a dysfunctional one, allowing for greater understanding and treatment of these pathologies.

The inflammatory response consists of macroscopic effects like redness, swelling, heat, and pain, and each of these symptoms is the result of stimulation of the immune system [6]. For instance, the redness observed in inflammation is the result of increased vasculature permeability due to signals such as bradykinin (a basic peptide which increases vascular permeability), whereas the tissue damage that occurs is primarily the result of the release of reactive oxygen and nitrogen intermediates triggered by signals from migrating neutrophils [7]. Leukocytes spend the majority of their time within the spleen and other various lymph nodes, however, these cells also migrate throughout the blood-stream. The time that these cells spend migrating or resting is optimized to allow for the maximum amount of coverage within the body, which allows the cells to best identify and react to threats as fast as possible [8]. Once a threat has been identified, signals such as cytokines and chemokines are used to recruit more immune cells to the site of injury, as well as stimulate changes in metabolism and the production of other types of leukocytes best suited to handle the threat [9]. Since perhaps one in ten thousand leukocytes is capable of recognizing any individual threat, the circulation time is extremely important: the faster a response can be mounted, the less damage overall will be caused by the pathogen as well as the acute inflammatory response.

Cytokine Storm

Once tissue damage or infection occurs, afflicted cells immediately release cytokines that express the need for an inflammatory response. These cytokines activate the

local endothelium, which then expresses a high degree of cell adhesion molecules (CAMs) that recruit neutrophils against the threat [10]. Neutrophil extravasation occurs in several steps: rolling, chemo attractant activation, adhesion and migration. The rolling step is mediated by selectins on the neutrophil surface which lightly bind to carbohydrates along the endothelium, causing the cell to "bounce" from one endothelial carbohydrate to the next [11]. The next step is mediated by chemokines (specifically IL-8 and MIP-1β), molecular signals which cause a conformational shift in the structure of the integrin molecules on the neutrophil. This shift causes them to firmly bind to the endothelial wall [12]. Once adhered, the neutrophils migrate between the endothelial walls, although the exact mechanisms for this migration are as of yet unknown. Though neutrophils are generally the first cells that respond to an inflammatory threat by extravasation in this manner, B and T cells are also recruited (although using different CAMs) by the same general mechanism [13]. What separates neutrophil extravasation to leukocyte extravasation is that for leukocytes, the chemokine attractants that are used in recruitment are tissue specific, allowing those leukocytes to hone in on the appropriate organ.

Because of their role in both leukocyte homing, and general neutrophil extravasation, chemokines are major regulators of traffic within the body. Not all chemokines are associated with the inflammatory response, and some are even necessary for homeostatic or developmental roles. However, regardless of purpose, most chemokines are selectively (and specifically) involved in the regulation of adhesion, chemotaxis, and activation of leukocyte populations [14]. The chemokines that are known to be involved in inflammation are usually produced in response to infection, and are stimulated either by contact with pro-inflammatory cytokines such as TNF-α, or by direct contact with

pathogens. These chemokines are locally expressed at the sites of inflammation, and recruit leukocytes by the process indicated previously. Once inside the tissues, these leukocytes move up the chemokine concentration gradient in order to assemble at the site of the infection [15]. Almost all chemokines structurally share a four cysteine motif, although in one branch of the chemokine family, this motif is bisected by another random amino acid. Although chemokines bind to their receptors with extremely high affinity, the receptors themselves are not specific to an individual chemokine, and will often adhere to multiple chemokines (CXCR2 for example recognizes 6 different chemokines with high affinity) [16]. Chemokines are also able to induce a response on the vascular endothelium extremely quickly, promoting adhesion and generating oxygen radicals within seconds of their release. The fact that chemokine receptors can bind to a specific number of chemokines with high affinity, and that each different kind of leukocyte produces a different receptor profile, coupled with the fact that each tissue releases a different cocktail of chemokines in response to a threat, allows for combinatorial regulation of the various populations of leukocytes. This allows each kind of immune cell to be specifically activated depending on the need of the issue involved, without cross recruitment or side effects.

Local vs Systemic Inflammation

Inflammatory responses can be observed either locally, or systemically, depending on the injury in question. Localized inflammatory responses are characterized by redness, swelling and a buildup of fluid [17]. Almost immediately, plasma enzymes are recruited to dilate the vasculature and induce clotting to seal the wound from the rest of the body. After a few hours, neutrophils are recruited to the injury site, and attack any pathogens present, while simultaneously releasing pro-inflammatory mediators, which include macrophage

attractants such as MIP-1α and MIP-1β [18]. After 6 hours, these signals recruit activated macrophages which in turn produce pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α, which are responsible for many of the local and systematic changes associated with inflammation. All of these cytokines primarily act locally, with TNF-α and IL-1 serving to increase the adhesiveness of the endothelium, as well as causing macrophages and endothelial cells to secrete the chemokines which neutrophils use to migrate to injury sites via chemotaxis [19]. Another pro-inflammatory mediator that is released, IFN-γ, acts with TNF-α to increase phagocytic activity and release more lytic enzymes into the injury site. This process clears bacteria, but also causes tissue damage at the injury site. Because of the damage that these lytic enzymes can cause [20] (along with the reactive oxygen and nitrogen radicals), the pro-inflammatory response can drive severe tissue damage if not limited: TGF-β has been shown to limit the inflammatory response, while simultaneously recruiting fibroblasts to create the extracellular matrix that is required if wound healing is to occur.

While the local changes described above are occurring, there is also an acute systemic change that occurs, which is designed to shift the host's mechanisms in the direction of defense. Some symptoms of the systemic acute inflammatory response are fever, synthesis of hormones such as ACTH and hydrocortisone, up regulation of white blood cell production, and the release of many pro-inflammatory acute phase proteins in the liver [21]. The fever response that occurs is mainly due to the actions of the aforementioned pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) along with OSM and LIF, which act on the hypothalamus to up regulate body temperature. These 5 factors have very redundant effects on the immune system, due to the fact that they share a common

pathway, which activates the transcription factor NF-IL6, which is similar in form to a liver specific transcription factor, C/EBP [22]. C/EBP is a transcription factor that is constitutively expressed, and regulates albumin and transthyretin, but as the systemic inflammatory response progresses, this transcription factor is down regulated in favor of NF-IL6. Thus, over time, serum levels of albumin and transthyretin decrease, while proteins associated with the inflammatory response (ie, IL-6) are up regulated.

Systemic Inflammatory Response Syndrome

Though the immune system has a strong line of defense against invading pathogens, it is not always successful in clearing them from the body in a reasonable amount of time. Because their consistent presence induces pro-inflammatory signals from any cell which comes in contact with them, these bacteria force the body to maintain an acute inflammatory response long after it remains advantageous to survival to do so [23]. This causes significant tissue damage, as well as hypermetabolism, and can eventually lead to organ failure and death. These symptoms are also associated with autoimmune diseases (in which the immune system self-perpetuates an inflammatory response by failing to recognize itself as non-foreign), as well as cancer, in which the buildup of tumor cells also induces an inflammatory response, but because the cells themselves are not foreign, the invasion is not cleared [24]. The definitive marker for chronic acute inflammation is the activation of macrophages, which often clump together in wound sites, forming massive single celled organisms that create scar tissue and prevent healing [25]. Two major signals involved in perpetuating chronic inflammation are TNF-α and IFN-γ, which are secreted by Th1 cells and macrophages, specifically. TNF-α in particular appears to be a significant contributor to the wasting that is associated with chronic inflammation, and along with

IFN-γ, this cytokine can initiate a full blown inflammatory response [26]. These signals cause the release of various interferons, which stimulate macrophages into increased microbicidal activities, including the release of hydrolytic enzymes and reactive oxygen and nitrogen species [27]. These agents are effective at destroying bacteria, however they cause significant tissue damage, and in the case of chronic inflammation, the damage is both continuous and extensive.

Sources of Sepsis

Sepsis represents a physiological state of heightened inflammation, but it can be driven by a variety of stimuli – injuries and infections – which drive inflammatory mediators and systemic inflammatory responses [28]. In particular, major clinical drivers of sepsis include severe burn injuries, and bacteremia. Mechanistically, it is believed that burns induce an inflammatory response, followed by an anti-inflammatory wound healing response that dominates the recovery phase. [29] This response to burn, which acts through the production of pro-inflammatory mediators (mainly from macrophages) compromises the immune system, allowing for any subsequent injuries to be much more severe [30]. When a subsequent infectious insult occurs during the anti-inflammatory phase, this relative immunosuppression combined with the physiologic response to the invading microorganism leads to a pathophysiological state recognized as sepsis [31]. Sepsis can either result in an overwhelming infection, or an overreaction hyperinflammatory response causing widespread damage through anti-microbial oxidative mediators [32]. In either setting, the end result is often multiple organ failure and, ultimately, death. However, there are multiple mechanisms for achieving the septic state in humans, and animals, including bacteremia – the presence of bacteria triggering immunological responses that often

originate from inside the gut microbiome and migrate to the blood and lymph system [33, 34]. Though the macroscopic symptoms of sepsis tend to converge in different injury systems, their specificity and underlying molecular mechanisms remain poorly understood.

The activation of the inflammatory response into a septic response is not only characterized by the production of circulating acute phase proteins, including cytokines and chemokines, and the activation of innate immune cells such as notably circulating neutrophils and macrophages, but also with major changes in metabolism. Due to its central role in producing serum proteins, regulating metabolism, and destroying foreign entities within the blood, the liver plays a central role in the development of the inflammatory response. In order to meet the energetic demands of the activated immune system, the liver is required to synthesize and export glucose through gluconeogenesis. This process occurs in addition to mounting an anti-bacterial defense through the production of proteins associated with the complement and coagulation cascades [35]. In order to meet these demands as well as maintain homeostasis of albumin levels and other major blood proteins, it is required to draw amino acids from peripheral tissues, both for the production of proteins and their catabolism for energy. This demand is extreme, and drives the loss of lean body mass that represents a major threat to the health of the patient. Because the liver is central to the onset and perpetuation of hyper metabolism [36], restoring liver homeostasis and function may represent a promising avenue of research towards improve clinical outcome following injury [37, 38]. The liver represents an experimental challenge for investigation, as its response to the stress of inflammation occurs across multiple regulatory levels, including, but not limited to altered gene expression and enzymatic activity. Assessing the drivers behind the hepatic metabolic and inflammatory response

requires multi-pronged investigative techniques that can assess transcriptional changes within the liver, which drive changes in liver metabolism and whole body homeostasis.

Animal Models

In order to decipher and investigate these septic responses, both in the liver and in other tissues, a number of animal models have been proposed and developed. One of the most commonly used of these models is the cecal ligation and puncture technique (CLP) in rodents [33, 34]. The rationale behind this technique is that an immunological response to bacterial infection can be driven from the host's own microbiome – a phenomenon not different from some clinical complications in sepsis [39] – through surgical intervention and perforation of the bowel. More specifically, the design involves midline laparotomy, exteriorization of the caecum, ligation of the caecum distal to the ileocecal valve and punctures of the ligated caecum [33]. This process creates a bowel perforation with leakage of fecal contents into the peritoneum, which establishes an infection with mixed bacterial flora and provides an inflammatory source of necrotic tissue. Following CLP, animals generally develop bacteremia, hypothermia, hypotension, and hypermetabolic and catabolic state at whole body level.

Another commonly used injury model for studying systemic inflammation in the context of animal systems is to induce the inflammatory response via burn injury [40]. This technique does not generally involve surgical intervention, but rather creates third degree burns. In detail, the systemic response can be induced by applying a full-thickness burn on an area of the dorsal skin corresponding to varying degrees of total surface body area (TBSA), depending on the experimental design: higher surface area leading to a more

severe response [41]. For 20% TBSA severe burns, the model has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant alterations on feeding patterns [42]. This allows for both acute responses and long term recovery processes to be assessed in tissues of interest. In particular, Burn injury is a useful model, because it characteristically causes a significant disruption of host's defenses, drastically depleting CD4+ T-cells [43] and disrupting the wound healing and coagulation cascades [44]. Further, burn injury induces significant polymorphonuclear neutrophil (PMN)-mediated microvascular damage which is highly exacerbated by subsequent infectious [45] episodes. Notably, burn-induced immune effects appear to be systemic and not related to localized tissue mast cell dysfunction [46]. Because burn injuries are clinically followed up with serious complications, this animal model can be combined with other infection models to interrogate the responsiveness of compromised systems to further insults, and assess the scope of the inflammatory response in those states.

Chapter I – Transcriptional Responses to Inflammatory Injuries within the Liver

Prior work

In order to interrogate the septic state, and its drivers, it was necessary to establish and characterize in house animal models capable of producing inflammatory responses. To this end, Dr. Orman developed three separate injury models, and analyzed them for an inflammatory response via cytokine and chemokine measurement and analysis [47]. These three models were a TBSA burn injury of 20%, a cecal ligation and puncture model (CLP), and a sham cecal ligation and puncture model (SCLP). Burn injury has already previously been associated with inflammatory cytokine release and an acute inflammatory response [40, 48], while CLP and its control, SCLP were also shown to induce potent inflammatory mediators, including TNF-α, IL-1 and IL-6 20 hours after stimulus [49, 50]. The purpose of these studies by Dr. Orman was to show an induction of an inflammatory response: notably, the burn model he developed and characterized showed significant up regulation of IL-12, IL-10, IL-18 and GCSF at the early stage while GRO/KC and MCP-1 were up regulated at the late stage. Meanwhile, the CLP model was notable for its down regulation of GMCSF, Leptin and IP-10 concentrations, and SCLP showed even more cytokine down regulation, including IFN, IL-1β, IP-10, Leptin and IL-17. Dr. Orman's work was very important in two major areas: firstly, it established a profile of systemic inflammatory changes within the rodent model system that would go on to be interrogated for metabolic and hepatic transcriptional changes that was baseline for the current in house system. One of the issues discussed in Dr. Orman's work, and by other sources in the literature, is that there is a wide range of reported responses on inflammatory cytokines and chemokines following injuries such as burn and CLP. Differences in rodent strain, experimental

technique (including the size of the burn injury, the number of cecal punctures, and even the width of the puncturing needle), and even rodent age and size are contributing factors [48, 51], and thus it is critical to establish a baseline response for the specific laboratory model that would be used for future experiments. The other major feature of Dr. Orman's cytokine work on these rodent injury models is the altered dynamics of metabolic and appetite associated hormones following injury. This is expected of a hypermetabolic model, and was critical for establishing that the injuries that will be used to interrogate other features of the injury response are also strongly impacting metabolic function. Overall, the methodologies laid out here are owed to Dr. Orman's hard work in establishing and first characterizing this injury model:

Animal Strain

Unless otherwise specified, all animals used in studies followed this protocol [2, 3, 47]. Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing between 150 and 200g were utilized for this study. The animals were housed in a temperature-controlled environment (25°C) with a 12-hour light-dark cycle and provided water and standard chow ad libitum. All experimental procedures were carried out in accordance with National Research Council guidelines and approved by the Rutgers University Animal Care and Facilities Committee.

Burn Protocol

Unless otherwise specified, all burn models were induced following this protocol [2, 3, 47]. A systemic hypermetabolic response was induced by applying a full-thickness burn on an area of the dorsal skin corresponding to 20% of the total body surface area

(TBSA) as described elsewhere [41]. This model was chosen because it has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant alterations on feeding patterns [42]. Rats were first randomized into two groups: burn and sham burn (control group). Rats were anesthetized by intraperitoneal injection of 80 to 100 mg/kg ketamine + 12 to 10 mg/kg xylazine, and all hair removed from the dorsal abdominal area using electric clippers. The animal's back was immersed in water at 100°C for 10 s to produce a full-thickness scald injury covering 20% TBSA. Immediately after burns, the animals were resuscitated with 50 mL/kg of saline injected intraperitoneally. Negative controls (sham burn) consisted of animals treated identically but immersed in warm water (37°C). Rats were single caged after burn or sham burn and given standard rat chow and water ad libitum until sacrifice. No post-burn analgesics were administered, consistent with other studies with this full thickness burn model since the nerve endings in the skin are destroyed and the skin becomes insensate [52]. Furthermore, after animals woke up, they ate, drank and moved freely around the cage, responded to external stimuli, and did not show clinical signs of pain or distress. Animal body weights were monitored daily and found to increase at the same rate in both groups.

Cecal Ligation and Puncture

Unless otherwise stated, all CLP injuries used for interrogating responses were done using this protocol [2, 3, 47]. Rats were first anesthetized, and then the analgesic buprenorphrine (0.01 to 0.05 mg/kg) and Bupivicaine (0.125% to 0.25%) were given subcutaneously. The abdominal cavity was cut open by a 2 cm midline incision. The cecum of the rat was exposed and ligated just below the ileocecal valve so that intestinal obstruction was not produced. Care was taken not to ligate the cecal branch of the ileocecal

artery, thus preserving viability of the cecum itself, in order to increase the survival rate. The cecum was punctured 4 times with a 20 gauge needle and replaced in the peritoneum. The abdominal incision was then sutured in layers using interrupted monofilament sutures. The animal received 10 mL/kg saline intraperitoneally for resuscitation. Negative controls (sham CLP or SCLP) consist of animals given identical surgical procedures, however, they were not ligated at the cecum and did not receive punctures. Rats were single caged after the treatments and given standard rat chow and water ad libitum until sacrifice.

Transcriptional Response to Cecal Ligation and Puncture [1]

The dynamics of liver function are critical to the overall robustness of the inflammatory response in complex organisms. Hepatic cells are responsible for maintaining protein production, metabolic integrity, and aiding in degrading necrotic and bacterial fragments in the blood. While the cytokine model established that both inflammatory mediators and metabolic hormones could be altered by injury stimuli, it is unclear which pathways in the liver might be responding to these signals. Because production of acute phase proteins is core to the liver's function in inflammatory environments, transcription is hypothesized to play a large role in defining the responsiveness of hepatic cells to various injuries.

In order to characterize the hepatic changes occurring in response to, and driving the inflammatory response in cecal ligation and puncture specifically, the expression profiles of more than 31,000 gene probes were measured at different time points over the first 24 hours post injury. Animals were given CLP and SCLP treatment as described by Dr. Orman, who collected tissue samples for microarray analysis prior to my work on the

project. The microarray analysis and analysis of differential expression and clustering that is included here for the sake of context was carried out by Dr. Yang, who then worked with me in functionally annotating the response and writing up the final publication.

Animals were sacrificed by Dr. Orman (starting at 9am) at different time points (0, 2, 4, 8, 16, and 24hr post-treatment) in each group and liver tissues were collected and frozen for microarray analysis (n=3 per time point per group). The tissues were lysed and homogenized using Trizol, and the RNAs were further purified and treated with DNase using RNeasy columns (Qiagen). Then cRNAs prepared from the RNAs of liver tissues using protocols provided by Affymetrix were utilized to hybridize Rat Genome 230 2.0 Array (GeneChip, Affymetrix) comprised of more than 31,000 probesets.

Transcriptional profiling of hepatic function following CLP injury has already been conducted [5, 41, 43, 53-56]. However, these studies either focus on a single time point (24 h post-CLP) [5, 41, 43, 53-55] therefore missing the critical early response, or only use pair wise comparisons between time points, rather than assessing the dynamics of the whole time scale as a changing function [56]. In addition, most studies normalize CLP against the SCLP control, which utilizes a surgical intervention, but omits the ligation and puncture of the cecum. However, the surgery on its own is a wound to the animal that will trigger its own independent result, and thus the experimental outcome of a CLP procedure is the combination of the surgical wound followed directly by bacterial infection in the peritoneum. Direct comparison of the effect of bacteria on the wound can therefore be obtained by the traditional design of SCLP as a control, but that does not characterize the entirety of the inflammatory response, and any dynamics that are common to the injury and the infection will be lost. Therefore, the experimental design in this study was to treat both

conditions as inflammatory stimuli: infection and trauma, compared against uninjured animals. The results therefore will both elucidate the role of bacteria in the gut as a modifier to the inflammatory response, while using bioinformatics techniques to characterize the transcriptional responses of both injuries as inflammatory stimuli to the liver.

Gene expression in the liver was compared in SCLP vs. sham and CLP vs. sham conditions at 0, 2, 4, 8, 16, 24h post treatment. In both injury models (CLP and SCLP), differentially expressed genes were identified by Dr. Yang utilizing an ANOVA analysis that compared time course profiles of each gene to identify those that are differentially expressed. These differentially expressed genes between SCLP and sham and between CLP and sham were then clustered into general expression patterns that characterize different large scale responses to injury. Simultaneous analysis of either SCLP or CLP with sham groups' expression profiles allowed for the characterization of dynamic patterns of both groups and revealed a comprehensive picture of coordinated hepatic gene expression relating to metabolism, inflammatory signaling and protein production and degradation.

Data Analysis

Analysis of CLP, SCLP and sham gene expression data includes normalization, filtering, combining the datasets and clustering, all of which was conducted by Dr. Yang, who was responsible for analyzing and filtering gene expression data. First, DNA chip analyzer (dChip) software was used with invariant-set normalization and perfect match (PM) model to generate expression values. Then normalized data sets corresponding to CLP vs. sham and SCLP vs. sham groups were investigated to identify the temporally and

differentially expressed probesets over time between each of the two conditions by applying EDGE for each gene [57]. The significance threshold for this test was set as q-value<0.001 and p-value<0.001. Finally concatenated data sets corresponding to differentially expressed probesets in CLP vs. sham or SCLP vs. sham groups were combined to form one single matrix, which was then clustered using the approach of "consensus clustering" [58]. The goal was to identify subsets of transcripts with coherent expression pattern in CLP and sham as well as SCLP and sham respectively. Then, the biological relevance of the intrinsic responses were characterized by evaluating the enrichment of the corresponding subsets by using the pathway enrichment function ($p<0.05$) in Ingenuity Pathway Analysis tools (Ingenuity Systems, Mountain View, CA) as well as analyzing the functions of each individual gene.

SCLP Characterization

The gene expression levels were measured at 0, 2, 4, 8, 16 and 24 h in the livers of rats following shams and SCLP treatment. By considering the time-dependent variations in the gene expression profiles of sham group, differentially expressed SCLP responsive genes that showed altered short term dynamic profiles were identified. Specifically, Dr Yang identified 1722 probesets in SCLP group exhibit altered gene expression patterns over time compared to the corresponding sham control. Consensus clustering in Dr Yang's analysis further determined 6 statistically significant clusters composed of 191, 389, 193, 52, 123 and 73 probesets respectively. The average expression patterns of the 6 clusters are depicted in **Figure 1** (right panel) while a heat map of all probesets is shown in the left panel. IPA pathway analysis as well as single gene ontology analysis was used to further elaborate the functional annotations of SCLP injury responsive genes.

1) In Cluster 1, SCLP induced a significant activation of response which peaks at 16h post treatment. The genes in this Cluster are mainly associated with riboflavin metabolism (*Ptprj, Sacm1l*), Sphingolipid metabolism (Ptprj, Neu2, *Sacm1l*), fatty acid metabolism (*Cyp2c6, Cyp1a1, Auh*), fructose and mannose metabolism (*Ptprj, Sacm1l*), tryptophan metabolism (*Cyp2c6, Cyp1a1, Auh*). Single gene ontology analysis indicates that other genes in this group are associated with production of steroid (*Cyp1a1, Cyp7a1, Lhb, Mzb1*), biosynthesis of terpenoid (*Abcg1, Cyp1a1, Cyp7a1, Lhb*), modification of lipid (*Abcg1, Cyb5b, Cyp1a1, Cyp2b1, Cyp7a1, Pip5k1b, Sacm1l*). In all, this Cluster is mainly related to the fatty acid metabolism, amino acid metabolism, and glucose metabolism.
2) The second SCLP induced-response exhibits a persistent suppression during the entire 24h post treatment. Genes in this critical response are enriched in pathways which include the protein ubiquitination pathway (*Usp7, Psmb2, Ube2n, Hspa9, Psma4, Psme2, Psmd14, Psmd1, Hspd1*), and fatty acid elongation in mitochondria (*Hadhb, Hadha*). In addition, genes related to cellular assembly and organization are also present in current Cluster including *Sec22b, Vcpip1, Kif5b, Pfn1, Ctnnb1, Loc643751, Gosr1, Picalm, Cdkn1a, Pafah1b1, Ube2n, Tinagl1, Tpm3, Eif3a, Csf3, Ldb3, Actr3, Cp110, Vcpip1, Pex19, Pfn1, Unc13d, Cebpz, Dag1, Immt.* Thus, the downregulation of the genes in this Cluster may indicate a decrease in protein degradation and the impairment of cellular structure and normal function.
3) The genes in Cluster 3 in SCLP condition are highly activated from the early beginning starting from 0 h which reaches its maximum at 8h following injury. Pathway analysis indicates that this Cluster is mainly relevant to pro-inflammation

which is specifically in IL-6 signaling (*Il6st, Il1rl1, Il6r, Lbp*), role of Jak family kinases in IL-6- type cytokine signaling (*Il6st, Il6r*) and acute phase response signaling (*Il6st, Il1rl1, Il6r*). Single gene ontology analysis indicates that various genes in this Cluster are directly related to inflammatory response (*Abl1, Bcr, Il6r, Il6st, Lbp, Il1rl1, Abca3, Cxcr4, Mtie, Ahr, Tpst1, Xbp1*). IL-6 is important modulator of the immune system and major inducer of the acute phase response both in vivo and in vitro [59]. The central role of IL-6 in inflammation makes it an important target for the management of inflammatory diseases. The tyrosine kinases of the Janus Kinase (JAK) family and signal transducers and activators of transcription (STAT) family are utilized by IL-6-type cytokines as the major mediators of signal transduction [60]. The acute phase response is a rapid inflammatory response which usually consists of fever, an increase in inflammatory mediators including pro-inflammatory cytokines, chemokines and a change in concentration of the acute phase proteins [61]. Thus, all these aforementioned pathways are relevant to the pro-inflammatory response.

4) Following SCLP injury, the genes in Cluster 4 show a persistent suppression within the first 24h. Genes in this Cluster are involved in metabolism of xenobiotics by cytochrome p450 (*Gstt2/Gstt2b, Cyp2d6*) and drug metabolism (*Cyp2d6, Maoa, Gstt2/Gstt2b*). Detoxification is one of the most important functions for the liver prior to injury, and plays an important role in liver function during sepsis [62]. The xenobiotic metabolic process is a series of reactions that serves to detoxify poisonous compounds by binding to functional groups and catalyzing their transformation into biologically degradable products [63]. Therefore, the

suppression of the expression of the genes may suggest the reduced detoxification effect.

5) The genes in Cluster 5 are mainly related to complement system (*C1r, C4b*), coagulation system (*F9, Thbd*), Cxcr4 signaling (*Rnd2, Rras, Pik3r6*), Jak/Stat signaling (*Rras, Pik3r6*), IL-4 signaling (*Rras, Pik3r6*) and acute phase response (*C1r, Rras, C4b*). The complement system is a cascade of enzyme activations that bridges the innate and acquired immune systems and attacks bacteria by rupturing cell membranes. Coagulation is a complex process that responds to injury by the rapid formation of a clot. All the proteins encoded by the genes in complement and coagulation cascades are important positive acute phase proteins (APP) which are diffusible inflammatory mediators [64-66]. Jak-STAT signaling forms a series of critical pathways involved in producing both cell-mediated and acquired immune responses, particularly in response to cytokine stimulation (ie, IL-6) [67]. Thus these gene groups represent a second inflammatory response that results in an increase in the synthesis of the acute phase proteins and important inflammatory pathways related to cytokine signaling.
6) Finally, Cluster 6 exhibits downregulation compared to the sham. Genes in this Cluster are primarily involved in cellular assembly and organization (*Psme3, Srf, Xiap, Cacna1a, Cgref1, Mus81, Nfu1, Pfdn6, Clint1, Chmp2a*). The downregulation of the same function is also observed in Cluster 2 in SCLP condition which indicates further damage to the normal structure and function of the cell.

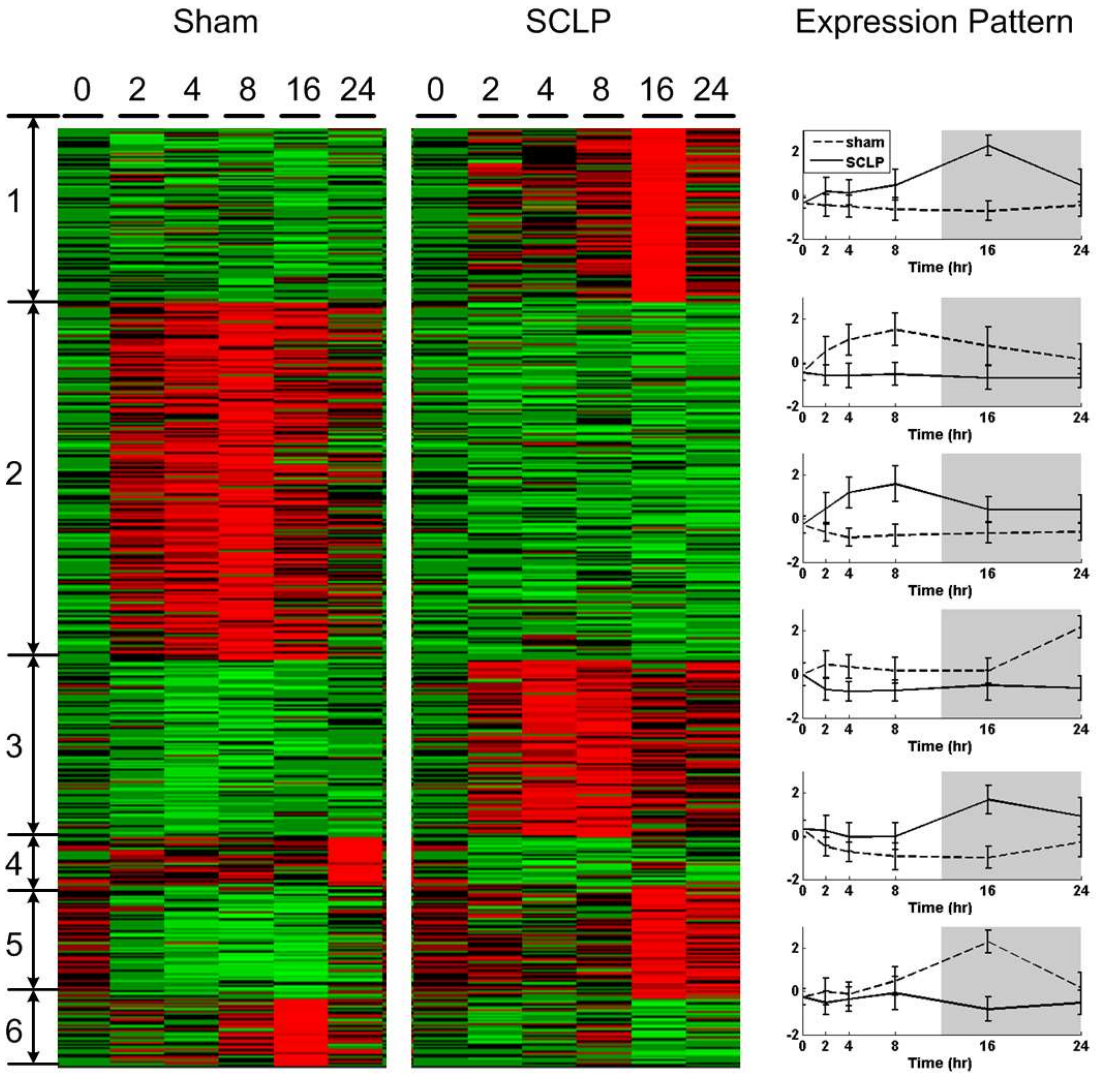

Figure 1. Short term gene expression profiles of rat livers in response to sham or SCLP injury generated by Dr. Yang and analyzed together.

Left Panel, expressions of 191, 389, 193, 52, 123 and 73 probesets in 6 clusters in sham rats and SCLP rats at 0, 2, 4, 8, 16, 24 h post-treatment are exhibited in a heat map.

Right Panels, the expression patterns are shown by plotting the average normalized (z-score) expression values of 191, 389, 193, 52, 123 and 73 probesets in 6 clusters in sham and SCLP groups (displayed as the means ± SEM).

SCLP Dynamics: Aseptic Inflammation from Surgery

SCLP treatment imposes a trauma to the host system which activates a series of inflammatory, metabolic and cellular alterations. By functionally characterizing each transcriptional profile, and by using single gene ontology, it is possible to obtain an overview of the major changes that occur in the cellular dynamics between rats that have been subjected to SCLP, and sham rats. These changes represent the major features of the host's systemic response to SCLP, which contains both unique and common elements with similar responses from CLP. The early upregulation of pro-inflammatory cytokines, chemokines, and their corresponding receptors in Cluster 3 peaking at 8h indicates the activation of the immune system and the first stage of a pro-inflammatory response (P1). This first response triggers a second pro-inflammatory wave (P2), manifested by the acute phase protein synthesis in Cluster 5 which reaches its maximum at 16h post SCLP. The most significant feature of Cluster 5 is the enhanced production of positive APPs. The acute-phase synthesis of complement and coagulation cascade proteins is a striking pro-inflammatory feature of innate immunity, but in this injury model only makes an appearance in P2 following the upregulation of the cytokines and their receptors in P1. It is reported that protein synthesis in liver tissue was increased by 42% following trauma [68]. In addition, the inhibition of the degradation of the protein is observed in Cluster 2. Thus, the increase of the protein synthesis coupled with the decrease in the protein degradation leads to significant net protein production. Previous studies suggest that ubiquitin-proteasome pathway is activated in muscle tissue which leads to muscle wasting in sepsis patient [69]. Thus, the requirement of amino acids (AA) to produce large amount of APP in liver may be satisfied by the increased flux of amino acids from the periphery

tissue to the liver, especially from the accelerated breakdown of muscle proteins. Furthermore, the enhanced production of the genes involved in metabolism, which include fatty acid metabolism, amino acid metabolism, and glucose metabolism, in Cluster 1 and Cluster 5 may be an indication of the hypermetabolism [70] following injury. The fuel for these changes would be energy and substrate sources from peripheral tissues, significantly contributing to muscle catabolism. The suppression of the genes involved in cellular assembly and organization in Cluster 2 and Cluster 6 suggests the damage induced by oxidative stress. Finally, the inflammation inhibits the genes related to xenobiotics biodegradation. Gene expression and activities of cytochrome P450 enzymes are also observed to be downregulated in the liver during the host response to inflammation which results in a change to reduced therapeutic or detoxification effect [71]. It is earlier speculated that the suppression is the pathophysiological consequence of the liver's need to devote its transcriptional machinery to the production of acute-phase proteins controlling the systemic inflammatory response [72]. However, since the maximum suppression of the P450 expression (~24h post SCLP) occurs later than the maximum enhanced production of the acute phase (~16h post SCLP), the downregulation of the synthesis of P450 related genes may suggest some other role for this gene pathway.

CLP Characterization

Identification and functional characterization of CLP regulated expression patterns

The gene expression levels at 0, 2, 4, 8, 16 and 24h following CLP were recorded. By considering the time-dependent variations in the gene expression profiles of sham group, differentially expressed CLP responsive genes that showed altered short term

dynamic profiles were identified. Dr. Yang identified 2039 probesets that were differentially expressed over time between sham and CLP condition. Dr. Yang also obtained 6 statistically significant clusters composed of 437, 295, 171, 154, 91 and 73 probesets respectively obtained by applying the consensus clustering method. The average expression patterns of the 6 clusters are depicted in **Figure 2** (right panel) while a heat map of all probesets is shown in the left panel. IPA pathway analysis as well as single gene ontology analysis was used to further elaborate the functional annotations of CLP injury responsive genes.

1) Cluster 1 is characterized by an early and persistent up-regulation during the first 24h post CLP injury which is related to inflammation. The genes in this Cluster are enriched in acute phase response signaling (*Il6st, Serping1, Hpx, C4bpa, Itih4, Il6r, C5, Lbp, Il6*), role of JAK family kinase in IL-6 type cytokine signaling (Il6st, Il6r, Il6), IL-6 signaling (*Hspb3, Il6st, Il6r, Lbp, Il6*), T helper cell differentiation (*Il6st, Il6r, Hla-Drb1, Hla-Dqb1, Il6*), crosstalk between dendritic cells and natural killer cells (*Prf1, Ifnb1, HLA-DRB1, Il6*), complement system (*Serping1,C4bpa, C5, Cfh, C8a*), coagulation system (*Bdkrb2, Kng1, F5*), N-glycan biosynthesis (*Dad1, Ddost, Mgat5, Mgat5b, and Rpn*2). The acute phase response signaling, role of jak family kinase in IL-6 type cytokine signaling and IL-6 signaling are also observed in previous SCLP- induced responses which are all the indicators of the activation of pro-inflammatory pathways. In addition, T helper cells include two types, Th1 and Th2. Importantly Th1 cells are responsible for phagocyte- dependent protective host response as well as producing pro-inflammatory cytokines including IFN-γ and IL-2 [73]. Natural killer (NK) cells and dendritic cells (DCs) represent two

distinct components of the innate immune system. NK cells kill the bacteria or virus releasing cytotoxic granule proteins which cause cells to die. The crosstalk between NK cells and DCs is required for optimal immune cell expansion and activation resulting in the production of cytokines from both cell types [74]. Thus, the T helper cell differentiation pathway and crosstalk between dendritic cells and natural killer cells synergize in order to create an anti-bacterial effect. Finally, N-linked glycans are extremely important in proper protein folding in eukaryotic cells [75]. Thus, combined with complement system and coagulation system, the last three pathways together represent a separate pro-inflammatory response resulting in an increase in the synthesis of the acute phase proteins and important cytokines. In addition, single gene ontology demonstrates that 36 genes in this Cluster are directly related to inflammatory response. Out of 36 genes, a group of them participate in activation of leukocytes (*Adam9, Blk, C5, Cfh, Ddost, F5, Hla-Dqb1, Infnb1, Il6, Kng1, Lbp, Mgat5, Nfkb1z, Prf1, Ptprj, Serping1,* and *Vcan*), inflammation (*Bdkrb2, C5, Cxcl2, Hpx, Infb1, Il11, Il13ra1, Il6, Il6r, Il6st, Kng1, Lbp,* and *Prf1*), infiltration by neutrophiles (*C5, CD36, Cfh, Cxcl2, Il6,* and *Il6r*), infiltration of granulocytes (*C5, Cd36, Cfh, Cxcl2, Il6, Il6r,* and *Kng1*), activation of monocytes (*Adam9, Blk, C5, Ddost, Hla-Dqb1, Infnb1, Il6, Lbp, Mgat5, Nfkb1z, Prf1,* and *Ptprj*), activation of phagocytes (*Adam9, C5, Cfh, F5, Ifnb1, Il6, Kng1, Lbp*, and *Serping1*). Five are associated with infection mechanism (*Ifnb1, Il6, Nfkb1z, Prf1,* and *Il11*) and twelve are associated with antigen presentation (*C5, C8a, Cfh, Cfhr1, Kng1, Masp1, Adam9, Ifnb1, Lbp, F5, Il6,* and *Serping1*).

2) In Cluster 2, the CLP induced response exhibits a constant expression pattern within 24h post treatment indicating a suppression comparing to the control. Genes in this major temporary class are critical in protein ubiquitination pathway *(Rbx1, Psmb5, Psmd7, Ub2n, Hspa9, Hspd1, Hsp90b1, HSP90AB1, PSMC6, PSMB2, PSMA4, HSP90AA1, PSMD14*), fatty acid elongation in mitochondria (*Acaa2, Hadha*). Besides these signaling pathways, annotation of gene reveals that this Cluster also includes genes involved in catabolism of protein (*Kiaa0368, Loc643751, Psmd14, Rbx1, and Ube2n*), folding of protein (*Canx, Hsp90aa1, Hsp90ab1, Hspd1, Pfdn2, St13, and Tcp1*), refolding of protein *(Hsp90aa1, Hspd1, St13*). In addition, genes associated with cellular assembly and organization (*Actr3, Loc643751, Srf, Kif5b, Pfn1, Prpf19, Smn1/ Smn2, Mapre1, Eif3a, Rhoa, Tcp1, Map7, Mre11a, Naa50, Hsp90aa1, Immt, Creb1, AbI1, Robo2, Copb2, Ftl, and Serp1*) are present in this Cluster. Therefore, the downregulation of this Cluster suggests the decrease in the protein degradation coupled with CLP induced- damage to cell.

3) Compared to the sham group, CLP treatment triggers a strong activation within the first 2h post injury in Cluster 3. The genes involved in this temporal profile are related to fatty acid metabolism, glucose metabolism and amino acid metabolism. Specifically, genes are enriched in fatty acid metabolism (*Adh7, Eci2, Adh1C, Acadsb, Cyp51a1, Aldh7a1*), bile acid biosynthesis (*Adh7, Adh1c, Aldh7a1, and Srd5a1*), glycolysis/ gluconeogenesis (*Acss2, Adh1, Adh7, and Aldh7a1*), metabolism of xenobiotics by cytochrome P450 (*Gstm1, Adh7, Adh1c, Cyp51a1*), steroid biosynthesis (*Cyp51, Ebp*), valine, leucine and isoleucine degradation (*Acadsb, Adh7, Aldh7a1*), propanoate metabolism (*Acss2, Aldh7a1*), tyrosine

metabolism (*Adh1, Adh7*), pyruvate metabolism (*Acss2, Aldh7a1*). As might be expected, healthy animals (sham) subjected to a light-dark schedule display pronounced rhythms in glycogen content, with a peak occurring late in the night, following the main period of food intake. This is in agreement with our result that metabolic functions, especially glucose metabolism, reach peak function during the dark phase in sham group. Significant evidence suggests that the metabolic rate in sepsis is extremely high due to immense energy requirements. The hypermetabolism is demonstrated by accelerated metabolic rates, increased nitrogen loss and loss of lean body mass, stimulated acute-phase protein synthesis in the liver, and abnormalities in lipid and carbohydrate metabolism [76]. Thus, our result may be an indication for the sepsis induced hypermetabolism. Besides these pathways, individual gene ontology analysis indicates that lipid metabolism are enriched in this Cluster including metabolism of lipid (*Acadsb, Acsm3, Acss2, Adh1c, Adh7, Cln3, Cyp51a1, Ebp, EcI2, Il1a, Mbtps1, Mvk, and Ptgs1*), quantity of lipid (*Abca3, Adora2a, IL1a, IL33, Lpgat1, Lrp1, Mbtps1, Ptgs1, Scd2, Srd5a1*), exposure of phospholipid (*Il1a, Lgals2*), binding of phosphatidic acid (*Gas6, Zfyve1*). In addition, thirty-two, and ten probesets in this Cluster are involved in lipid metabolism, small molecular biochemistry, and vitamin and mineral metabolism. Thus, this Cluster is mainly related to metabolic changes within the liver.

4) CLP induces a persistent suppression in Cluster 4 which is mainly involved in mitochondrial dysfunction (*Ndufb8, Cyc1, Ndufs3, Maoa*), insulin signaling pathway (*Calm3, Slc2a4, Trip10*), oxidative phosphorylation (*Ndufb8, Cyc1,*

Ndufs3), DNA replication (*Mcm5, Pold2, Rfc4*), mismatch repair (*Pold2, Rfc4*), purine metabolism (*Pde4a, Pd37a, pold2, Trm1*) and pyrimidine metabolism (*Pold2, Trm1, Tyms*), DNA replication, recombination and repair (*Ckap2, Hbxip, Kpnb1, Tubb, Arrb1, Pde4a, and Axin2*). Mitochondria is an organelle generating most of the cell's supply of ATP, which make them the primary consumers of oxygen in a cell and contain a multitude of redox carriers capable of transferring single electrons to oxygen. Mitochondrial dysfunction occurs when the ROS-mediated oxidative stress overpowers the antioxidant defense system indicating the tissue undergoing an oxidative stress condition [77, 78]. Insulin is an anabolic hormone which promotes the storage of substrates in liver by stimulating lipogenesis, glycogen and protein synthesis [79]. Thus, downregulation of the genes involved in the insulin signaling pathway suggests a potential mechanism to explain the onset of a hypercatabolic state which is characteristic of hypermetabolism. In addition, the strategy of adaptive circadian clocks could be strategy on the part of UV-sensitive cellular processes to occur at night to avoid UV-induced damage [80]. Our analysis showed that functions related to DNA replication/repair is suppressed by the CLP injury.

5) The response induced by CLP exhibits a much higher elevation response compared to sham in Cluster 5. The molecular and cellular functions in this Cluster include lipid metabolism (*Fabp4, Fabp5, Far1, Plau, Por, and Dhrs4*), small molecule biochemistry (*Fabp4, Fabp5, Far1, Plau, Por, Dhrs4, Gucy2c, Rundc3a, Galnt2, Pd310a, and Mmp14*). Sepsis is a common surgical problem which can induce profound changes in the plasma concentrations of cytokines and hormones, leading

to a catabolic state. Hypertriglyceridaemia and increased fat oxidation are the main features of altered fat metabolism encountered in this state [70].

6) Finally, the Cluster 6 includes the genes relevant to cellular assembly and organization (*Chmp5, Vcpip1, Col5a2, Mtss1, Mapkapk2, Smoc2, Pdzd2*), cellular function and maintenance (*Adrbk1, Unc13c, Vcpip1, and Adcyap1r1*), tissue development (*Hnrnpa2b1, Adcyap1r1, Tob1, Chst11, Sulf2, and Adrbk1*), protein degradation (*Tmprss8, Edem1, Senp6, March 6, and Trib1*). Thus, the downregulation of the genes involved in cellular assembly and organization suggest the damage of the injury to normal cellular function and structure.

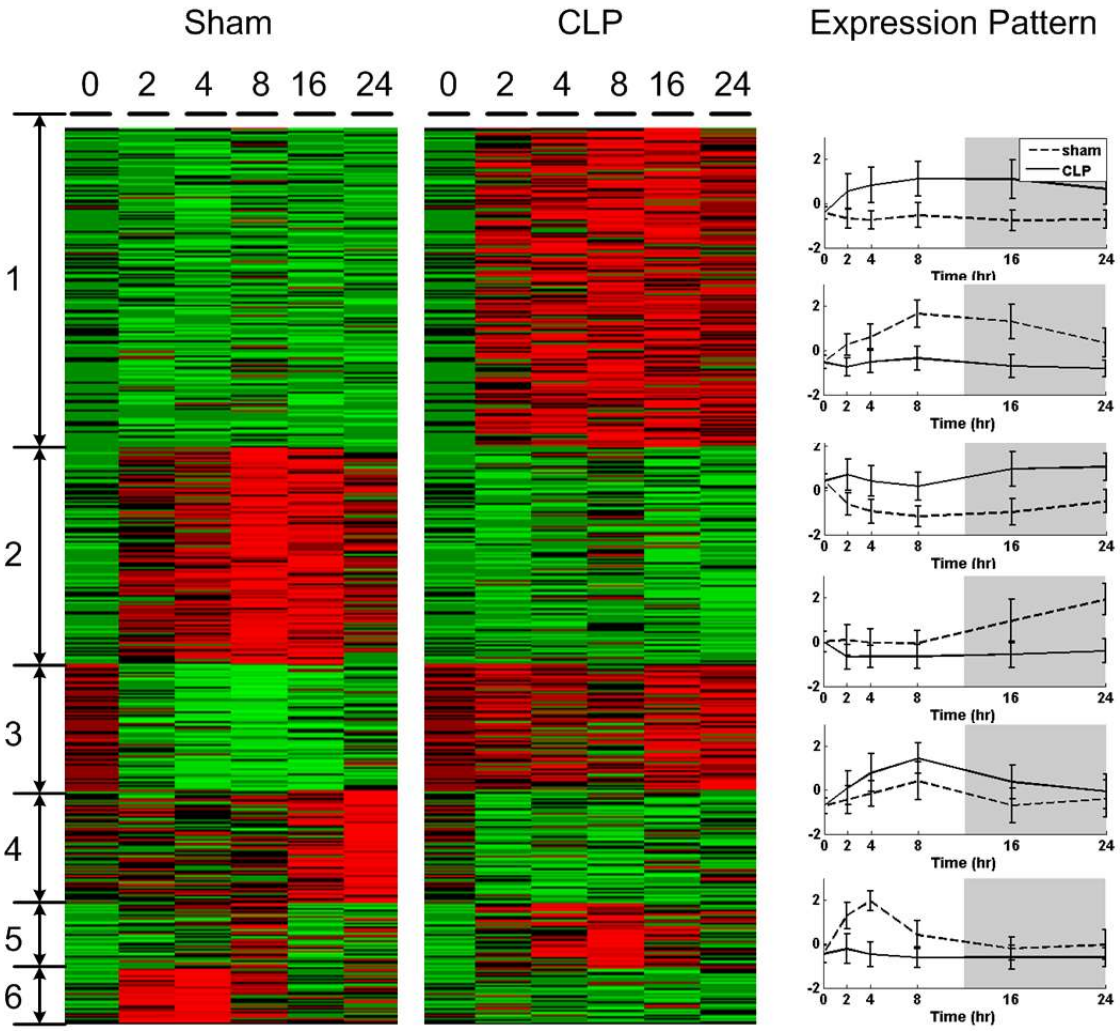

Figure 2. Gene expression profiles of rat livers in response to sham or CLP injury generated by Dr Yang and analyzed together.

Left Panel: Expressions of 437, 295, 171, 154, 91 and 73 probesets in 6 clusters in sham rats and CLP rats at 0, 2, 4, 8, 16, 24 h post-treatment are exhibited in a heat map. Right Panels: the expression patterns are shown by plotting the average normalized (z-score) expression values of 437, 295, 171, 154, 91 and 73 probesets in 6 clusters in sham and CLP groups (displayed as the means ± SEM).

CLP Dynamics: Systemic Response to Surgically Induced Sepsis

Similar to SCLP, CLP treatment imposes a surgical trauma to the host system, but also has the additional effect of releasing bacteria into the peritoneal space. This elicits additional responses from the host's systemic response, which must activate pathways that can clear the infection. Functionally characterizing the transcriptional profiles, along with single gene ontology, can display the major changes that occur in the cellular dynamics between CLP treated rats and sham rats. Though these changes are involved in the inflammatory response, just as in SCLP, it contains elements which are unique in ontology and dynamics, owing to the septic form of injury.

Pro-inflammatory cytokines are critical mediators of the immune and metabolic response during sepsis and elevation of these cytokines are associated with the initiation and propagation of the inflammatory response. The activation of pro-inflammatory cytokines and chemokines in Cluster 1 indicates the activation of the immune system and a pro-inflammatory response. Interestingly, following CLP injury, there is no delay in the onset of acute phase protein synthesis, manifested by the enhanced production of proteins in the complement and coagulation cascades. The addition of the activation of T helper cell differentiation and NK cell in the Cluster 1 by the release of the pro-inflammatory mediators is unique to CLP, presumably aiming to kill bacteria and protect the host from infection. Just as in the SCLP condition, our results indicate that the increased acute phase protein synthesis in Cluster 1 as well as decreased protein degradation in Cluster 2, leads to an up regulation of total protein synthesis, resulting in enhanced hepatic uptake of amino acids and protein synthesis in the liver. In the CLP model, protein synthesis in liver tissue

was increased by 164% following trauma and sepsis [68]. Thus, the amino acids (AA) required in order to produce large amount of APP in liver may be supplied by the increased flux of amino acids from the accelerated breakdown of muscle proteins. Interestingly, many of these changes are also observed in the hyperdynamic phase of human sepsis, which CLP is known to recreate [34]. Inflammation induced downregulation of the insulin signaling pathway in Cluster 4 loses the promotion of the storage of substrates in liver thereby leading to the increase of the degradation of fatty acid, amino acid and glycolysis in Cluster 3 and Cluster 5. Therefore, the impaired insulin signaling pathway, starting from the beginning and putatively as a result of the alterations in inflammatory gene expression, is expected to further strengthen the catabolism in muscle, and increase energy output by the liver in order to meet demand. Besides the transcriptional alteration in inflammation and metabolism, genes expression related to other functions also change expression patterns in comparison to the sham animals. The persistent downregulation of the genes functioning in DNA replication, mismatch repair, purine and pyrimidine metabolism are observed in CLP-induced response in Cluster 4. This is consistent with Almendro's study, which shows an induction of DNA fragmentation in rat skeletal muscle following the onset of the septic state [81]. Thus, the reduced expression of DNA replication and repair may explain the increased DNA damage. In addition, downregulation of the normal cellular assembly and organization indicates significant cell damage induced by oxidative stress. The increased mitochondrial dysfunction as well as the decrease in oxidative phosphorylation suggests that energy production is declining. These findings correlated with the notion that mitochondrial dysfunction resulting in bioenergetic failure may be an important factor in the pathophysiology of sepsis-associated multiorgan failure [77].

Interestingly, mitochondrial dysfunction and the insulin signaling pathway are in the same Cluster sharing the same expression patterns, indicating coregulation in these pathways. Following the CLP injury, the downregulation of the production of the energy produced by oxidative phosphorylation may be a sign of serious hepatic dysfunction. In order to avoid this, the low energy production state may be compensated by production of the quick energy through increased degradation of the substrates in liver, caused by the dampened insulin signaling pathway. Thus, there is likely a fine-tuned relationship between various stimuli that allow for adaptation to various different types of insults (septic vs aseptic) and different degrees of energetic dysfunction by upregulating various metabolic pathways.

Aseptic vs. Septic Acute Responses

The results obtained by Dr Yang and analyzed by myself indicate that both CLP and SCLP induce the activation of a pro-inflammatory response that encompasses enhanced synthesis of acute-phase proteins, as well as increased metabolism and tissue damage. However each injury shows significant differences in the dynamics. The discrete P1 and P2 phases present in the pro-inflammatory SCLP response contrast strongly with the combined P1 and P2 phases present in the pro-inflammatory CLP response. This indicates that these transcriptional modules are at least partially independently regulated. Genes triggered in CLP which are directly in response to bacteria removal are absent in SCLP injury, indicating that infection was successfully produced in the CLP animal model. A group of genes relevant to oxidative stress induced damage are unique in CLP injury which may be due to the difference in the severity of the two injuries. The same functions with individual dynamics, such as the metabolic changes spread over three clusters in CLP, indicate that these functions may be regulated by different transcription factors or

regulatory mechanisms. The published work done in collaboration with Dr. Yang goes into detail identifying putative transcription factors, and showing significant differences between CLP and SCLP (data not shown) [2]. In particular, the temporal nature of the study allows investigators to observe regulatory differences in injury models that are not apparent from static snapshots of gene profiles – including the metabolic implications of reactive oxidative species in the infectious response.

Chapter II: Transcriptional Response to Cecal Ligation and Puncture in Long Term Injury models [2]

Clinically, the inflammatory response to sepsis was previously thought to be represented by a strong, acute phase, anti-bacterial cascade that activated the immune system, and eventually caused systemic damage within the host [82]. However, recent evidence has emerged for a compensatory anti-inflammatory response [29], which hypothesized that the wave of early acute phase immune activation is followed by a wave of anti-inflammatory mediators, which suppress immune function to control the inflammatory response. Furthermore, the incidence of long term immune suppression in clinical settings [31], known as immunoparalysis, provides evidence that such an imbalance in this second wave can be just as clinically detrimental to pathogenic outcome as an imbalance in the early inflammatory mediators. Therefore, healthy outcomes do not depend solely on the strength of the pro-inflammatory response, or the anti-inflammatory response, but rather the balance between the two over a prolonged healing period [83]. The previous section, which covers the short term response in CLP and SCLP is a critical, but incomplete piece of the puzzle. Identifying long term factors in the resolution of each injury type can put into context the observed differences in gene pathways previously observed, and show whether the putatively predicted differences in gene regulation lead to an overall difference in outcome.

In order to have a proper spectrum of progression in our injury model from short to long term care, animals were given the exact same CLP and SCLP treatments as in the short term CLP analysis, all of which were collected by Dr. Orman. Typical literature

rodent responses to CLP include bacteremia, hypothermia, hypotension, and whole body hypermetabolic and catabolic states [84]. One notable phenotypic outcome of the long term study into the effect of CLP was that 100% survival in animals was observed after the sham and CLP procedures. Although some CLP models include fatalities, the goal of this long term analysis was to avoid biasing the analysis towards less severe responses by having a loss of animals prior to the final sample point. Because hypermetabolism is a condition that clinically manifests in a prolonged way rather than as an acute response, and because the liver is the primary organ responsible for both whole body metabolism and blood protein synthesis [85], the characterization of these long term hepatic gene responses to injury can aid in understanding the drivers and characteristics of this phenomenon.

Animal Model and Data Analysis

Animals were housed and treated with cecal ligation and puncture exactly as described earlier in the section save that animals were sacrificed at 9am in each group on days 1, 2, 5 and 8 post injury. All animal data collected in this study was performed by Dr. Orman.

Data analysis of the long term response differed from the short term data analysis during the consensus clustering steps and the post clustering data treatment. Analysis of CLP and SCLP gene expression data includes normalization, filtering, combining the datasets and clustering, all of which was conducted by Dr. Yang. First, DNA chip analyzer (dChip) software was used with invariant-set normalization and perfect match (PM) model to generate expression values. Then normalized data sets corresponding to CLP vs. sham and SCLP vs. sham groups were investigated to identify the temporally and differentially expressed probesets over time between each of the two conditions by applying EDGE for

each gene [57] as was done in the previous study. The significance threshold for this test was set as q-value <0.001 and p-value<0.001. Finally concatenated data sets corresponding to differentially expressed probesets in CLP vs. SCLP were combined to form one single matrix, which was then clustered using the approach of "consensus clustering" by Dr. Yang [58]. The goal was to identify subsets of transcripts with coherent expression pattern in CLP and sham as well as SCLP and sham respectively. Then, we characterized the biological relevance of the intrinsic responses by evaluating the enrichment of the corresponding subsets by using the pathway enrichment function ($p<0.05$) in Ingenuity Pathway Analysis tools (Ingenuity Systems, Mountain View, CA) as well as analyzed the functions of each individual gene. By using the concatenated SCLP and CLP clusters, the covariance between the 10 time points was generated, and then eigenvectors and eigenvalues were calculated. I then transformed the data into principal components, which were plotted in a 2 dimensional plot showing PC1 and PC2, which account for 65% of the total data.

Identification of CLP Related Patterns and Characterization of Cluster Function

Hepatic gene expression levels were measured at 0, 1, 2, 5 and 8 days post injury in the livers of rats following CLP and SCLP treatment. The differentially expressed genes whose profiles differed between the CLP and SCLP groups were identified, through the methods outlined previously. A total of 1057 probesets were found to be differentially expressed in CLP ($q = .01$), when compared to SCLP. These genes were further culled using consensus clustering by Dr. Yang, which allowed for the identification of three major motifs within the differentially expressed gene set, with the clusters containing 152, 200, and 56 probesets respectively. The genes contained in each of the clusters, as well as the

average Cluster patterns, are shown in **Figure 3**, where the right hand panel contains the average expression level of the clusters, while the left contains a heat map of all the genes contained in those clusters. By using IPA pathway analysis, combined with further identification of relevant genes through single gene ontology, each of the three clusters was characterized below:

1) Cluster 1 is comprised of 152 probes which were clustered into a single motif, shown in **Figure 3**. In the first Cluster, the genes in CLP have a minimum compared to SCLP following the first day, rise to a much larger fold change on day 5, and then finally fall back to the SCLP baseline on day 8. Utilizing the KS test (shown in the manuscript [2]) to determine whether the distributions of the two profiles are different at each point allows for the identification of the 1 day, and 5 day points as statistically different, while the points at days 2 and 8 pass the K-S test between the two conditions. Using pathway analysis, the genes in this Cluster are primarily associated with the Pentose Phosphate Pathway (*Gpi, G6pd*), Insulin Signaling (*Accn2, Prkag2, Acly*), p38 MapK Signaling (*Pla2g6 ,Fas*), Fatty Acid Metabolism (*Acaca*), and Calcium Signaling (*Tpm1, Myh3, Prkag2, Asph*). Single gene ontology analysis indicates that other genes in this group are associated with the innate immune response (*Il11, Ccl20*), mitochondrial respiration (*Cox6a23*), DNA damage (*Ddit4l*) and NADPH production (*Me1*). This Cluster is primarily related energy production through fatty acid degradation, the production of anti-oxidant species, innate immunity signaling via calcium channels and traditional MapK signaling pathways. This Cluster is up regulated early in SCLP, and suppressed

over the long term, but suppressed early in CLP, and up regulated over the long term.

2) Cluster 2 is comprised of 200 probes which were clustered into a single motif, shown in **Figure 3**. The CLP genes that have been differentially expressed display a maximum in the first day, when compared to SCLP. However, following the first day, those genes return to baseline by Day 2, and then are significantly suppressed compared to SCLP on Day 5. The relative activation of SCLP genes returns to baseline by Day 8, though the CLP genes overshoot their SCLP counterparts. Utilizing the KS test (shown in the manuscript [2]) to determine the statistical significant of differences between the two points gives the identification of the 1 day, 5 day and 8 day points as significantly different, while the point at day 2 passes the K-S test between the two conditions. Pathways which are enriched by genes within this Cluster include NF-κB signaling (*Ntrk2,Bmpr2,Tnf*), Cell cycle regulation (*Hdac7,Hdac10*), HIF-1α signaling (*Mmp24,Slc2a3*) and amino acid degradation (*Papss2, Ahcy, Kat2b, Smyd3*). Single gene ontology has identified further relevant genes, including innate immune responses via the ERK pathway (*C1ql3, Il3ra, Sh2d2a*) and cell cycle and gap junction based genes (*Gja5, Mmd2, Ptpn3*). This Cluster contains pro-inflammatory gene ontologies, which are suppressed early in SCLP, and then up regulated at later time points, but show up regulation early in CLP, and suppression at the later time points.

3) Cluster 3 is comprised of 56 probes which were clustered into a single motif, shown in **Figure 3**. The genes in this Cluster, unlike Clusters 1 and 2, display a suppression compared to the Day 0 baseline, which persists through Days 1 and 2. Following

this, the CLP genes exhibit an extreme down regulation compared to SCLP, which peaks at Day 5, and overshoots the baseline at Day 8, with CLP emerging slightly greater than SCLP. Utilizing the KS test test (shown in the manuscript [2]) to determine the statistical significant of differences between the two points gives the identification of the 1 day, 5 day and 8 day points as significantly different, while the point at day 2 passes the K-S test between the two conditions. Pathway analysis indicates that the genes in this Cluster are related primarily to fatty acid metabolism and amino acid metabolism within the mitochondria (*Echs1, Gcdh*), DNA Repair (*Xrcc6*), Toll-like Receptor Signaling (*Tlr7, Egfr*), and Nitrogen metabolism (*Ca5a*). Single gene ontology has further identified genes that are related to central carbon metabolism and anti-oxidant production (*G6pc3, Gstm3, Ndufc2*). Though the genes in this Cluster are much fewer in number than those in Clusters 1 and 2, the ontologies contain critical genes which include toll like receptors, which have long been associated with sepsis [86], glucose-6-phosphatase, which represents a critical final step in gluconeogenesis for liver glucose output, and glutathione transferase, which is the traditional liver defense against peroxidation [87]. This Cluster also contains pro-inflammatory gene ontologies, and while both conditions manifest an early suppression, SCLP manifests a strong up regulation at Day 5, which is then suppressed at Day 8, relative to CLP.

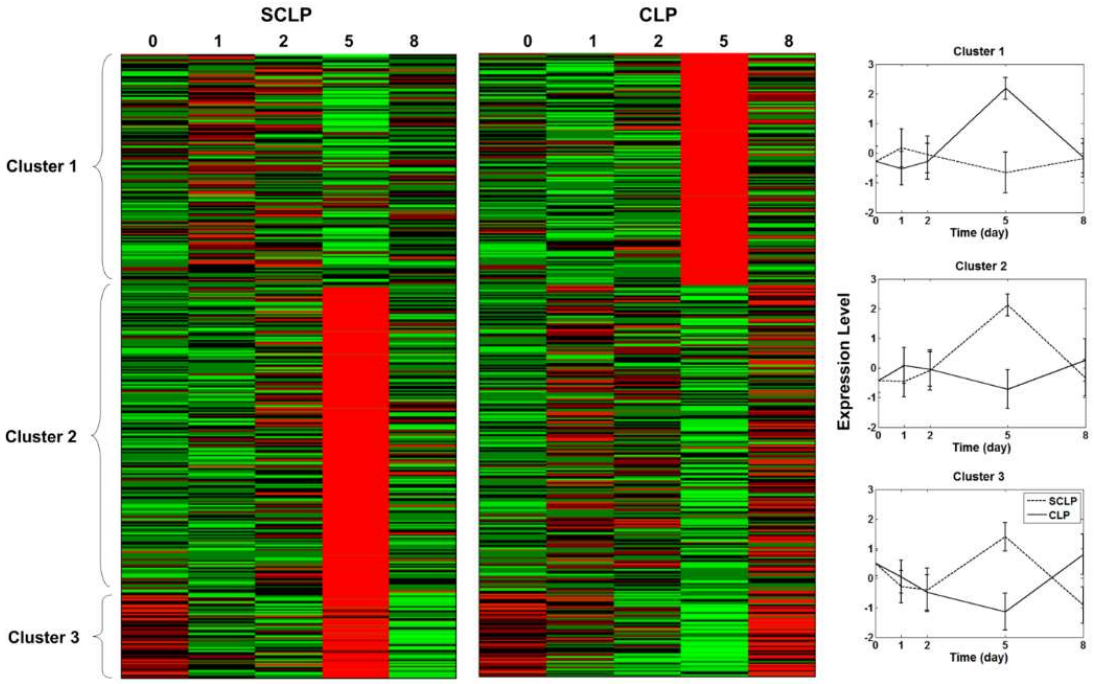

Figure 3. Long Term gene expression profiles of rat livers in response to sham or SCLP injury generated by Dr. Yang and analyzed together

Left Panel: expressions of 152, 200 and 56 probesets in 3 clusters in CLP rats compared to SCLP rats at 1, 2, 5 and 8 days post injury

Right Panels: the expression patterns are shown by plotting the average normalized (z-score) expression values of 152, 200 and 56 probesets in 3 clusters in CLP rats compared to SCLP rats (displayed as the means ± SEM).

Characterization of Response Progression through Principal Component Analysis

By concatenating the SCLP and CLP datasets together, and only taking into account the genes which were identified as belonging to Clusters 1, 2, and 3, it was possible to use principal component analysis (PCA) to identify the major features of these three clusters, and the places where the response within CLP and SCLP diverge. Shown in the top panel of **Figure 4** is a heat map which plots the contribution of all 408 genes against the 10 total principal components which were calculated. The genes have been segregated by Cluster, and it can be seen that Cluster 1 is the primary driving force behind the first principal component, while Cluster 3 is the primary driving force behind the second principal component. Shown in the lower panel of **Figure 4** is a plot of the coefficients for the CLP and SCLP data for each day, with linear connections between the days in chronological order. Two major features dominate this plot: firstly, there is a large degree of symmetry between the CLP and SCLP responses, where both conditions behave identically on the y-axis, corresponding to principal component 2 (PC2), but have mirrored responses on the x-axis, corresponding to principal component 1 (PC1). Furthermore, the first two days following the injury appear to be primarily characterized by changes in PC2, with shifts occurring in the y-axis (though Day 1 has shifts along PC1 as well). Day 5, however, is characterized by a massive change in the first principal component, which then returns back in Day 8. The other major feature of this plot is that at the end of the profile, CLP appears to return back to a state close to Day 1, while SCLP appears to return back to a state closer to Day 2, with neither returning to their Day 0 profiles.

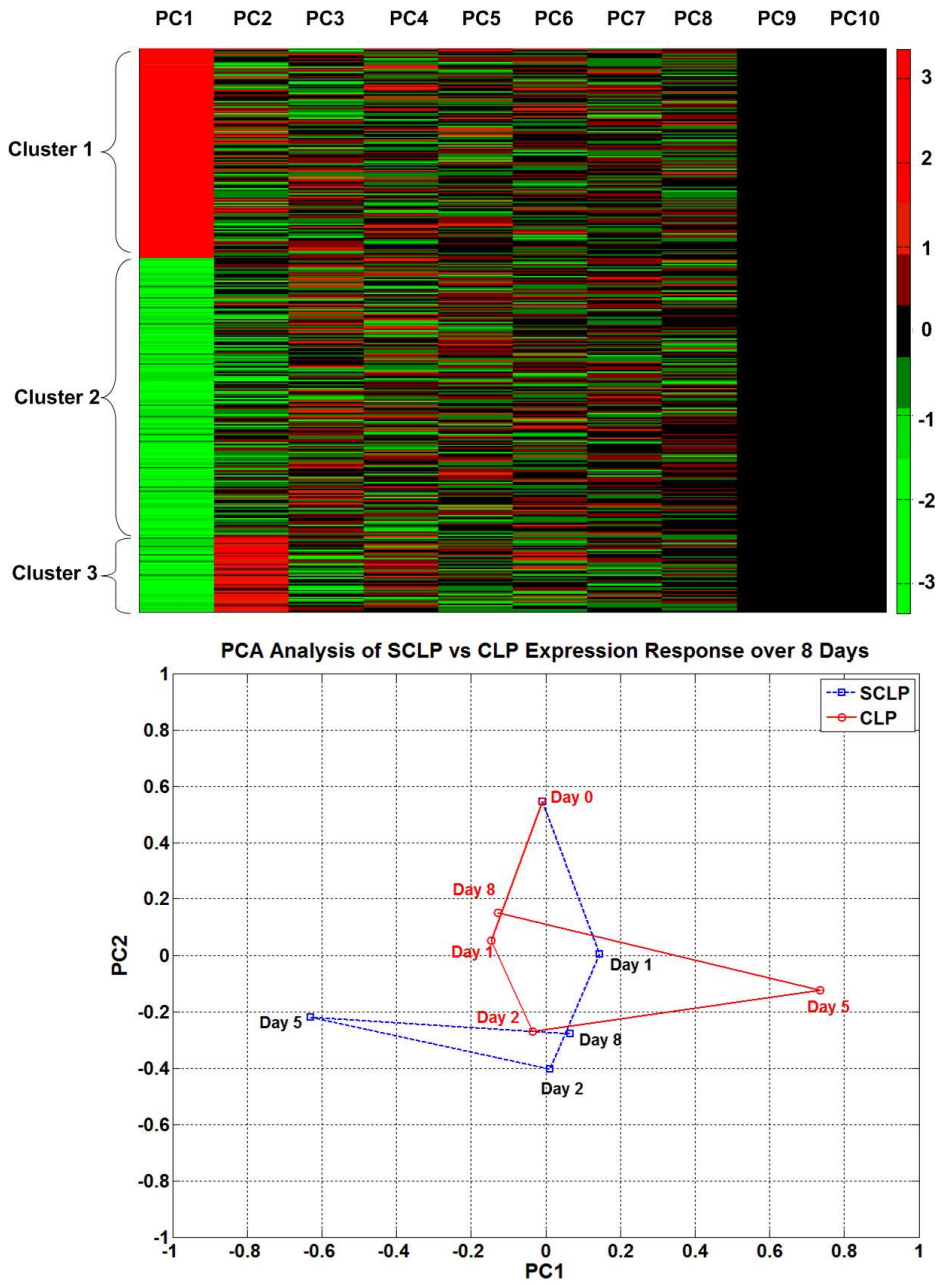

Figure 4. Principal Component Analysis of the Clustered Genes in SCLP and SCLP. Top Panel: The contributions of all 408 probes that make up Clusters 1, 2 and 3 are shown to each of the principal components. Bottom Panel: The two principal components (PCs) that best represent the CLP and SCLP data are plotted on each day to show the trajectory of each injury. These two PCs make up 65% of the information dataset, where red represents CLP, and blue represents SCLP.

Short Term Differences Manifest in Long Term Disease Progression: CLP vs. SCLP

Though CLP and SCLP share common surgical stressors, CLP is a significantly different injury in that, by releasing bacteria into the peritoneal space, the rats enter a state of sepsis, as opposed to trauma. The long term impact of bacteria on the response of the system may play a significant role in the resolution of the disease, as in clinical cases, this bacterial translocation occurs without the trauma of the SCLP surgery. While many of the traditional pro-inflammatory markers are missing from the gene ontologies, it can be observed in the supplementary data [2] that these markers show dynamic expression common in both CLP and SCLP, owing to the fact that they are both inflammatory stimuli. However, there are significant differences that manifest in the three clusters that indicate that although both injuries share a common core response, they are distinguished by outlying inflammatory mediators and metabolic stressors.

The early mild depression and late strong activation of genes within Cluster 1 following CLP, which includes ontologies relevant to the pentose phosphate pathway, insulin signaling, MapK signaling, fatty acid metabolism, and calcium signaling is indicative of an anti-oxidant response. It has been shown that the pentose phosphate pathway is critical in the regeneration of NADPH, an important reducing agent that removes reactive oxygen species and lowers the redox state of cells [88]. Insulin acts as an anabolic hormone, stimulating lipogenesis, glycogen and protein synthesis in the cell in order to promote the storage of excess metabolites [79]. Its presence in this Cluster indicates that under septic conditions, the liver burns more macromolecules early through the down regulation of this hormone's signaling pathway, but then restores them more vigorously compared to SCLP, potentially to make up for the excess burning. It is

interesting that p38 MapK signaling pathways would be present in Cluster 1, as it appears that this pathway is suppressed early, and activated late, acting as an anti-inflammatory signal. However, the dynamics of the p38 MapK pathway have been shown to regulate both the pro- and anti-inflammatory portions of the immune response [89], and thus the portion of the signaling pathway which is suppressed early may be related to the regulation of anti-inflammatory pathways. The gene which is involved in fatty acid metabolism, *Acaca*, encodes the protein Acetyl-CoA carboxylase, which represents the rate limiting step in fatty acid biosynthesis [90]. Combined with the up regulation of insulin signaling pathways, this is an indication for the restoration of macromolecule production in the liver following 2 days after sepsis, though this response fades at 8 days. Calcium signaling has previously been characterized as a potent activator of innate immunity [91], and thus the early suppression of proteins related to calcium signaling but not sepsis (such as *Tpm1*, which encodes a critical actin skeletal protein [92]) may be an effort by liver cells to avoid the side effects of alterations in calcium intended to facilitate innate immune signaling. The up regulation of these genes later indicates that this is no longer a threat, and that calcium sensitive proteins need to be restored for cell function. Overall, Cluster 1 makes the case for an anti-inflammatory response in CLP rats which is more potent than the aseptic SCLP treatment. This response is further characterized by the presence of anti-oxidant genes, which will lower the redox ratio of the cell. These observations are further supported by the single gene ontologies, which show further production of anti-oxidant species, and evidence of DNA damage.

Cluster 2 is primarily related to pro-inflammatory signaling pathways, with significant activity within the NF-κB signaling pathway, the cell cycle, HIF-1α and the

degradation of various amino acids. Interestingly, the NF-κB signaling pathway is identified in this Cluster, though the p38 MapK pathway was identified in Cluster 1. Since these two clusters show opposite dynamics, it is likely that the genes within this pathway, which include *Tnf* among others, act in an antagonistic manner with those in Cluster 1, where the Cluster 2 genes promote an inflammatory response, while the Cluster 1 genes divert the signal away from one. NF-κB signaling has been well characterized as a pro-inflammatory response [93], which indicates a stronger early response in CLP compared to the bacteria free SCLP insult. The presence of cell cycle identified proteins, which are primarily histone modifiers, represents a twofold change in the transcriptional dynamics of the cell, as well as an inhibition of cell cycle progress in cells: it has been shown in the lung that the inhibition of these modification proteins can attenuate injury [94], likely due to the suppression of anti-inflammatory check points. The early activation and late suppression of these proteins, therefore, indicates a more severe septic response, with additional transcriptional changes. The presence of HIF-1α signaling within Cluster 2 is a phenomenon which links the presence of stronger pro-inflammatory signals to markers of oxidative stress. HIF-1α is a transcription factor family which is well known for its response to oxidative stress [95], and since septic conditions have been shown to produce increased levels of reactive oxygen species in the liver [96], likely is acting in response to the increased concentration of those species. It is worth noting that biomarkers of oxidative stress are also present in Cluster 1, in the form of anti-oxidant species, which aim to scavenge these reactive molecules. The final major pathway identified in Cluster 2 relates to amino acid metabolism, and is critical to the hyper metabolic response. Since protein synthesis in the liver has been reported to increase by over 160% following CLP [68], the

maintenance of amino acid supplies are critical to maintain this output. Since CLP appears to be up regulating hyper metabolic genes that degrade certain selenoamino acids, along with lysine, the corresponding degradation of peripheral tissue might be increased to make up for demand. These changes, which manifest themselves in the form of muscle wasting, and increased urea production, are hallmarks of human sepsis, which CLP is designed to mimic [34]. Furthermore, Cluster 2 shows signs of oxidative stress through transcription factor biomarkers, indicating that oxidative stress is part and parcel of the pro-inflammatory response. This is further supported by the single gene ontologies, which show the activation of complement proteins, and cytokine receptors. Further regulation of transcriptional machinery and gap junction integrity is also prominent, indicating that the genes are activated in order to recruit immune cells to the appropriate locations.

Similar to Cluster 2, Cluster 3 has pathways which correspond strongly to a pro-inflammatory stimulus, however, the unique dynamics in each indicate that these aspects of the pro-inflammatory response may be regulated by separate mechanisms. One of the prominent pathways present in Cluster 3 is fatty acid degradation, which includes genes involved in beta oxidation within the mitochondria, and the degradation of lysine. The mitochondria is well associated with the production of energy, which is ideally a goal of hypermetabolism, but it is also the primary site for the production of reactive oxidative species within the cell [97]. Because of this, the presence of these genes within Cluster 3 indicates not only a stronger hyper metabolic response in CLP, as evidenced in Cluster 2, but also provides a mechanism for the oxidative stress responses that are observed in the activation of HIF-1α, and later, the activation of anti-oxidant pathways within Cluster 1. Furthermore, the presence of DNA repair enzymes indicates that the generation of these

reactive species is causing damage [98], especially considering that many transcriptional reading frames are open, in order to generate mRNA for acute phase proteins. The presence of the Ca5a gene, which was associated with Nitrogen metabolism is also further evidence of oxidative function. This gene encodes mitochondrial carbonic anhydrase, which is primarily based in the liver, and is critical for ureagenesis, and gluconeogenesis [99]. Both of these processes are well established outcomes of sepsis, with the liver providing energy to the innate immune response through glucose production by dismantling amino acids to create urea for energy. Finally, the presence of Toll-like receptor signaling pathways within this Cluster confirm it as an anti-bacterial, pro-inflammatory Cluster whose form mimics Cluster 2, and whose functions are similar in intent. Toll like receptors are well known for their ability to recognize bacteria, or pieces of bacteria, and activate the innate immune response [100]. The presence of these receptors within the CLP response in Cluster 3 is evidence of recognition of the bacterial threat that is posed by the CLP condition. Gene ontologies further support this Cluster's characterization as a pro-inflammatory, hypermetabolic gene profile, as G6pc3 encodes glucose-6-phosphatase, which is an enzyme located only in the liver, and is the critical final step for gluconeogenesis. Furthermore, the presence of NADH dehydrogenase within the single gene ontology shows increased mitochondrial function, while glutathione transferase production indicates a response from the cell to rising oxidative stress levels.

CLP and SCLP Dynamics and Characterization

In the first day post injury, the genes within Cluster 1 appear to be slightly suppressed in the CLP condition as compared to the SCLP condition, however, this response quickly resolves and both conditions return to the time 0 baseline at Day 2.

Following Day 2, the Cluster under SCLP remains suppressed, and does not appear to deviate significantly from the baseline, while the same genes under CLP undergo a massive activation, which peaks at Day 5. This process appears to have completely resolved itself at Day 8, with the distributions of SCLP and CLP profiles being identical under the KS test. Since Cluster 1 is an anti-inflammatory, anti-oxidant that is also responsible for macromolecule biosynthesis, possibly to restore energy repositories following hypermetabolism, the early suppression of this Cluster under CLP is indicative of the acute phase response and resolution, where the bacterial infection causes anti-inflammatory mechanisms to be suppressed. However, the late activation of this Cluster following the CLP injury, both in comparison to SCLP, and to the healthy baseline at time 0, indicate that the long term response following CLP is predominantly anti-inflammatory. The anti-inflammatory nature of the long term response to the septic injury model has significant implications for susceptibility to further infections within the rats, as their immune defenses are compromised during this anti-inflammatory period. This is in agreement with recent clinical studies, which indicate that immunosuppression may play a strong role in the severity of clinical sepsis [31], and indicates that the traditional view whereby sepsis causes damage through a vigorous inflammatory response may only be valid in the acute, short term phase of the injury.

Unlike Cluster 1, the genes within the second Cluster are slightly activated in the CLP injury when compared to the SCLP injury, though, like Cluster 1, both return to the time 0 baseline at Day 2. Following Day 2, the genes within Cluster 2 do not deviate from the time 0 baseline under the CLP condition; however, they are strongly up regulated on Day 5 following the SCLP injury. Though the KS metric determines that the CLP and

SCLP profiles are not identical at Day 8, it should be noted that the Day 8 distributions show significantly less difference than the other days, and microarray data is known to be noisy [101]. Since the dynamics of Cluster 2 mirror those of Cluster 1 in reverse, and Cluster 1 returns to its baseline, it is possible that Cluster 2 returns to baseline at Day 8, despite failing the K-S metric, though without later time points, this remains unconfirmed. Cluster 2, overall, displays hallmarks of increased pro-inflammatory activity, which is manifested primarily through the NF-κB signaling pathway, HIF-1α and signs of hypermetabolism. It is therefore appropriate that Cluster 2 behave opposite to Cluster 1, since the CLP response in Cluster 1, which is significantly anti-inflammatory, acts as a suppressant for the pro-inflammatory Cluster 2 genes, which do not deviate from their baseline after the second day. Similarly, the SCLP genes in Cluster 2 show a significant pro-inflammatory stimulus, while the SCLP genes in Cluster 1 remain suppressed. Thus, the long term activation of pro-inflammatory genes within the SCLP condition, when compared both to CLP, and to the time 0 baseline, indicates that the SCLP injury illicit a long term pro-inflammatory response. Since SCLP represents aseptic trauma, including a gash in the abdominal skin, the maintenance of a long term strong pro-inflammatory response may represent the priming of anti-bacterial defenses at the site of injury. This contrasts significantly with the CLP response, and may represent a fundamental difference in the long term response of patients who suffer trauma but do not develop sepsis.

Unlike both Clusters 1 and 2, Cluster 3 shows a similar response between CLP and SCLP during the first two days of injury, where both show a decrease relative to the time 0 baseline, and come together at Day 2. Following Day 2, however, the SCLP response is strongly activated relative to the baseline, while the CLP response continues to decline.

Between Days 5 and 8, the clusters switch dynamics again, and where the CLP profile returns approximately to the time 0 baseline, the SCLP profile decreases dramatically, both in relation to the CLP profile, and the baseline. It is worth noting that the short term CLP response, characterized by an early pro-inflammatory response, does not include genes from Cluster 3, as this Cluster is suppressed under both CLP and SCLP at Day 1, relative to the baseline. However, in the long term response, the CLP response is fairly consistent with that of Cluster 2, showing suppression followed by a return to the time 0 baseline. In contrast, the SCLP response is consistent with the previous two clusters over the first 5 days, with early suppression of pro-inflammatory markers followed by up regulation, but is characterized by an unusual drop in expression levels at Day 8. Cluster 3 represents pro-inflammatory, reactive oxygen species and is partly responsible, in conjunction with Cluster 2, for innate immunity signaling, and hyper metabolic effects, including amino acid and fatty acid degradation. Thus, the early suppression of the response in both clusters may indicate that these genes are not affected by the injury unique mediators in the early response. However, following the second day, there is significant deviation between the two injuries, with SCLP maintaining a pro-inflammatory response consistent with Cluster 2. Consistent with the resolution observed in Clusters 1 and 2, the CLP response returns to the time 0 baseline on Day 8, however, the failure of the SCLP injury to do so may indicate that its long term response has not yet resolved. This is to be expected, as the animals have not fully healed from the injury yet, and are possibly entering a prolonged recovery phase.

In order to formally represent the differences between the response of the animals between CLP, and SCLP, principal component analysis (PCA) was used in order to characterize the state of all three clusters of the animal on each day (shown in the right

panel of **Figure 3**). While both conditions begin at the same point, they rapidly diverge following injury, splitting across the y-axis. Previously, the interesting symmetry between Clusters 1 and 2 has been discussed, and this symmetry carries over to the principal components. Given that the most striking difference between the two conditions occurs at Day 5, with CLP having a strongly positive value for principal component 1 (PC1), while SCLP has a strongly negative value for the same, it can be inferred that PC1 represents the strength of the anti-inflammatory response. Thus, CLP's strong anti-inflammatory behavior in Cluster 1 is the primary contributor to its positive value; while SCLP's strong pro-inflammatory behavior in Cluster 2 is the primary contributor to its negative value. Principal component 2 (PC2) is harder to characterize, however, the left panel of **Figure 3** shows that it is primarily controlled by the activity of Cluster 3. Thus, the changes in PC2 from Day 0 to Day 2 represent the fact that although Clusters 1 and 2 have symmetrical behavior during this time, Cluster 3 shows down regulation in both conditions, which does not return to the baseline. Thus, PC2 represents the asymmetrical pro-inflammatory contribution of Cluster 3 to the profile. It is worth noting that neither CLP nor SCLP return back to their Day 0 condition at the end of the experiment. This indicates that liver's response to injury may not have resolved itself following the 8 days, and there may yet still be ramifications to the condition over an even longer timescale. Furthermore, at Day 8, CLP has returned to a point that is close to its Day 1 condition, while SCLP has returned to a point closer to its Day 2 condition. This may represent a break in symmetry in the two response progressions that indicates different pathways of eventual resolution in the future.

Overall, the three clusters that distinguish CLP and SCLP represent complementary halves of the inflammatory response. Specifically, Clusters 1 and 2 show gene ontologies

and dynamics which are diametrically opposed: in both SCLP and CLP, the up regulation of one Cluster is accompanied by the down regulation of the other. This indicates that while SCLP and CLP have fundamentally different ontologies, they manifest these changes in a two stage process, where an acute phase resolves into a long term phase, whose dynamics are opposed to the initial response. Particularly in the case of CLP, it appears that the dynamics of the response mimic those found in clinical sepsis [102]: an early immune activation is resolved after 2 days following injury, with an anti-inflammatory immunosuppressive response manifesting after the second day, and only resolving after the eighth day. Because the animals have a 100% survival rate within this study, it can be hypothesized that the measured responses represent a balanced outcome between the acute and long term responses that allows the animal to resolve both the infection, and the response, and eventually heal. It is worth noting then, that the magnitude of the fold changes within the acute phase response, which manifests at Day 1, appears to be significantly less than those in the long term response that manifests most strongly at Day 5. Thus, while an overreaction of the long term anti-inflammatory response can cause immunoparalysis [29], the healthy resolution also appears to require a significant magnitude difference between the responses at a transcriptional level, suggesting that in the future, the characterization of the relative size of the long term response may be a relevant parameter towards determining clinical outcomes.

It is interesting to note that biomarkers of oxidative stress go hand in hand with biomarkers for inflammation, and also appear to increase in severity following the CLP treatment. It is distinctly possible, due to the presence of oxidative stress responses within Clusters 2 and 3 (HIF-1α and glutathione transferase) that feedback mechanisms exist

which modulate the oxidative stress levels in order to create feedback and switch to an anti-inflammatory mechanism. Oxidative stress has been shown previously to affect gene expression and alter regulatory dynamics [103], and since it is present in all three clusters, and follows similar patterns to the anti- and pro-inflammatory dynamics, may act as a controller for these responses. Intervention at critical time points, where the pro-inflammatory and anti-inflammatory clusters cross over, may allow for a suppression of the pro-inflammatory response by allowing the anti-inflammatory genes to become dominant, due to loss of reactive oxygen species. While anti-oxidant treatments have previously been shown to be ineffective in a clinical setting [104], it may be due to the therapeutic agents being applied at times when oxidative stress is low, thus giving the therapeutic agent no active target to utilize. Intervention at critical time points, when oxidative stress is high, may allow for the alleviation of the damaging aspects of oxidative stress, while simultaneously dampening the pro-inflammatory response, thereby improving patient outcomes.

In the previous chapter on short term work [2], we were able to identify functions that were characteristic of the acute phase response to both CLP and SCLP. We were able to identify that while SCLP had several pro-inflammatory clusters, the CLP response had one single Cluster that represented a much stronger pro-inflammatory response during that acute phase. This finding is reflected within the long term data, which directly compares CLP and SCLP gene expression, since CLP has an increase over SCLP in Cluster 2 (which is pro-inflammatory), while being slightly suppressed in Cluster 1 (which is anti-inflammatory). Furthermore, the identification of oxidative stress biomarkers in only the CLP response during the short term study is interesting, as the long term dynamic response

shows an anti-inflammatory, anti-oxidative stress profile, with significant up regulation of Cluster 1, and suppression in Clusters 2 and 3 at Day 5. It may be that the presence of oxidative stress in the short term triggers the anti-inflammatory wave that is observed most strongly at Day 5, and that the onset of a more severe acute phase response is then followed by a much stronger protective long term response. In our short term study, Dr. Yang was also able to use putative transcription factor identification to show that the clusters under these two conditions were not regulated by the same machinery. This finding is critical for understanding of the long term response, as these differences in regulation may be responsible for the massive variation between the two functions following Day 2, where the profiles significantly diverge.

By identifying differentially expressed genes, and using consensus clustering methods using similar methodologies to the short term CLP analysis, Dr. Yang was able to find three major profiles from the microarray probes. The first Cluster represents an anti-inflammatory response, with anti-oxidative properties, which is suppressed early in the CLP condition compared to the SCLP condition, and later up regulated. The second and third clusters represent pro-inflammatory responses that promote oxidative stress, although Cluster 2 appears to be focused on signaling and amino acid metabolism, while Cluster 3's gene ontology is related to toll like receptor signaling and hyper metabolism. While Cluster 2 demonstrates an early activation, and Cluster 3 remains suppressed in the early phase, these two clusters are both suppressed in the long term response following CLP. The early resolution at Day 2 for each response implies that the acute phase pro-inflammatory response has finished, which indicates that fundamental changes may have occurred in the system. This fundamental change may be associated with the elimination of the bacterial

pathogen from the system; however, this remains to be investigated. The long term response, where the anti-inflammatory genes are up regulated, persists for 6 days after, indicating that the resolution of the innate immune response is on a significantly longer timescale than the resolution of the initial acute phase response. This indicates a two wave response that is consistent in both injuries, with a turnover point at Day 2 and a resolution by Day 8. In particular, the balance between Clusters 1 and 2 indicates that these two functions act in concert, and represent classical pathophysiological responses to sepsis, both anti- and pro-inflammatory. Though Clusters 1and 2 return to the time 0 baseline, indicating resolution of both the anti- and pro-inflammatory response, the SCLP response in Cluster 3 does not return, and shows persistent down regulation. By using principal component analysis to decompose the primary contributors of the gene clusters, it is possible to view the progression of the disease, and observe that while both CLP and SCLP share a qualitatively similar response, the inflammatory direction is diametrically opposed. Furthermore, this analysis of the system indicates that neither CLP nor SCLP has returned back to its Day 0 condition. This indicates that both responses have not resolved following an 8 day period, and the SCLP response may be transitioning from a pro-inflammatory response to an anti-inflammatory trend, based on the behavior of Cluster 3. The unique dynamics of these survival injuries, and their overlap with metabolic functions, particularly in the region of reactive oxygen species, provide clues into the nature of the hepatic response to various injuries – although they both could be classified as sepsis, a closer analysis reveals a much more divergent and unique dynamic.

Chapter III: Transcriptional Response to Burn Priming in Long term Cecal Ligation and Puncture Models [3]

The previous Chapters have focused on CLP as an animal model for clinical sepsis, which has been verified by Dr. Orman's initial cytokine experiments [47], and further by Dr. Yang's microarray measurements and our analysis together [2]. However, in true clinical settings, bacterial infection and sepsis do not emerge de novo, but often occurs through some other traumatic stimulus. For example, the classical response to burn injuries, which contribute the most to patient morbidity, are a series of systematic responses which activate both pro- and anti-inflammatory cascades, causing hypermetabolism, muscle wasting, organ dysfunction, and immune suppression [105]. In particular, the immune suppression caused by the burn injury, in conjunction with increased permeability in the intestinal barrier, allow for bacteria to cross into the abdominal space, and create a second, internal insult that leads to sepsis [106]. This second injury is hypothesized to contribute to the long term deterioration of patient health, and leading to total immune dysfunction [107] and multiple organ failure [108]. Though the inflammatory stimuli that have been traditionally studied in a clinical setting are of an acute nature [53, 109, 110], the progression of the disease within human patients occurs over a large timescale [106], often with weeks of deterioration. It has also been shown that anti-inflammatory responses often have multiple phases, that correspond to an acute pro-inflammatory stimulus followed by an anti-inflammatory response to resolve the condition [29]. It has been hypothesized that an imbalance between these two responses is what leads to pathophysiological sepsis [31], however, in order to understand what generates this imbalance, it is necessary to understand the mechanisms which generate and maintain the balance.

According to the ‘two hit’ theory [30], major trauma (such as burn injury) increase the production of pro-inflammatory mediators, mainly from macrophages. This in turn, compromises the immune system of the host, making the host vulnerable to a second hit (i.e., infection), leading to multiple organ failure and, ultimately, to death. Due to the relative severity of the sepsis cases following burn, it is believed that burn acts as a priming mechanism which compromises the immune system and causes it to either fail to remove the bacteria, or overreact to the bacterial presence and cause damage through anti-microbial oxidative mediators [32]. Furthermore, because the condition of clinical sepsis occurs over a long time period, the ways in which this priming manifests itself may occur over both the short and long term, following injury.

Despite the severity of the double hit injury within clinical settings, the condition of sepsis has been primarily studied in an isolated manner, with animals receiving cecal ligation and puncture to mimic a septic injury [84]. The only previous double hit study published has been Banta’s study which compares both the gene expression levels with metabolic fluxes of rats between burn primed septic rats and control rats within the liver [41]. This study only focuses on a single time point, 4 days following injury, which allows a snapshot of the response. By creating a time series of gene expression data within the liver, matching the scope of the previous Chapters, and comparing the burn followed by CLP to simply CLP, it is possible to characterize the magnitude and function of the effects of burn’s priming, as well as the dynamics which may indicate key time points where the specific condition of burn followed by CLP is significantly more severe than its septic counterpart.

Animal Model and Data Analysis

Animals were housed and treated with either burn or sham treatment followed by cecal ligation and puncture exactly as described in Chapter I according to the schedule shown in **Figure 5**. All animal data collected in this study was performed by Dr. Orman. Data analysis of the long term response was performed by Dr. Yang, and was identical to the CLP short and long term data, save that consensus clustering was not used. In order to characterize the probesets which show significant fold change at each time point, the method of statistical analysis of microarrays (SAM) was used [111]. This method compares the gene expression of the two response variables, BCLP and SCLP. Briefly, by computing a statistic g_i for each gene i, the strength of the relationship between the response variable (BCLP) and the standard (SCLP) is measured. False discovery is controlled via the random permutation of the gene response sets, in order to calculate the probability that the observed response is statistically significant. Following this, we characterize the biological relevance of the genes found to be statistically significant through SAM by evaluating the enrichment of the corresponding subsets in circadian rhythm specific pathways using the pathway enrichment function ($p<0.05$) in Ingenuity Pathway Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA) as well as analyzing the functions of individual genes extensively.

Due to the reduced stringency of not including consensus clustering as a filtering step for genes of interest, RT-PCR of a small subset of identified genes was used by Dr. Yang to confirm microarray trends. The RNA samples were amplified with Nugen WT Pico Kit (Part# 3300-A01) (NuGEN Technologies Inc, San Carlos, CA, USA). RNA was reversed transcribed into DNA. Then cDNA samples were diluted by 100 times. All the

samples were tested in triplicates. qPCR was performed using the ABI 7900 HT Sequence Sequence Detection System (TaqMan; Applied Biosystems, Foster City, CA, USA) using standard fluorescent chemistries and thermal cycling conditions. Primer and probe sequences were designed for each experimental gene's mRNA sequence using Primer Express software (Applied Biosystems) as shown in **Table 3**. For the gene of interest, 6-10 ng of cDNA was mixed with 20 μM FWR/Rvse and 10 μM (UPL) Probe (UPL). The expression level of the housekeeping gene GADPH [112] was used as an internal reference, and all fold changes are displayed as comparisons between BCLP and SCLP levels of the gene at each time point

GAPDH was added into Rodent GAPDH control kit ABI (Part No.4308313) (TaqMan; Applied Biosystems, Foster City, CA, USA) with 10 μM FWR/Rvse and 20 μM Probe. Thermal cycling conditions were as specified by the manufacturer: 50°C for 2 min, 95°C for 10 min, and 40 cycles as follows: 95°C for 15 s, ramp to 60°C for 1 min. For the analysis of the RT-PCR results, averaged normalized data for each experimental gene was compared between BCLP groups and SCLP groups using the $2^{-\Delta\Delta C_T}$ method [113].

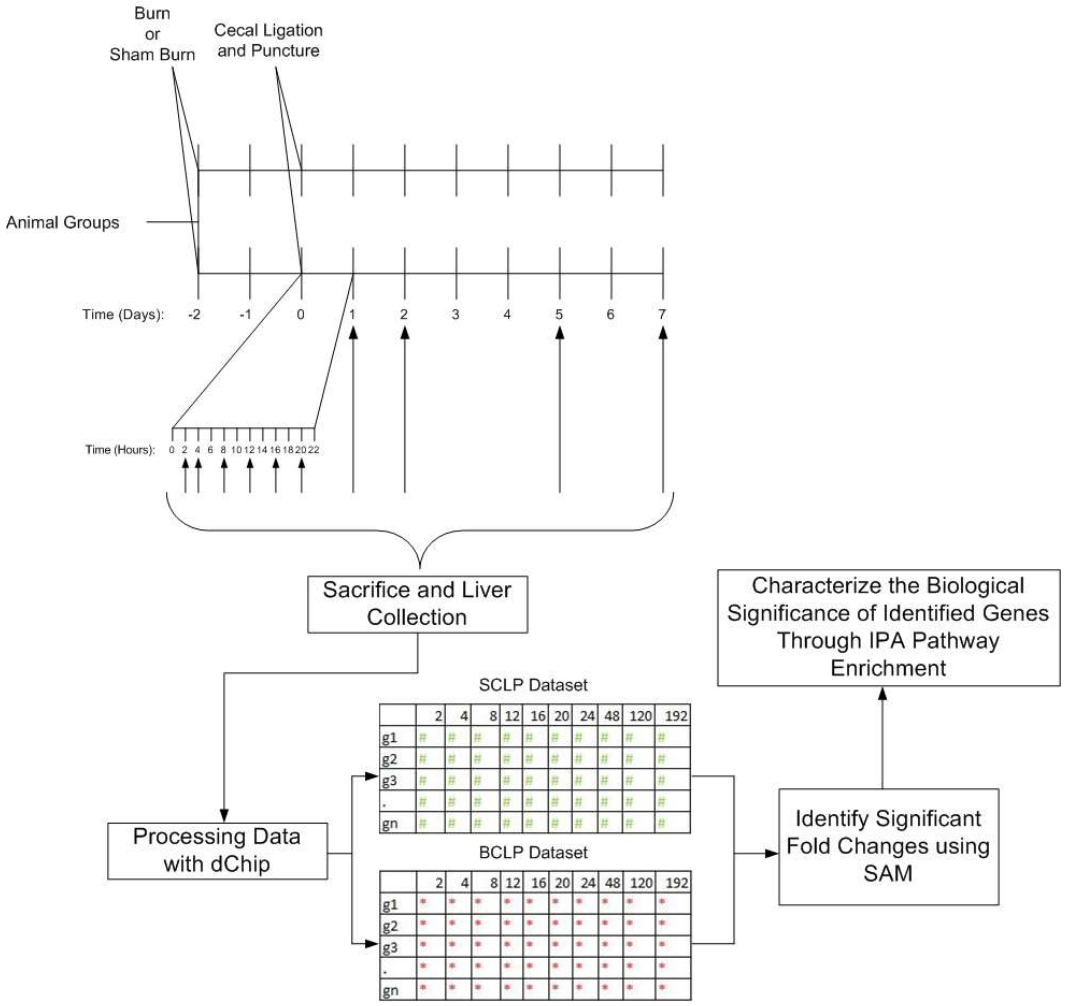

Figure 5. Schematic Overview of the Experimental Design – credit to Dr Yang for her contribution to the bottom half of this figure. Animals were first subjected to burn or sham burn treatments, and then 48 hours later, were subjected to cecal ligation and puncture. Sacrifice occurred at 2, 4, 8, 12, 16, 20, 24, 48, 120, and 192 hours post CLP. Microarray data from the liver was preprocessed using dChip software, and then compared using SAM (Statistical Analysis of Microarrays) to determine genes with significantly altered expression. This subset of genes was then processed using Ingenuity Pathway Analysis enrichment software in order to characterize the biological differences between the two injury models.

Identification of BCLP Related Patterns and Characterization of Per Day Changes

Hepatic gene expression levels were measured 2, 4, 8, 12, 16, 20, 24, 48, 120 and 168 hours following BCLP and SCLP treatment. Differences in gene expression in BCLP, when compared to SCLP, were identified at each time point. The complete list of genes identified can be located in the supplemental material [3] of the published work described as up regulated or down regulated categories, relative to SCLP. Interestingly, for time points 16, 20, 48, and 168 hours post-injury, no significant gene expression differences were identified indicating statistically identical gene expression between the BCLP and SCLP conditions for those time points. The full list of genes which passed IPA pathway analysis with p values less than 0.05 are also shown in the supplemental material of the published work, along with specific IPA identified pathways. The most relevant to innate immunity and hepatic metabolism following injury are summarized below, and in **Table 1**.

Innate Immunity Related Pathways

Over the 7 days post injury, 9 total pathways related to innate immunity showed significant changes in expression between BCLP and SCLP, indicating a change in transcriptional output between the two injuries. Specific genes associated with these pathways can be found in **Table 1**. Pathways which were down regulated in the first four hours include **Communication between Adaptive and Innate Immune Systems, Interferon Signaling** and **NF-κB activation**, which all correspond to key cytokines and intracellular proteins that recognize and propagate the inflammatory response. The **IL-6/IL-10 Signaling** pathway, related to the activity of key pro-inflammatory cytokines, had

similar dynamics, but remained suppressed 4 hours longer than previous pathways. The **ERK/MAP signaling** pathway, associated with G protein activation of the immune response, was similarly suppressed early, but also showed suppression at one day post injury. The **JAK/STAT signaling** pathway, associated in this case with suppression of cytokine signaling, does not follow a similar regulatory pattern, but is instead suppressed halfway through the first day. In addition, the chemokine receptor related pathway called **RXR Activation** was elevated midway through the first day as well. The **acute phase response signaling/complement signaling** pathway, associated with anti-bacterial acute phase protein production, was activated both early in the time course, but also very late, at 5 days post injury. The final pathway is associated with **Cell Cycle Check Point Regulation**, which controls cell proliferation both during the immune response, and shows similar patterns to the complement signaling pathway, with early suppression of anti-proliferators in the first day of injury, and late activation of anti-proliferators on the fifth day of injury . Overall, significant activity is observed very early in the time course, as well as at the first day, and the fifth day marks.

Immune Function	
Pathways	Genes
Communication between Adaptive and Innate Immunity	*Cxcl10, Il1a, Il1, Ccl3l1/Ccl3l3 , Il1b, Cd83* and *Ccl5*
Inteferon Signaling	*Ifit3, Mx1 , Jak2 , Stat* and *Irf1*
NF-KB Activation	*Nra, Tnfrsf14, Fos, Nfkbia* and *Nf*
IL-6/IL-10 Signaling	*Fos, Il1a, Nfkbia, Nfkbie, Il1b* and *Jak2*
ERK/MAP Signaling	*Myc, Ppp1r3d, Dusp6, Ppp1r14a* and *Prkag1*
JAK/STAT Signaling	*Cish* and *Socs2*
RXR Activation	*Cyp2b6* and *Cd36*
Acute Phase Response Signaling/Complement Signaling	*Il33, Serping1, Ftl, C9, Cfb* and *S100a9*
Cell Cycle Check Point Regulation	*Cdkn1b, Hdac4, Cdkn2b, Myc , Slk* and *Plk2*
Metabolic Function	
Pathways	Genes
PPAR-γ Signaling	*Cd36, Ppargc1a , Nfkbia, Gpd2, Nfkbie, Acvr1, Il1b, Jak2, Adipor2* and *Prkag1*
Xenobiotic Metabolism	*Aldh1b1, Nras, Ugt2b, Fmo1, Ces1 , Cyp4a22, Gsta4, Aldh1l1,* and *Cyp51a1*
Pyruvate/Propanoate Metabolism	*Aldh1b1, Hk2, Pklr* and *Pfkm*
Glycine, Threonine, and Serine Metabolism	*Sardh, Gnmt, Chdh, Alas1* and *Elovl6*
Arginine, Proline, Aspartate and Alanine Metabolism	*Ass1, Asl, Amd1* and *Prodh*
Amino Acid Metabolism	*Got, Tat, Aox1* and *Vars*
Fatty Acid Biosynthesis	*Pnpla, Akr1b7 , Fasn, Acaca, Me1, Acat1* , and *Elov6*
Protein Ubiquitination	*Hspa8, Usp15, Hsph1, Hsp90aa1, Ube2e2, Anapc11, Dnaja1 , Me2* and *Adh4*
NRF2 Mediated Oxidative Stress Response	*Sod2* and *Gstm3*

Table 1. Genes that show significant fold change following burn priming when compared with CLP within key metabolic and immune pathways. All listed genes were identified by SAM to be significantly different between both conditions, and in agglomerate found by IPA to be significant in their associated pathways.

Metabolism Related Pathways

In addition to pathways associated with innate immunity, 9 pathways related to hepatic metabolic changes showed significant changes following injury in BCLP compared to SCLP over a 7 day time course. Specific genes associated with these pathways can be found in **Table 1**. Pathways with heavy activity early in the time course include **PPAR-γ Signaling** and **Xenobiotic Metabolism**, which both are metabolic functions that crosstalk with innate immunity to degrade foreign bodies, and are activated early, then suppressed, or suppressed and then activated, respectively. **Pyruvate/Propanoate Metabolism** is a pathway critical to central carbon metabolism which is suppressed over the first four hours of injury. Specific amino acid pathways related to **Glycine, Threonine, and Serine Metabolism** and **Arginine, Proline, Aspartate and Alanine Metabolism** are activated 8 hours post injury, and in the case of the latter, persist to 12 hours post injury, at a time when previous pathways are winding down in activity. More general pathways related to **Amino Acid Metabolism** (including branched chain amino acid activity) were consistently activated early in the time course, and again as late as 5 days post injury, while the **Fatty Acid Biosynthesis** pathway is suppressed at those same time points. The **Protein Ubiquitination** pathway, which is related to the catabolism of proteins into their component amino acids, was activated by 8 hours post injury, but then suppressed at 24 hours post injury. The final metabolic pathway observed is related to **NRF2 Mediated Oxidative Stress Response**, which shows suppression midway through the first day, and whose functions primarily relate to the scavenging of potentially damaging reactive oxygen species within the cell. Similar to the innate immune pathways, genes associated with

metabolic function appear to show activity early in the time course, or later, at 1 day or 5 days post injury.

RT-PCR Confirmation of Expression Levels

In order to confirm the experimental microarray results, select genes were chosen by Dr. Yang for RT-PCR analysis that had passed both the SAMS tests and the false positive tests, and were significant biologically in the context of observed pathways. The full list of genes that were selected are *Slc1a4*, *Angptl4*, *Pcolce*, *Nfkbia*, *Stam2* and *G6pd*, which were up and down regulated at 2, 4 and 168 hours respectively. *Slc1a4* and *G6pd* are metabolic genes that are responsible for producing proteins responsible for amino acid transport into the cell, and central carbon metabolism respectively, while *Angptl4* and *Pcolce* are genes that encode acute phase proteins produced by the liver. *Nfkbia* is a component of the NF-κB transcription factor, which is well known for its activity in acute inflammation, and *Stam2* is a component of a downstream cytokine receptor signaling pathway. These genes reinforce trends, for example, RT-PCR results for *Nfkbia*, and *G6pd*, which encode a sub unit of the critical immune response transcription factor NF-κB and central carbon metabolism through Glucose-6-phosphate dehydrogenase respectively, lend significant weight to the more general observations of immune suppression and lowered carbon based metabolic activity. The results are shown in **Table 2**, where the trends in fold change match between the microarray experimental data and the RT-PCR data in every case, although the magnitude of the fold changes sometimes differs between the two experimental methods.

			Mean Fold Change	
Time	Gene Symbol	Gene Name	Affymetrix	RTPCR
2h post CLP	Slc1a4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2.69	3.47
	Angptl4	Angiopoietin-like 4	0.33	0.36
	Pcolce	Procollagen C-endopeptidase enhancer	3.05	6.43
4h post CLP	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.49	0.77
Day 7 post CLP	Stam2	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	2.49	1.07
	G6pd	Glucose-6-phosphate dehydrogenase	0.42	0.54

Table 2. RT-PCR Confirmation of Affymetrix Gene Expression Fold Change by Dr. Yang. Above are shown the 6 genes that were selected for RT-PCR confirmation: mean fold change compares the average BCLP expression levels to those of the SCLP condition, in both the microarray and the RT-PCR.

Impact of Burn Priming on the Inflammatory Response

While the innate immune system usually responds to systemic threats with both pro-inflammatory and anti-inflammatory phases [31], recent evidence suggests that the magnitude of the inflammatory response is highly dependent on the status of the host at the time of injury [114].

In previous work [2], key motifs were identified in the short term liver response of rodents to CLP when compared to uninjured animals, including a predominantly pro-inflammatory Cluster, with multiple cytokines, cytokine signals, complement, and coagulation proteins consistently upregulated. Therefore, fold changes observed between Burn-CLP animals and CLP animals represent deviations from this acute pro-inflammatory trend. During the first 4 hours after the septic insult, many of the immune regulated genes were suppressed under BCLP compared to SCLP. These genes are primarily involved in cytokine production, NF-κB signaling, and innate and adaptive communication through chemokines and other motifs. The suppression appears to be significantly stronger at the 4 hour time point, with critical inflammatory genes suppression (including *IL1, STAT1,* and *JAK2)* after thermal priming. In contrast the subsequent 4 hours post injury (the 8 hour time point) show significant up regulation of the immune system including several cytokines such as IL-33, a known Th2 cell attractor [54]. Further, at this time point, pro-inflammatory complement proteins (anti-bacterial acute phase reactants), are elevated in contrast with the suppression of inflammatory mediators observed previously. The observed early suppression of pro-inflammatory functions is likely caused by the burn priming: in single injury animals, burn injuries caused the animal to enter an immunosuppressive state during the first 24 hours, which would potentially dampen the

acute response to CLP. This suppression gives way to an overshoot of pro-inflammatory mediators not long after, indicating that burn does not promote pro- or anti-inflammatory mediators, but instead disrupts the balance between both in the healthy CLP response. In this case, this "spring back" of pro-inflammatory mediators is dampened back down to a normal CLP response before 24 hours, however, in a more severe injury, the first 8 hours may represent a critical time period where an overshoot may overwhelm anti-inflammatory forces that are designed to counterbalance it.

In previous studies, the hepatic response to single injuries was compared between those that involved a surgical trauma with septic complications (CLP), to a surgical trauma that did not have septic complications (sham-CLP: a procedure involving the same incisions, but with no puncture of the cecum), and discovered that these injuries, despite their similarities, provoked huge differences in the hepatic response over the short and long term [2]. In contrast to those results from the previous Chapter, the temporal nature of the burn priming of CLP appears to affect the strength of the pro-inflammatory and anti-inflammatory stimuli, rather than shifting the dynamic entirely. While there is early immune suppression relative to the CLP condition, the number of genes observed as suppressed that have innate immune functions in the burn-CLP case are much smaller than the size of the pro-inflammatory clusters observed in the single injury CLP results, and thus, the observed trend is more a dampening of the acute pro-inflammatory CLP response, as opposed to an immune suppressive response.

Furthermore, although acute phase protein production and cytokine production have been analyzed together in innate immune function due to their common functionalities, they are traditionally produced by heterogeneous cell types in the liver, which cannot differentiate. Though it is most likely that acute phase protein production originates from hepatocytes, while cytokine production originates from Kupffer cells, further investigations are required to understand how these cells communicate between one another to produce the observed response.

Previous work on the short-term metabolic response following CLP indicates that the induced sepsis is associated with a shift toward increased metabolism via central carbon metabolism, fatty acid metabolism, and amino acid metabolism [2]. In contrast, the priming of the burn injury does not appear to increase net metabolism, but instead shifts the emphasis of consumed metabolites towards nitrogen based sources. Almost immediately following CLP injury, we observed significant differences in metabolic response in burn-primed animals compared to the unprimed CLP group, particularly with respect to amino acid metabolism. Genes associated with amino acid metabolism are up regulated over the first 8 hours following injury, and then return to normal. Furthermore, the up regulation of amino acid degradation coincides with a down regulation of fatty acid energy sources, indicating that the burn injury predisposes the host to utilize an amino acid based energy source for the acute phase response. Given that amino acid degradation, through the urea cycle, is the primary mechanism by which the liver enters a negative nitrogen balance[115], this metabolic shift can be characterized as a move towards a state of hypermetabolism following burn injury, despite a suppressed immune response. Interestingly, there is no indication in our previous works that the single burn injury shifts metabolism towards

nitrogen sources at the expense of carbon based energy [1]. The phenomenon of an energy shift towards amino acids is remarkably different from the single injuries, which simply increase metabolic activity through multiple channels, and bears further investigation.

In addition to catabolic changes, xenobiotic metabolism continued to increase over the first 8 hours following injury, while NRF2 mediated-oxidative responses were down regulated. A similar increase in xenobiotic metabolism is also observed halfway through the first day, corresponding to moderately increased in immune function. This implies that systemic oxidation is higher post burn at this time point than seen with CLP alone, though to what degree remains unknown. Our previous characterizations of the hepatic response following CLP as a single injury [1] have also linked xenobiotic metabolism through cytochrome P450 to inflammation, indicating that this metabolic pathway is at least partially co-regulated with innate immunity. It is not clear how interconnected these elements of xenobiotic metabolism are with other metabolic pathways within hepatocytes, but their communication may represent an important link between inflammatory signaling elements and energy regulation that has hitherto been overlooked.

One of the interesting observations that came out of the previous Chapter's results on the long term effects of CLP in a single injury model was the emergence of significant anti-inflammatory gene expression at the 5 day mark, which occurred following a return to baseline at day 2 [2]. Interestingly, while most of the long term data points show no difference following burn priming, activity at the 5-day mark does occur, indicating that there is "memory" of the burn injury that persists through into the long term recovery response for CLP. Observed gene changes in this peak following burn priming include the activation of further wound repair, the down regulation of fatty acid synthesis, and the up

regulation of amino acid synthesis. The preference for amino acid synthesis over fatty acid synthesis in the long term is likely the direct result of burn injury priming: creating a preference for amino acid *degradation* at the expense of lipid degradation in the short term response. These findings may be compensatory for relatively larger loss of amino acids in the early phase leading to increased synthesis later. None of the observed genes indicate a suppression of the anti-inflammatory wound healing response, and to the contrary, they appear to enhance it. Due to the scarcity of data around this point, further investigations centering around 5 days post injury would be required to understand whether the wound healing response observed at this time point is persistent, and whether it is a mechanistic function of burn, or just indicative of increased injury to be repaired.

Overall, the priming with thermal injury before CLP creates an early immune suppression characteristic of the burn injury, followed by a resurgent inflammatory response that overshoots the baseline CLP response through a "spring back" mechanism. Pathways critical to this mechanism include NF-κB activation, IL-6/IL-10 signaling, acute phase response signaling, and communication between the adaptive and innate immune systems. Further, priming with thermal injury exacerbates post-CLP hypermetabolism through significantly increased amino acid catabolism at the expense of carbon catabolism through pyruvate and fatty acid metabolism pathways, a phenomenon unique to the double hit injury, while xenobiotic metabolism appears to be closely linked with immune functions. This indicates that excess urea production associated with amino acid degradation may be a byproduct of this metabolic shift which involves pathways not observed in the clinical pathologies. Further, we identify that the 5 day long term response, previously identified as critical in single injuries, remains a hitherto unexplored point of

resurgent gene expression that promotes wound healing and an immune suppressive state over the long term. Thus the immune suppressive state imposed by burn creates a more severe acute CLP response that has potentially severe metabolic and inflammatory effects, which appear to be partially co-regulated. Mitigating this immune suppression from burn may therefore be a viable clinical intervention for burn-sepsis related pathologies.

Conclusion

Overall, the work presented in this thesis aims at understanding gene expression changes associated with inflammation as a stressor. With tremendous aid from Dr. Yang and Dr. Orman, animal models were developed that could interrogate 3 injuries: Burn, Cecal Ligation and Puncture, and Sham Cecal Ligation and Puncture. Literature analysis of the hepatic response to these injuries was previously focused on pairwise comparisons between specific time points post injury. Due to the clinically fluid nature and progression of systemic inflammatory response syndrome and hypermetabolism, time course data provided much higher resolution into the dynamics associated with the acute and prolonged responses to these injuries. Analysis of the first 24 hours post CLP and sham CLP showed inflammatory responses in both conditions, however the dynamics of the response, and the genes involved diverged significantly, indicating that these two conditions represented two unique injury stimuli with separate regulators and drivers. The key characteristics of CLP driven sepsis seem to be the presence of a pro-inflammatory response, which drives hypermetabolism, immune cell activation, and damage from oxidative stress. This contrasts with SCLP, which shows an altered inflammatory response, leading to no immune cell activation, decreased detoxification potential, and hyper metabolism. Many of the identified transcription factors that drive the CLP response are unique to the injury, indicating that the differences in gene expression patterns reflect different underlying regulatory structures.

In order to understand how these two injury models might resolve over the long term, the CLP model, designed with survival in mind, was extended out to 8 days post injury, wherein three major clusters of gene expression were obtained. The first Cluster,

which is mainly related to genes of anti-inflammatory response and antioxidative properties, is suppressed early in the CLP condition and later upregulated compared to the SCLP condition. Cluster 2 represents pro-inflammatory responses and signaling, along with amino acid metabolism. Cluster 3 is also associated with pro-inflammatory response. The genes of Toll-like receptor signaling and hypermetabolism were identified in this Cluster as well. Clusters 2 and 3 are both suppressed in the long-term response following CLP. Clusters 1 and 2 acting in concert return to the time 0 baseline in both groups, indicating resolution of both the anti-inflammatory and pro-inflammatory response; however, the SCLP response in Cluster 3 shows persistent downregulation.

Principal component analysis of this data showed distinct trajectories for the two conditions, including a profoundly anti-inflammatory long term trajectory for the CLP condition, which may be indicative of immunosuppression. Although these data show that hepatic gene expression is remarkably different between CLP and SCLP, clinical sepsis does not often occur from de novo infection, but rather as the result of a different stimulus. Since CLP represents the SCLP injury plus the additional bacterial stimulus, yet shows a gene profile that is different not just in scale, but also in form and regulation, a double hit model was investigated, where CLP primed 48 hours prior with a burn injury was compared with CLP alone over the acute and long term responses. Genes found significantly enriched within pathways related to innate immune signaling through cytokines and NF-κB were co regulated with xenobiotic metabolism genes and acute phase protein genes, were suppressed early, and then activated at later time points. Furthermore it was identified that amino acid metabolism, pyruvate metabolism, fatty acid metabolism and NRF-2 mediated oxidative stress genes were down regulated over the time course in the burn primed injury

compared to the unprimed CLP. Overall, these observed trends within the double hit burn-sepsis model represent unique immune and metabolic pathways and dynamics not found in the CLP injury alone, including an early suppression followed by overreaction of pro-inflammatory mediators, and an increase in amino acid metabolism at the expense of central carbon pathways.

These studies together show that the phenotype represented by systemic inflammation and even hyper metabolism are the result of complex interactions between multiple pathways. Although these studies focused exclusively on the liver, other organs play a critical role in this response, and may show similarly unique dynamics at the transcriptional level in response to different injuries. Furthermore, although the overall phenotype is similar, the underlying genes driving it appear to be significantly different between different injury types, suggesting that there is no singular pathway towards alleviating systemic inflammation from a hypermetabolic standpoint. In addition to inflammatory mediators, many metabolic genes were found to be expressed within the liver in many of these conditions. These suggest that metabolism may act not just as a symptom of systemic inflammation, but also as a driver, creating pressure on the hepatic system that can lead to distress and dysfunction.

Finally, future work should look at aiming to validate hepatic phenotypes within these systems, although that may prove to be a challenging task. One of the major difficulties with animal models of this kind is the need to balance the severity of the injury (and thus the mortality rate of the animal) with the ability to reliably collect data without biasing results towards the exceptional responders at later time points. It is possible that, in the future, more severe injuries need to be considered, as injuries with 100% survival

rates may be able to provide information about the liver response, but are unlikely to seriously tax the homeostasis of the animals, represented by their universally healthy end points. Overall, it appears clear that any hepatic driven interventions into this disorder must approach the issue from both a metabolic and an inflammatory angle, as the two processes are intrinsically linked within the liver's response to all of the injuries assayed by these studies.

References

1. Yang, Q., et al., Dynamics of Hepatic Gene Expression Profile in a Rat Cecal Ligation and Puncture Model. J Surg Res, 2011.

2. Mattick, J.S., et al., Long-term gene expression profile dynamics following cecal ligation and puncture in the rat. J Surg Res, 2012. **178**(1): p. 431-42.

3. Mattick, J.S., et al., Impact of burn priming on immune and metabolic functions of whole Liver in a rat cecal ligation and puncture model. Int J Burns Trauma, 2013. **3**(1): p. 55-65.

4. Bone, R.C., et al., Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. Chest, 2009. **136**(5 Suppl): p. e28.

5. Dombrovskiy, V.Y., et al., Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. Crit Care Med, 2007. **35**(5): p. 1244-50.

6. Kindt, T.J., et al., Kuby immunology. 2007, New York: W.H. Freeman.

7. Kobayashi, S.D., et al., Neutrophils in the innate immune response. Arch Immunol Ther Exp (Warsz), 2005. **53**(6): p. 505-17.

8. Luster, A.D., R. Alon, and U.H. von Andrian, Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol, 2005. **6**(12): p. 1182-90.

9. Kim, M.Y. and J.Y. Cho, Molecular association of CD98, CD29, and CD147 critically mediates monocytic U937 cell adhesion. Korean J Physiol Pharmacol, 2016. **20**(5): p. 515-23.

10. Rastogi, D., A.J. Ratner, and A. Prince, Host-bacterial interactions in the initiation of inflammation. Paediatr Respir Rev, 2001. **2**(3): p. 245-52.

11. Barkalow, F.J., et al., Brain endothelium lack one of two pathways of P-selectin-mediated neutrophil adhesion. Blood, 1996. **88**(12): p. 4585-93.

12. Galkina, E.V., et al., Interactions of C-Reactive Protein and Serum Amyloid P Component with Interleukin-8 and Their Role in Regulation of Neutrophil Functions. Russ J Immunol, 2000. **5**(4): p. 363-374.

13. Franklin, R.A., P.A. Atherfold, and J.A. McCubrey, Calcium-induced ERK activation in human T lymphocytes occurs via p56(Lck) and CaM-kinase. Mol Immunol, 2000. **37**(11): p. 675-83.

14. Said-Sadier, N. and D.M. Ojcius, Alarmins, inflammasomes and immunity. Biomed J, 2012. **35**(6): p. 437-49.

15. Sozzani, S. and A. Del Prete, Chemokines as relay signals in human dendritic cell migration: serum amyloid A kicks off chemotaxis. Eur J Immunol, 2015. **45**(1): p. 40-3.

16. Gunda, S.K., et al., Structural investigations of CXCR2 receptor antagonists by CoMFA, CoMSIA and flexible docking studies. Acta Pharm, 2012. **62**(3): p. 287-304.

17. Maksic, D., et al., Systemic and intraperitoneal proinflammatory cytokines profiles in patients on chronic peritoneal dialysis. Med Pregl, 2007. **60 Suppl 2**: p. 53-7.

18. Khawar, M.B., M.H. Abbasi, and N. Sheikh, IL-32: A Novel Pluripotent Inflammatory Interleukin, towards Gastric Inflammation, Gastric Cancer, and Chronic Rhino Sinusitis. Mediators Inflamm, 2016. **2016**: p. 8413768.

19. Martin, P.L., et al., Reviews Preclinical Safety and Immune-Modulating Effects of Therapeutic Monoclonal Antibodies to Interleukin-6 and Tumor Necrosis Factor-alpha in Cynomolgus Macaques. J Immunotoxicol, 2005. **1**(3): p. 131-9.

20. Saner, F.H., et al., Pharmacology, clinical efficacy and safety of terlipressin in esophageal varices bleeding, septic shock and hepatorenal syndrome. Expert Rev Gastroenterol Hepatol, 2007. **1**(2): p. 207-17.

21. Vincent, J.L. and T. Gustot, Sepsis and cirrhosis: many similarities. Acta Gastroenterol Belg, 2010. **73**(4): p. 472-8.

22. Alter, J. and E. Bengal, Stress-induced C/EBP homology protein (CHOP) represses MyoD transcription to delay myoblast differentiation. PLoS One, 2011. **6**(12): p. e29498.

23. Carney, D.E., K. Matsushima, and H.L. Frankel, Treatment of sepsis in the surgical intensive care unit. Isr Med Assoc J, 2011. **13**(11): p. 694-9.

24. Linton, A., et al., Inflammation in malignant mesothelioma - friend or foe? Ann Cardiothorac Surg, 2012. **1**(4): p. 516-22.

25. Li, H., B. Manwani, and S.X. Leng, Frailty, inflammation, and immunity. Aging Dis, 2011. **2**(6): p. 466-73.

26. Faloon, P.W., et al., Identification of Small Molecule Inhibitors that Suppress Cytokine-Induced Apoptosis in Human Pancreatic Islet Cells, in Probe Reports from the NIH Molecular Libraries Program. 2010, National Center for Biotechnology Information (US): Bethesda (MD).

27. Yu, J.H. and H. Kim, Oxidative stress and inflammatory signaling in cerulein pancreatitis. World J Gastroenterol, 2014. **20**(46): p. 17324-9.

28. Bone, R.C., et al., Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference

Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest, 1992. **101**(6): p. 1644-55.

29. Adib-Conquy, M. and J.M. Cavaillon, Compensatory anti-inflammatory response syndrome. Thromb Haemost, 2009. **101**(1): p. 36-47.

30. Deitch, E.A., Multiple organ failure. Pathophysiology and potential future therapy. Ann Surg, 1992. **216**(2): p. 117-34.

31. Hotchkiss, R.S., et al., The sepsis seesaw: tilting toward immunosuppression. Nat Med, 2009. **15**(5): p. 496-497.

32. Jaeschke, H., Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol, 2011. **26 Suppl 1**: p. 173-9.

33. Rittirsch, D., et al., Immunodesign of experimental sepsis by cecal ligation and puncture. Nature Protocols, 2009. **4**(1): p. 31-36.

34. Wichterman, K.A., A.E. Baue, and I.H. Chaudry, Sepsis and septic shock--a review of laboratory models and a proposal. J Surg Res, 1980. **29**(2): p. 189-201.

35. Jeschke, M.G., The Hepatic Acute Phase Response to Thermal Injury, in Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins, F. Veas, Editor. 2011, InTech.

36. Sax, H.C., et al., Amino acid uptake in isolated, perfused liver: effect of trauma and sepsis. J Surg Res, 1988. **45**(1): p. 50-5.

37. Jeschke, M.G., R.E. Barrow, and D.N. Herndon, Extended hypermetabolic response of the liver in severely burned pediatric patients. Arch Surg, 2004. **139**(6): p. 641-7.

38. Williams, F.N., et al., What, how, and how much should patients with burns be fed? Surg Clin North Am, 2011. **91**(3): p. 609-29.

39. Alverdy, J.C., R.S. Laughlin, and L. Wu, Influence of the critically ill state on host-pathogen interactions within the intestine: gut-derived sepsis redefined. Crit Care Med, 2003. **31**(2): p. 598-607.

40. Gauglitz, G.G., et al., Characterization of the inflammatory response during acute and post-acute phases after severe burn. Shock, 2008. **30**(5): p. 503-7.

41. Banta, S., et al., Contribution of gene expression to metabolic fluxes in hypermetabolic livers induced through burn injury and cecal ligation and puncture in rats. Biotechnol Bioeng, 2007. **97**(1): p. 118-37.

42. Jayaraman, A., et al., Gene Expression Profiling of Long-Term Changes in Rat Liver Following Burn Injury. Journal of Surgical Research, 2009. **152**(1): p. 3-17.

43. Fazal, N. and W.M. Al-Ghoul, Thermal injury-plus-sepsis contributes to a substantial deletion of intestinal mesenteric lymph node CD4 T cell via apoptosis. Int J Biol Sci, 2007. **3**(6): p. 393-401.

44. Ravindranath, T.M., et al., Tissue factor pathway inhibitor and thrombin activatable fibrinolytic inhibitor plasma levels following burn and septic injuries in rats. Clin Appl Thromb Hemost, 2004. **10**(4): p. 379-85.

45. Goto, M., et al., Burn injury exacerbates hemodynamic and metabolic responses in rats with polymicrobial sepsis. J Burn Care Res, 2006. **27**(1): p. 50-9.

46. Xu, P.B., et al., Gene expression profiling reveals the defining features of monocytes from septic patients with compensatory anti-inflammatory response syndrome. J Infect, 2012. **65**(5): p. 380-91.

47. Orman, M.A., et al., Comparison of the cytokine and chemokine dynamics of the early inflammatory response in models of burn injury and infection. Cytokine, 2011. **55**(3): p. 362-71.

48. Barber, R.C., et al., Increasing percent burn is correlated with increasing inflammation in an adult rodent model. Shock, 2008. **30**(4): p. 388-93.

49. Walley, K.R., et al., Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect Immun, 1996. **64**(11): p. 4733-8.

50. Ertel, W., et al., The complex pattern of cytokines in sepsis. Association between prostaglandins, cachectin, and interleukins. Ann Surg, 1991. **214**(2): p. 141-8.

51. Klein, D., et al., Differences in the hepatic signal transcription pathway and cytokine expression between thermal injury and sepsis. Shock, 2003. **20**(6): p. 536-43.

52. Valenti, L.M., et al., High levels of endogenous nitric oxide produced after burn injury in rats arrest activated T lymphocytes in the first G1 phase of the cell cycle and then induce their apoptosis. Experimental Cell Research, 2005. **306**(1): p. 150-167.

53. Cobb, J.P., et al., Application of genome-wide expression analysis to human health and disease. Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4801-6.

54. Komai-Koma, M., et al., IL-33 is a chemoattractant for human Th2 cells. European Journal of Immunology, 2007. **37**(10): p. 2779-2786.

55. Li, Z.J., et al.,[Research of gene expression profile of liver tissue in rat sepsis model]. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue, 2007. **19**(3): p. 156-9.

56. Chinnaiyan, A.M., et al., Molecular signatures of sepsis: multiorgan gene expression profiles of systemic inflammation. Am J Pathol, 2001. **159**(4): p. 1199-209.

57. Scheff, J., et al., Assessment of Pharmacologic Area Under the Curve When Baselines are Variable. Pharmaceutical Research, 2011: p. 1-9.

58. Nguyen, T.T., R.S. Nowakowski, and I.P. Androulakis, Unsupervised selection of highly coexpressed and noncoexpressed genes using a consensus clustering approach. Omics, 2009. **13**(3): p. 219-37.

59. Bader, A., et al., A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes. Biotechnology Progress, 1992. **8**(3): p. 219-225.

60. Puthier, D., R. Bataille, and M. Amiot, IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. Eur J Immunol, 1999. **29**(12): p. 3945-50.

61. Baumann, H. and J. Gauldie, The acute phase response. Immunol Today, 1994. **15**(2): p. 74-80.

62. Wannemacher, R.W., Jr., et al., Role of the liver in regulation of ketone body production during sepsis. J Clin Invest, 1979. **64**(6): p. 1565-72.

63. Pollack, G.M., et al., Chronic stress impairs oxidative metabolism and hepatic excretion of model xenobiotic substrates in the rat. Drug Metab Dispos, 1991. **19**(1): p. 130-4.

64. Haque, R., et al., Alterations in systemic complement component 3a and 5a levels in patients with cerebral arteriovenous malformations. J Clin Neurosci. **18**(9): p. 1235-9.

65. Iyer, A., et al., Inhibition of inflammation and fibrosis by a complement C5a receptor antagonist in DOCA-salt hypertensive rats. J Cardiovasc Pharmacol.

66. Zeerleder, S., C1-inhibitor: more than a serine protease inhibitor. Semin Thromb Hemost. **37**(4): p. 362-74.

67. Rawlings, J.S., K.M. Rosler, and D.A. Harrison, The JAK/STAT signaling pathway. J Cell Sci, 2004. **117**(Pt 8): p. 1281-3.

68. Hasselgren, P.O., et al., Changes of protein metabolism in liver and skeletal muscle following trauma complicated by sepsis. J Trauma, 1984. **24**(3): p. 224-8.

69. Mitch, W.E. and A.L. Goldberg, Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. N Engl J Med, 1996. **335**(25): p. 1897-905.

70. Samra, J.S., L.K. Summers, and K.N. Frayn, Sepsis and fat metabolism. Br J Surg, 1996. **83**(9): p. 1186-96.

71. Morgan, E.T., Regulation of cytochrome p450 by inflammatory mediators: why and how? Drug Metab Dispos, 2001. **29**(3): p. 207-12.

72. Morgan, E.T., Suppression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. Mol Pharmacol, 1989. **36**(5): p. 699-707.

73. Romagnani, S., T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol, 2000. **85**(1): p. 9-18; quiz 18, 21.

74. Carnaud, C., et al., Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J Immunol, 1999. **163**(9): p. 4647-50.

75. Helenius, A. and M. Aebi, Intracellular functions of N-linked glycans. Science, 2001. **291**(5512): p. 2364-9.

76. Biolo, G., et al., Metabolic response to injury and sepsis: changes in protein metabolism. Nutrition, 1997. **13**(9 Suppl): p. 52S-57S.

77. Brealey, D., et al., Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2004. **286**(3): p. R491-R497.

78. Brealey, D. and M. Singer, Mitochondrial Dysfunction in Sepsis. Curr Infect Dis Rep, 2003. **5**(5): p. 365-371.

79. Saltiel, A.R. and C.R. Kahn, Insulin signalling and the regulation of glucose and lipid metabolism. Nature, 2001. **414**(6865): p. 799-806.

80. Nikaido, S.S. and C.H. Johnson, Daily and circadian variation in survival from ultraviolet radiation in Chlamydomonas reinhardtii. Photochem Photobiol, 2000. **71**(6): p. 758-65.

81. Almendro, V., et al., Sepsis induces DNA fragmentation in rat skeletal muscle. Eur Cytokine Netw, 2003. **14**(4): p. 256-9.

82. Dominioni, L., et al., Sepsis score and acute-phase protein response as predictors of outcome in septic surgical patients. Arch Surg, 1987. **122**(2): p. 141-6.

83. Bone, R.C., Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS). Ann Intern Med, 1996. **125**(8): p. 680-7.

84. Hubbard, W.J., et al., Cecal Ligation and Puncture. Shock, 2005. **24**: p. 52-57.

85. Namba, M., Function of the liver cells in the short-term and the long-term cultures. I. Albumim production of the liver cells in vitro. Acta Med Okayama, 1966. **20**(6): p. 251-9.

86. Lenert, P.S., Classification, mechanisms of action, and therapeutic applications of inhibitory oligonucleotides for Toll-like receptors (TLR) 7 and 9. Mediators Inflamm, 2010. **2010**: p. 986596.

87. Mosialou, E. and R. Morgenstern, Activity of rat liver microsomal glutathione transferase toward products of lipid peroxidation and studies of the effect of inhibitors on glutathione-dependent protection against lipid peroxidation. Arch Biochem Biophys, 1989. **275**(1): p. 289-94.

88. Smith, P.F., D.W. Alberts, and G.F. Rush, Menadione-induced oxidative stress in hepatocytes isolated from fed and fasted rats: the role of NADPH-regenerating pathways. Toxicol Appl Pharmacol, 1987. **89**(2): p. 190-201.

89. Chi, H., et al., Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2274-9.

90. Lehner, R. and A. Kuksis, Biosynthesis of triacylglycerols. Prog Lipid Res, 1996. **35**(2): p. 169-201.

91. Sayeed, M.M., Alterations in calcium signaling and cellular responses in septic injury. New Horiz, 1996. **4**(1): p. 72-86.

92. Gunning, P., R. Weinberger, and P. Jeffrey, Actin and tropomyosin isoforms in morphogenesis. Anat Embryol (Berl), 1997. **195**(4): p. 311-5.

93. Matsusaka, T., et al., Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8.

94. Zhang, L., et al., Histone deacetylase inhibitors attenuate acute lung injury during cecal ligation and puncture-induced polymicrobial sepsis. World J Surg. **34**(7): p. 1676-83.

95. Sparkenbaugh, E.M., et al., The role of hypoxia-inducible factor-1alpha in acetaminophen hepatotoxicity. J Pharmacol Exp Ther, 2011. **338**(2): p. 492-502.

96. Fernandez-Sanchez, A., et al., Inflammation, oxidative stress, and obesity. Int J Mol Sci. **12**(5): p. 3117-32.

97. Hoffsten, P.E., et al., Formation of "lipid peroxide" under conditions which lead to swelling and lysis of rat liver mitochondria. Biochem Biophys Res Commun, 1962. **7**: p. 276-80.

98. Collado, R., et al., Early ROS-mediated DNA damage and oxidative stress biomarkers in Monoclonal B Lymphocytosis. Cancer Lett, 2011.

99. Nagao, Y., et al., Human mitochondrial carbonic anhydrase: cDNA cloning, expression, subcellular localization, and mapping to chromosome 16. Proc Natl Acad Sci U S A, 1993. **90**(16): p. 7623-7.

100. Matesic, D., A. Lenert, and P. Lenert, Modulating Toll-like Receptor 7 and 9 Responses as Therapy for Allergy and Autoimmunity. Curr Allergy Asthma Rep, 2011.

101. Simon, R., et al., Pitfalls in the Use of DNA Microarray Data for Diagnostic and Prognostic Classification. Journal of the National Cancer Institute, 2003. **95**(1): p. 14-18.

102. Hotchkiss, R.S. and I.E. Karl, The Pathophysiology and Treatment of Sepsis. New England Journal of Medicine, 2003. **348**(2): p. 138-150.

103. Apel, K. and H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol, 2004. **55**: p. 373-99.

104. Grau-Carmona, T., et al., Effect of an enteral diet enriched with eicosapentaenoic acid, gamma-linolenic acid and anti-oxidants on the outcome of mechanically ventilated, critically ill, septic patients. Clin Nutr. **30**(5): p. 578-84.

105. Dasu, M.R., et al., Gene expression profiles of livers from thermally injured rats. Gene, 2004. **327**(1): p. 51-60.

106. Sasse, K.C., et al., Long-term survival after intensive care unit admission with sepsis. Critical Care Medicine, 1995. **23**(6): p. 1040-1047.

107. Billiar, T.R., et al., Intestinal gram-negative bacterial overgrowth in vivo augments the in vitro response of Kupffer cells to endotoxin. Ann Surg, 1988. **208**(4): p. 532-40.

108. Deitch, E.A. and R. Berg, Bacterial translocation from the gut: a mechanism of infection. J Burn Care Rehabil, 1987. **8**(6): p. 475-82.

109. Baracchi, F., et al., Sepsis-induced alterations in sleep of rats. Am J Physiol Regul Integr Comp Physiol, 2011. **301**(5): p. R1467-78.

110. Remick, D.G., et al., Role of Interleukin-6 in Mortality from and Physiologic Response to Sepsis. Infection and Immunity, 2005. **73**(5): p. 2751-2757.

111. Tusher, V.G., R. Tibshirani, and G. Chu, Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences, 2001. **98**(9): p. 5116-5121.

112. Barber, R.D., et al., GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiological Genomics, 2005. **21**(3): p. 389-395.

113. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001. **25**(4): p. 402-8.

114. Castanon-Cervantes, O., et al., Dysregulation of Inflammatory Responses by Chronic Circadian Disruption. The Journal of Immunology, 2010. **185**(10): p. 5796-5805.

115. Kamin, H. and P. Handler, The metabolism of parenterally administered amino acids. II. Urea synthesis. J Biol Chem, 1951. **188**(1): p. 193-205.