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DYNAMICS OF GENE EXPRESSION PROFILING IN LIVER FOLLOWING THERMAL
INJURY AND SEPSIS

by

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ABSTRACT OF THE DISSERTATION

Dynamics of Gene Expression Profiling in Liver Following Thermal Injury and Sepsis

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Burn injury leads to a prolonged inflammatory response in the body. Moreover, severe burn injuries are always associated with bacterial infections which cause more persistent inflammatory response, resulting in prolonged hyper-metabolism and hyper-catabolism on systemic level. Despite significant advances in patient care, morbidity and mortality remain high in those patients. The difficulty in developing new and more effective medications is due, in part, to our incomplete understanding of the underlying pathophysiology of the disease. Liver, the main organ regulating both the inflammation and metabolism plays a key role in responding to external injuries. Thus, analyzing the responses in liver to burn, infection and “double hits” injury from global perspective as well as in a timely manner may offer a molecular framework for study on the pathophysiology of systemic inflammation induced by injuries.

The overall studies were divided into single injury in which the animals were subjected to single burn injury or single cecal ligation and puncture (CLP) injury individually and “double hits” injury in which the rats were subjected to a burn injury and subsequent CLP injury. Animals were sacrificed at various time points, and whole liver samples were analyzed using Affymetrix Rat

Genome 230 2.0 Arrays. After identifying differentially expressed probesets in injury rats vs. sham over time, the concatenated data sets corresponding to these differentially expressed probesets in injury and sham were combined and analyzed using a “consensus clustering” approaches. Ingenuity Pathway Analysis (IPA) was used to functionally annotate genes, and RT-PCR was used to confirm microarray trends.

Both single burn and CLP injury induces the activation of pro-inflammatory response, anti-inflammatory response, and enhanced synthesis of acute-phase proteins, increased metabolism and tissue damage. Genes which are directly in response to bacteria removal are only triggered in CLP injury. In double hits study, burn priming prior to CLP disrupts the transcriptional response in the liver to septic injury in the rat by altering the onset of anti-bacterial functions in the liver. In addition, burn enhanced hyper metabolic conditions through aggressive amino acid degradation at critical time points.

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TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION	ii
ACKNOWLEDGEMENT	iv
LIST OF FIGURES	xii
1. INTRODUCTION	1
2. LITERATURE REVIEW	3
2.1. Postburn Inflammation.....	3
2.1.1. Burn-Induced Inflammation In Liver.....	3
2.1.2. Bacterial Translocation-Induced Inflammation In Liver Following Burn Injury	5
2.1.3. The Pathophysiology Of Burn Induced ‘Two Wave’ Inflammation.....	6
2.2. Alterations in Gene Expression Levels in Liver post Burn Injury and Sepsis.....	7
2.2.1. Hepatic Gene Expression Post Burn	7
2.2.2. Gene Expression Post Infection	8
2.2.3. Gene Expression Post ‘Double Hits’	10
3. DYNAMICS OF SHORT TERM GENE EXPRESSION PROFILING IN LIVER FOLLOWING THERMAL INJURY	11
3.1. Introduction.....	11
3.2. Material and Methods	13
3.2.1. Animal Model	13
3.2.2. Data Analysis	14

3.3.	Results and Discussion	16
3.3.1.	Functional Characterization Of Major Clusters	18
3.3.2.	Assessing And Interpreting The Differences In Transcriptional Dynamics Between Sham And Burn	24
3.4.	Conclusions.....	27
4.	Dynamics of Short Term Gene Expression Profiling In Liver Following CLP	29
4.1.	Introduction.....	29
4.2.	Materials and Methods.....	31
4.2.1.	Animal Model	31
4.2.2.	Data Analysis	32
4.2.3.	Promoter Extraction And Computational Prediction Of Putative Transcriptional Regulators	34
4.3.	Results.....	35
4.3.1.	Identification And Functional Characterization Of SCLP Regulated Expression Patterns	35
4.3.2.	Identification Of Putative Regulators Of SCLP-Specific Transcriptional Responses	39
4.3.3.	Identification And Functional Characterization Of CLP Regulated Expression Patterns	42
4.3.4.	Identification Of Putative Regulators Of CLP-Specific Transcriptional Responses	48
4.4.	Discussion.....	51

4.4.1.	SCLP Dynamics: Aseptic Inflammation from Surgery	51
4.1.2.	CLP Dynamics: Systemic Response to Surgically Induced Sepsis.....	52
4.1.3.	Comparison Between SCLP and CLP- Induced Response	55
4.1.4.	Dynamics Of Induced Transcriptional Responses	59
4.1.5.	Transcriptional Regulation In SCLP And CLP Conditions	61
4.5.	Conclusions.....	65
5.	DYNAMICS OF LONG TERM GENE EXPRESSION PROFILING IN LIVER FOLLOWING CLP	68
5.1.	Introduction.....	68
5.2.	Materials and Methods.....	72
5.2.1.	Animal Model	72
5.2.2.	Data Analysis	73
5.3.	Results.....	75
5.3.1.	Identification of CLP Related Patterns and Characterization of Cluster Function	75
5.3.2.	Characterization of Response Dynamics	79
5.4.	Discussion.....	82
5.5.	Conclusions.....	92
6.	PRIMING EFFECTS OF BURN INJURY ON CECAL LIGATION AND PUNCTURE IN RATS	94
6.1.	Introduction.....	94
6.2.	Materials and Methods.....	96

6.2.1.	Animal Model	96
6.2.2.	RT-PCR Analysis.....	98
6.2.3.	Data Analysis	98
6.3.	Results.....	100
6.3.1.	Identification Of BCLP Related Patterns And Characterization Of Per Day Changes	100
6.3.2.	RT-PCR Confirmation of Expression Levels	104
6.3.3.	Identification of Critical Gene Expression Motifs Between BCLP and SCLP Conditions	105
6.4.	Discussion	107
6.4.1.	The Effect of Burn Priming on Immune Function	108
6.4.2.	The Effect of Burn Priming on Metabolism	111
6.4.3.	The Effect of Burn Priming on the Long Term Response	113
6.5.	Conclusion	115
7.	CONCLUDING REMARKS AND FUTURE DIRECTIONS	116
7.1.	CONCLUSIONS.....	116
7.1.1.	Short term burn injury.....	116
7.1.2.	Short Term CLP and SCLP injry	117
7.1.3.	Long Term CLP injury.....	119
7.1.4.	Double Hits injuries	121
7.2.	Future Directions	122

7.2.1.	The Effect Of Circadian Rhythm	122
7.2.2.	Mathematical Modeling	124
References.....		127

LIST OF TABLES

Table 3.1 Information of critical genes in each of the four clusters	18
Table 4.1 Predicted TFs relevant to the transcriptional regulation of some activated functions in SCLP injury	40
Table 4.2 Predicted TFs relevant to the transcriptional regulation of some activated functions in CLP injury.....	49
Table 5.1 Comparison of functional transcription clusters following CLP and SCLP	77
Table 6.1RT-PCR confirmation of Affymetrix gene expression folds change.....	105

LIST OF FIGURES

Figure 3.1 Schematic overview of the microarray data analysis	16
Figure 3.2 Gene expression profiles of rat livers in response to sham- burn or burn injury	17
Figure 3.3 Proposed network of changes in the liver following burn injury	24
Figure 4.1 Schematic overview of the microarray data analysis	33
Figure 4.2 Gene expression profiles of rat livers in responses to sham to SCLP injury	36
Figure 4.3 Gene expression profiles of rat liver in response to sham or CLP injury	43
Figure 4.4 Comparison between the SCLP- and CLP- induced responses	56
Figure 4.5 Proposed network of changes in the liver following SCLP injury	60
Figure 4.6 Proposed network of changes in the liver following CLP injury	61
Figure 4.7 Injury- specific transcriptional regulation of inflammatory- relevant functions	63
Figure 5.1 Schematic overview of the microarray data analysis	74
Figure 5.2 Gene expression profiles of rat livers in response to sham or SCLP injury	76
Figure 5.3 Principal component analysis of the clustered genes in SCLP and CLP	81
Figure 6.1A time line displaying when significant genes were altered following the double hits injury	101
Figure 6.2 Gene expression profiles of rat liver in response to S_CLP and B_CLP injury	106

CHAPTER 1

1. INTRODUCTION

In the United States, over 1.2 million burn injuries are reported annually (1) and despite significant advances in patient care morbidity and mortality remain high in those patients (2, 3). Responses to thermal injury are both local and systemic, involving highly activated production of pro- and anti- inflammatory cytokines, cellular protection mechanisms, hypermetabolism, prolonged catabolism, organ dysfunction and immune-suppression (4). Severe burn and trauma is generally associated with bacterial infections which cause more persistent inflammatory response with an ongoing hypermetabolic and catabolic state. Depending on the severity of the injury and septic complications, hyper-metabolism and other changes associated with the systemic inflammatory response can progress to multiple organ dysfunction syndromes, which can have a mortality rate as high as 90-100% (5).

Though much is known about the molecular and physiological pathway of the acute inflammatory response induced by thermal injury and subsequent bacterial translocation induced inflammation, this knowledge has not lead to effective therapies. One reason may be the traditional approach of sequentially studying individual gene products which lack of the global identification of the overall response from transcriptional level. Another reason may be that the complex nature of the response renders targeting isolated components an ineffective strategy (6). Thus, the complex nature of burn-induced inflammation makes appealing system approaches that explore hypotheses for deciphering complex modes of action. Therefore, it has been hypothesized that dynamic analysis of time series gene expression data based on proper statistical and data mining metrics may provide insights into the global dynamics of the inflammatory process.

To this end, the DNA microarray analysis is a proper approach which allows the characterization of interactions among different cellular pathways simultaneously that so far are considered separately. Microarray technique has emerged as one of the promising high-throughput genomic technologies due to its ability to monitor the changes on expression level of thousands of genes in a cell or tissue sample (7). Advances in transcriptomics, provides us with a genome scale phenotype which is analogous to a state vector descriptive of the cell for a given condition.

It is proclaimed that liver is the most susceptible and vulnerable organ during inflammation and multiple organ failure (8). Liver is an organ that plays a critical role in modulating immune function, inflammatory processes and that acute phase response in the attempt to restore homeostasis. The increase in serum levels of acute phase proteins synthesized in the liver is prominent among the acute phase response following burn injury which is believed to be of particular important for the adjustment of physiological process of stress response (9). In addition, liver is one of the most important players in systemic hyper metabolism since it is the main organ controlling circulating levels of metabolites and proteins. It is the major site for gluconeogenesis and disposal of amino acid nitrogen as urea. During catabolic state, muscle protein is converted into amino acids which are then released into the blood stream, where they are taken up by the liver. Thus, rat liver is the selected as the subject of this research.

The purpose of this research effort is to investigate the changes in gene expression spectrum of liver tissue in rats following burn injury, CLP injury, and “double hits” injury in order to elucidate the hepatic transcriptional responses to burn injury and subsequent sepsis. Thus, from global perspective as well as in a timely manner, the research may offer a molecular framework for study on the pathophysiology of systemic inflammation induced by injuries.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Postburn Inflammation

2.1.1. Burn-Induced Inflammation In Liver

Animal and human studies proposed that burn-induced systemic alterations are probably mediated by the orchestrated release of inflammatory mediators from the burn wound and by stress hormones (10). TNF- α , IL-1 β , IL-6 are demonstrated by research to be the three main proinflammatory mediators in the burn-induced inflammation. TNF- α , tumor necrosis factor- α , a critical cytokine involved in systemic inflammation, is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF- α is the regulation of immune cells. TNF- α is essential for the complete initiation of inflammation during invasion, and self-limited inflammation is normally characterized by decreasing TNF- α activity (11). In addition, IL-1 β and IL-6 are also important modulators of the immune system and major inducers of the acute phase response both *in vivo* and *in vitro* (12).

P38 mitogen activated protein (MAP) kinase signaling pathway and nuclear factor NF- κ B system are two important signaling pathways in burn-induced inflammation. p38 MAP kinase activation has been known to be one of the most important aspects of the signaling event that may mediate the release of TNF- α and IL-1 β and contributes to burn-induced liver injury (13). Activation of p38 MAP kinase leads to induction of a group of proteins central kinase to inflammatory process which will finally induce cytokine secretion. Previous studies have suggested that Kupffer cells (KCs) were a major source of *in vivo* TNF- α and IL-1 β post burn injury and activation of p38

MAP kinase played a critical part in modulating the production of these proinflammatory cytokines by KCs (14). Adversely, inhibition of p38 MAP kinase has been demonstrated to depress the release of TNF- α and IL-1 β and reduce the liver injury in models of acute pancreatitis, and ischemia reperfusion injury (15, 16). On the other hand, NF- κ B system is another major signaling pathway responsible for proinflammatory cytokine release post burn trauma. Though the relationship between p38 MAP kinase and NF- κ B system in the proinflammatory cytokines release post thermal injury has not yet been addressed. Several studies tried to identify the relationships between the two. Madrid *et al.* found that the p38 MAP kinase promoted direct transactivation of NF- κ B, through I κ B kinase (17). However, in (18), the results indicates that p38 MAP kinase does not affect the activation of NF- κ B directly in the liver of severely burned rats.

Acute-phase response (APR) is one of the major mechanisms in mammalian organisms responding to infection or trauma (19). In liver, the APR is characterized by significant alterations in its gene expression, finally leading to the up-regulation of acute-phase proteins (APP), such as, α 2-macroglobulin, hepatglobin and C-reactive protein, as well as in the down-regulation of transferrin and albumin. The cytokines which are produced during and participate in inflammatory responses such as IL-6, IL-1 β , and TNF α are the main activators of the production (19, 20). Though the acute phase response has the ability to assist the system return to the homeostasis, it is a cascade of events contributing to hypermetabolism and long lasting catabolism. It was previously believed to persist for only a short time after injury. However, now new evidence suggests that systemic hypermetabolism and catabolism associated with burn injury persevere for a longer time. A persistent and enhanced acute phase response has been shown to be potentially lethal to the host, with the uncontrolled and prolonged release of proinflammatory cytokines and acute phase proteins being associated with catabolism and hypermetabolism, resulting in the compromise of vital organs and thus to multiple-organ failure (MOF) and

increased morbidity and mortality (21-27). Down-regulation of constitutive hepatic proteins is probably further exacerbating these detrimental effects (21-23). The metabolic rate in host following burn injury is extremely high in liver; energy requirements are immense which are met by the mobilization of proteins and amino acids from peripheral tissues. Increased protein turnover, degradation, and negative nitrogen balance are all characteristic of this severe critical illness (25, 28). Consequently, the structure and function of essential organs including the liver, skeletal muscle, skin, immune system, and cellular membrane transport functions, are compromised (26, 27). The severe thermal injury induces the proinflammatory acute phase response for an extended period. Recent studies (29) have revealed that following a severe burn, resting energy expenditure and peripheral protein catabolism are increased for more than 9 months. Thus, the hepatic acute phase response in liver plays a more important part during hypermetabolism and catabolism post burn trauma than the previously believed (30).

2.1.2. Bacterial Translocation-Induced Inflammation In Liver Following Burn Injury

It is well known that hosts with severe burn injury are exceedingly susceptible to bacterial infections caused by deficiencies in host immune defenses, intestinal bacterial overgrowth, increased permeability or damage to the intestinal mucosal barrier due to thermal injury (31). Not only bacterial infection from the injured area but also bacterial translocation from the gut cause septic complications in the hosts (32). Post burn injury, mesenteric lymph nodes and liver indeed contain bacteria in mice (33). Seventy percent of bacteria which enter the bloodstream are identified to accumulate in liver and are trapped by Kupffer cells and hepatocytes in liver from the blood, suggesting the essential role of the liver in host defense as a reticuloendothelial organ (34, 35).

It is generally accepted that the reduced resistance to infection post severe injury is associated with abnormalities of both innate and adaptive immunity. Indigenous bacteria are continuously translocating in low quantity from the GI tract even in healthy hosts. They are usually killed by the host reticuloendothelial system in route or *in situ* in lymphoid organs therefore MLN and other extraintestinal sites normally remain sterile. Thus, persistent translocation of indigenous bacteria does not usually cause infection in the MLN or other lymphoid organs of healthy animals, despite continuous translocation from the GI tract. However, primary injury as burn has already altered the host immune system under which circumstance the bacteria translocation is detrimental. Thus, bacterial translocation has been postulated to play a role in the development of further immunologic alterations (36) and ultimately multiple organ failure (37), which may complicate severe thermal injury.

2.1.3. The Pathophysiology Of Burn Induced ‘Two Wave’ Inflammation

A common cause of persistent hypermetabolism and catabolism is severe trauma and burns, especially when patients are associated with complications, such as nosocomial infections. Although the first stimulation is sufficient to trigger a systemic inflammatory response affiliated with hypermetabolism, it is believed that a secondary insult post the primary injury, due to infection or other insult, plays a major part in causing a prolonged inflammatory response with an ongoing hypermetabolic and catabolic state leading to severe loss of lean body mass and increased risk of multiple organ dysfunction syndrome (38). The ‘two-hits’ phenomenon describes the cumulative impact of serial stimulations on an organisms in which the initial insult primes the leukocyte cellular components, leading to a greatly enhanced host pro-inflammatory response to the second insult (39). This phenomenon has been advanced by the clinical finding that in patients with multiple physiologic insults, relatively minor subsequent insults are tolerated (40), while others have shown an amplified response (40). According to the ‘two hit’ theory (39),

the primary injury (such as burn trauma) increase the production of proinflammatory mediators, mainly from macrophages. This in turn, compromises the host immune system, rendering the host vulnerable to a second hit such as infection, leading to multiple organ failure and, finally, to death. It is generally conceded that a primary injury such as burn trauma alters immune function such that a secondary bacterial insult increases morbidity and mortality over that observed with either insult individually.

2.2. Alterations in Gene Expression Levels in Liver post Burn Injury and Sepsis

2.2.1. Hepatic Gene Expression Post Burn

Liver is one of the important players in the systemic inflammation since it is the main organ controlling the circulating levels of metabolites and production of acute phase proteins. It is known that inflammatory mediators as well as metabolic changes in the circulation results in persistent alterations in gene expression levels in the liver. Therefore, liver is one of the target organs to understand the underlying molecular mechanisms of the disease state and to propose therapeutic approaches. In general, applying classical RT-PCR technique to analyze a specific gene expression, numerous studies have already elucidated that upregulation of some important receptors (such as CD14 receptors, protease activated receptors, histamine H-1 and H-2 receptors), transcription factors (NF- κ B, Stat3, and C/EBP- β) and other proteins or kinases (such as ERK, JNK, and p38) involved in the MAPK, Jac/STAT, and Ik-B/NF-kB signaling pathways has been observed in the liver during the inflammation (41-49). Recently, microarray technology and transcriptional profiling have been used to elucidate genome-wide changes in the liver following the burn injury (50-52). Vemula and co-workers (50) analyzed the gene expression alterations in rat livers during the first 24 h post burn trauma. Functional analysis of differentially expressed

339 genes revealed that metabolism and inflammation accounted for nearly 42%. The results indicated that the inflammatory genes that were altered included several classic acute phase response markers, and others involved in the complement, kinin, clotting, and fibrinolytic protein systems. On the other hand, metabolic genes expression changes showed that fatty acid oxidation increased after burn to meet the enhanced energy demands. The same group (52) also studied the gene expression profiling of long term (1, 2, 4 and 7 day) changes in rat liver following burn injury. They identified that 60 % of 740 differentially expressed genes showed significant changes either on day 1 or on day 7 postburn. Detailed analysis of the data also revealed that fatty acids are used in the liver as energy substrates for the first 4 days after the injury but not at later time point. Dasu et al. (51) also analyzed the gene expression profiles in the livers of rats at different time points (2h, 6h, 24h, 240h) after a 40 % TBSA burn. They identified that approximately 39 genes out of 8700 genes on each array across all the time points showed a significant change in the expression patterns.

2.2.2. Gene Expression Post Infection

Cecal ligation and puncture is an animal model that mimics the physiological changes in human sepsis (53) and is more clinically relevant than animal models of endotoxemia or gram- negative bacteremia(54). Following CLP, animals develop bacteremia, hypothermia, hypotension, and damage to multiple organ systems. The CLP model is considered the gold standard for sepsis research (53, 55).

There are numerous studies regarding the individual gene expression analysis where RT-PCR and immune-histochemical methods have been utilized. The liver proteins that changed in abundance after sepsis had a range of functions such as acute phase proteins, coagulation, ER stress, oxidative stress, apoptosis, mitochondrial proteins and nitric oxide metabolism. It is found that cyclophilin increased in abundance after CLP which play an critical role in sepsis-induced acute

renal failure (56). STAT1 activity increased rapidly in the liver, lungs and small intestine post CLP, peaking at 6-12h, while increased slowly, and still kept at mild level from 2 to 48h in the kidneys. Compared with STAT1, lower STAT3 activities were detected only in the liver and lungs, with negative detection in the small intestine and kidneys. HMGB1 mRNA levels significantly increased in liver, lungs and small intestine at various time points after CLP respectively (57).

Although there are significant numbers of studies in the literature on specific gene expression alteration in the liver, limited studies on gene expression changes post CLP by using microarray technology are reported. In general, 7 studies relating CLP (CLP is the only stressor) induced sepsis gene expression analysis by using microarray technology are reported. Among them, only one measures gene expression level at 4 different time points (6, 12, 18, 24 post CLP) (58) and multiple organs (lung, liver, thymus, spleen, kidneys and brain). The rest 6 other focuses on single time point at 24h post CLP on kidney (59), heart (60, 61), blood (62), liver (63, 64). Li et al., (63) explored the gene expression profile of liver tissue in rat liver at 24h post CLP and identified 522 genes in rat sepsis model changed gene expression accounting for 12.7%, among them 244 gene expression down-regulated, and 278 gene expression up-regulated. Multiple organ dysfunction syndrome (MODS) induced by sepsis involves a series of gene differential expressions, such as cell apoptosis genes, immunity related genes, cell cycle and control related genes, energy metabolism related genes, blood system related genes, cancer related genes, growth factor genes, acute stress reaction related genes. Cobb revealed that very little overlap was observed in the septic gene expression profiles of spleen and liver. Most of the genes identified have previously been linked to regulation of the inflammatory response (64). However, these studies either focus on a single time point (24 h post-CLP) (63, 65) which probably misses the critical early response or do not take the time scale into account (58), i.e. the inherent ordering and spacing provided by the time points are ignored.

2.2.3. Gene Expression Post ‘Double Hits’

Hepatic gene expression analysis following “double hit” by applying microarray technique could only be found in Banta’s study which compare both the gene expression with metabolic flux (66) in rat liver. In this study (66), systemic hypermetabolic response was induced in rats by applying a moderate burn injury followed 2 days later by CLP to produce sepsis. On day fourth after burn, alterations in gene expression levels in liver have been analyzed. Dual injury model revealed that mRNA levels of genes involved in the urea cycle, the respiratory chain, gluconeogenesis, the metabolism of some amino acid and the specific transporters of glutamine and arginine were significantly up-regulated. In order to get a better understanding of the dynamic changes of the hepatic gene expression following the double hits model, time series data should be collected and analyzed properly.

CHAPTER 3

3. DYNAMICS OF SHORT TERM GENE EXPRESSION PROFILING IN LIVER FOLLOWING THERMAL INJURY

3.1.Introduction

Thermal injury, one of the most severe forms of trauma, triggers a number of physiological responses including local and systemic inflammation, hyper-metabolism, immune-suppression, and eventually organ dysfunction (51). Clinical studies have shown that an uncontrolled and prolonged action of inflammatory cytokines, which is evidenced by a sustained release of acute phase proteins, may contribute to detrimental complications (67). Liver is an important player in the modulation of the inflammatory response since it largely controls circulating levels of metabolites and the production of acute phase proteins. It is known that inflammatory mediators as well as metabolic changes in the circulation result in alterations in gene expression levels in the liver (50, 51). Therefore, understanding the liver response at the molecular level is critical to understanding the systemic inflammatory disease, as well as its potential as a target for therapeutic approaches.

Prior studies using classical RT-PCR to analyze gene expression in liver have shown that inflammation upregulated specific receptors (such as CD14 receptors, protease activated receptors, histamine H-1 and H-2 receptors), transcription factors (NF- κ B, Stat3, and C/EBP- β) and other proteins or kinases (such as ERK, JNK, and p38) involved in the MAPK, Jac/STAT, and Ik-B/NF-kB signaling pathways (41-49). Recently, microarray technology and transcriptional profiling have been used to elucidate genome-wide changes in the liver following the burn injury

(50-52). Vemula and co-workers (50) analyzed gene expression changes in rat livers during the first 24 h following the burn injury. Functional analysis of differentially expressed genes revealed that metabolism and inflammation accounted for the majority of the differentially expressed genes. Altered inflammatory genes included several classic acute phase response markers, and other genes involved in the complement, kinin, clotting, and fibrinolytic protein systems. On the other hand, metabolic genes showed that fatty acid oxidation increased after burn presumably to meet the enhanced energy demands. Dasu et al. (51) also analyzed the gene expression profiles in rat livers at different time points (2h, 6h, 24h, 240h) after a more severe burn than in the prior studies.

In general, unsupervised hierarchical clustering was applied in order to identify specific patterns of gene expression in the liver associated with burn injury. A limitation of the aforementioned studies is that the control sham-burn group was defined as the initial pre-burn condition corresponding to the 0 h time point. However, gene expression in a healthy animal liver naturally fluctuates over time due to circadian rhythms (68). In order to obtain a better resolution of the dynamics of the injury response, it is therefore necessary to account for the dynamics of the sham group as well.

In this study, we used a standard burn injury model of the rat to compare the dynamics of gene expression in liver in burn vs. sham conditions during the first 24 h. The differentially expressed genes between sham and burn condition over time whose expression patterns were significantly altered following burn were identified and clustered. Simultaneous analysis of both burn and sham-burn groups' expression profiles enabled to characterize the dynamic patterns of both groups and reveal a comprehensive picture regarding the temporally coordinated inflammatory and metabolic changes in the liver following burn injury.

3.2. Material and Methods

3.2.1. Animal Model

Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing between 150 and 200 g were used. 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.

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 (66). This model was chosen because it has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant alterations on feeding patterns (52). 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 lukewarm 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 (69). 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.

Microarray experiments to generate liver gene expression data have been explained elsewhere (50). Briefly, animals were sacrificed (starting at 9am) at different time points (0, 2, 4, 8, 16 and 24hr post-treatment, i.e., sham burn and burn) and liver tissues were collected, snap frozen in liquid nitrogen and stored at -80°C (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 probe sets.

3.2.2. Data Analysis

In this study gene expression data analysis includes data preprocessing, filtering for “between class temporal differential expressions”, combining the datasets and clustering as seen in **Figure 3.1**. First, DNA chip analyzer (dChip) software (70) was used with invariant-set normalization and perfect match (PM) model to generate expression values. Microarray outlier filter analysis (71) identified that there were approximately 10% outliers in sham and burn gene expression data, which is typically observed in a microarray data. The outliers were replaced by the mean of the replicates (72). Then the data sets corresponding to burn and sham groups were investigated to identify the differentially expressed probesets by using the method (EDGE) proposed by Storey et al. (73). The statistical test used is analogue to an F statistics which compares the goodness of fit of the model under the null hypothesis to that under the alternative hypothesis. The null hypothesis model is obtained by fitting a time-dependent curve to the two or more groups combined, and the alternative hypothesis model is obtained by fitting a separate curve to each group. The significance threshold for this analysis was set as *q-value* <0.01 and *p-value* <0.01. This step determined a set of probesets whose expression patterns were significantly altered following the treatment considering the temporal differences between the control and treatment

groups. Finally the data sets corresponding to those differentially expressed probesets in either burn and/ or sham groups were combined to form one single matrix, which was then clustered using the a approach “consensus clustering” (74), in an unsupervised manner. This provided a set of burn responsive genes, which is significantly different than that of control group. We further applied one-way ANOVA test ($p < 0.01$) independently for each gene in each cluster and animal group in order to verify if the gene has been differentially expressed across the time only. Moreover, t-test was used additionally for pair-wise comparison of burn and sham genes identified in the clusters at each time point in order to estimate the activation time of a certain response related to burn injury. We characterized the biological relevance of the intrinsic responses by evaluating the enrichment of the corresponding gene subsets using the KEGG database through ARRAYTRACK (75) as well as analyzing the functions of each individual gene (76).

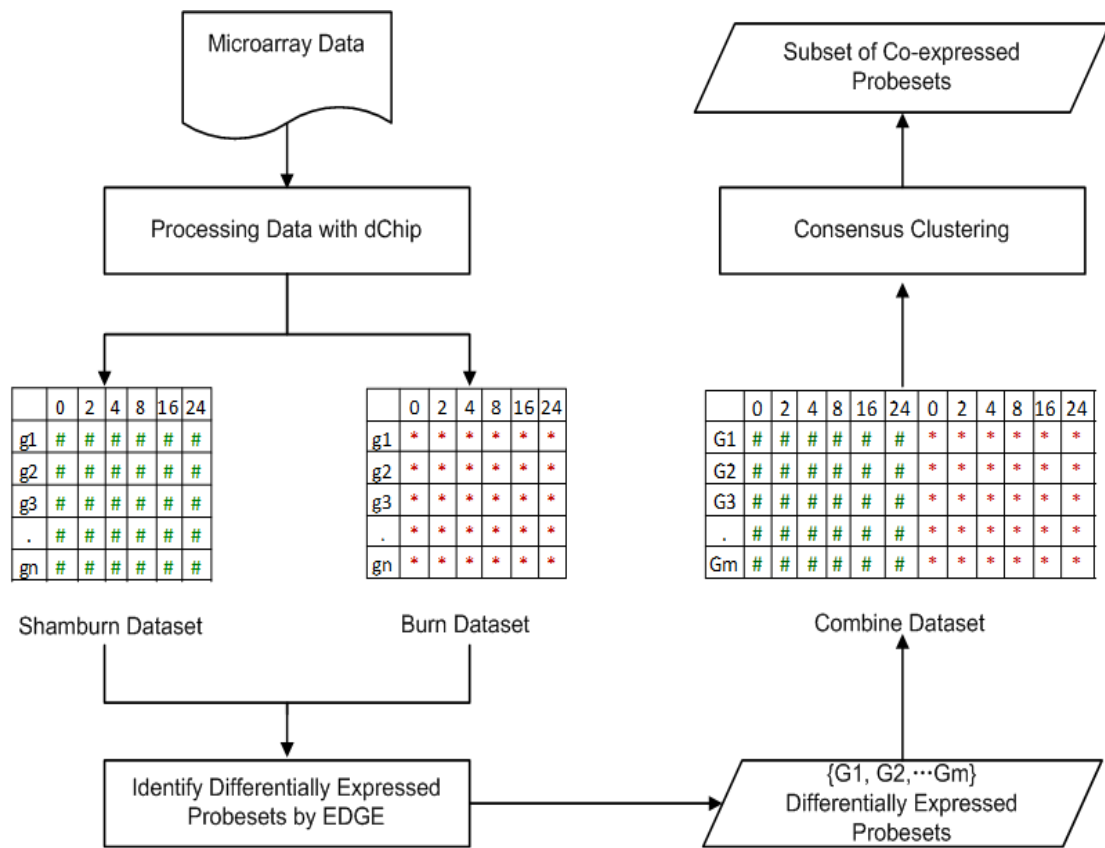


Figure 3.1 Schematic overview of the microarray data analysis

Microarray data was preprocessed by using dChip. Then, two data sets corresponding to burn and sham groups, respectively, were analyzed to identify the differentially expressed probesets by using EDGE with ‘between classes’ option under the statistical threshold $q < 0.01$, $p < 0.01$. Finally, the data sets corresponding to those differentially expressed probesets in burn and sham groups were combined to form one single matrix, which was then clustered using the approach of “consensus clustering” with threshold $p < 0.01$.

3.3.Results and Discussion

We examined liver-specific gene expression levels at 0, 2, 4, 8, 16, and 24 h post-treatment consisting of a 20% total body surface area (TBSA) burn or sham-burn. 1534 temporally

differentially expressed, i.e., burn responsive, were identified and subsequently clustered, using a consensus clustering approach, leading to identification of sub-set of genes assigned to 4 dominant expression patterns composed of 62, 82, 404, and 73 probe sets respectively. The average expression patterns of each dominant cluster are depicted in **Figure 3. 2** (right panel) while a heat map of all probe sets is shown in the left panel. A subset of critical genes is listed in **Table 3. 1**. ArrayTrack, as well as single gene ontology analysis, was used to further elaborate the functional annotations of burn injury responsive genes.

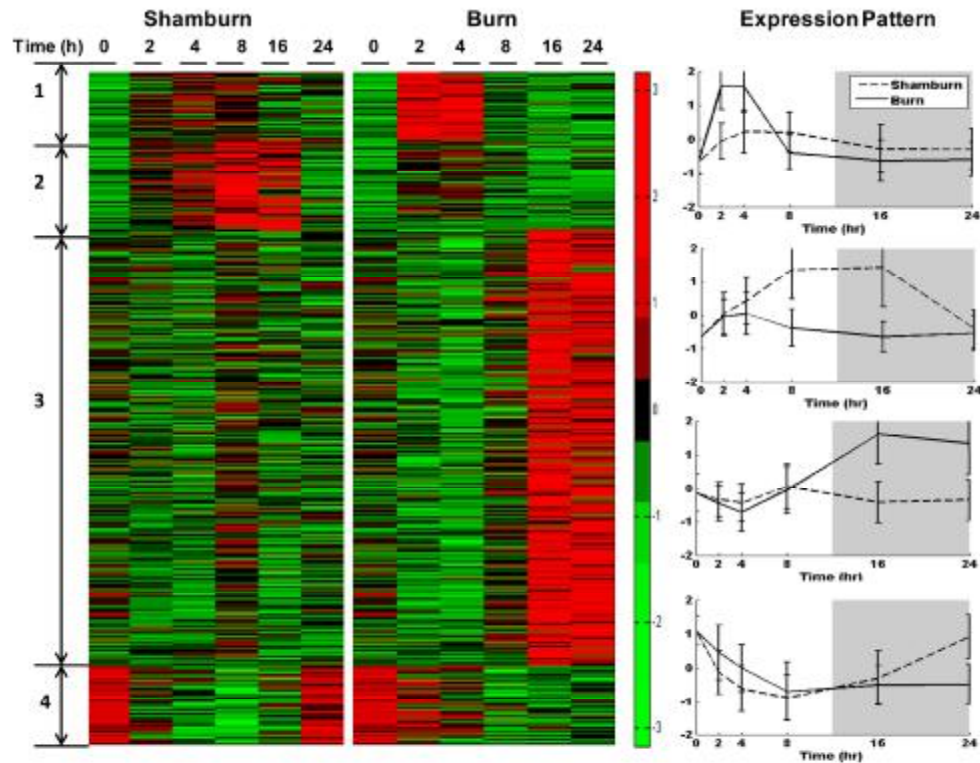


Figure 3.2 Gene expression profiles of rat livers in response to sham- burn or burn injury

Left Panel, expressions of 62, 82, 404 and 73 probesets in 4 clusters in sham-burn rats and burn rats at 0, 2, 4, 8, 16, 24 h post-treatment are exhibited in a heatmap.

Right Panel, the expression patterns are shown by plotting the average normalized (z-score) expression values of 62, 82, 404 and 73 probe sets in 4 clusters in sham-burn and burn groups (displayed as the means $\pm$ SEM).

Table 3.1 Information of critical genes in each of the four clusters

Cluster	Function	Gene name
Cluster 1	Pro-inflammatory Cytokine	<i>IL1a</i>
	Chemokine	<i>Cxcl16, Ccl11, Ccl9</i>
	Adaptive immune response regulation	<i>Ceacam1</i>
Cluster 2	Unsaturated fatty acid biosynthesis	<i>Acot1, Acot2, Acot3</i>
	Fatty acid metabolism	<i>Acaa2, Cpt1a, Dci</i>
	Synthesis of ketone bodies	<i>Hmgcs2</i>
	Lipid metabolism and lipid transport	<i>Eci1, Pigo, cyp4b1, Adfp, Pnpla8,</i>
	Cell-cell junctions	<i>Ablim3, Acer2, Cdh17</i>
	Complement and coagulation cascade	<i>C2, C4bpa, C8a, Cfh, Masp1, Serping1</i>
	N-glycan biosynthesis	<i>B4galt1, Dad1, Ddost, Dpagt1, Ganab,</i>
	Ribosome	<i>Rps25, Rps2</i>
	Jak-STAT signaling	<i>Il13, Il4, Il7, Jak3</i>
	TLR4 signaling	<i>IRAK1, LBP, TRAF3</i>
	Anti-inflammatory cytokine	<i>Il13, Il4</i>
	Transcription	<i>Brca1, Mcm7, Tcf25, Kdm1, Nfyb, Tef,</i>
	Translation	<i>Atpif1, Mrps21, Rps25, Rps2, Mrps11</i>
	Protein folding	<i>Dnajb11, Ppib, Hyou1, Edem2, Sep15,</i>
	Protein degradation	<i>Pcolce, Cpn1, caspase 12, Cdc34,</i>
	Protein target	<i>Tmed3, Ssr4, Tram1, Sec61a1, Rrbp1,</i>
	Bile acid production	<i>Idi1, tmem97, Npc2, Hsd17b4</i>
Cluster 4	Insulin signaling pathway	<i>Gck, Irs1, Mknk2, Trip10</i>
	Phenylalanine metabolism	<i>Ddc</i>
	Glycine, serine and threonine	<i>Bhmt</i>
	Galactose metabolism	<i>Gck</i>

3.3.1. Functional Characterization Of Major Clusters

Cluster 1 (**Figure 3.2**) exhibits an early up-regulation during the first two hours following thermal injury. One-way ANOVA ($p < 0.01$) indicates that the majority of the probesets in this cluster are

not differentially expressed in sham-burn, clearly indicating that their activity is the result of the burn injury. Burn injury induces a rapid, but transient, up-regulation of the genes in this cluster which resolves within 8 hrs post-burn. Functional annotation and characterization of cluster 1 identifies cytokines, chemokines and chemokine receptors as well as genes related to the modulation of innate and adaptive immune responses, including: IL-1 α , a pro-inflammatory cytokine playing a central role in the regulation of the immune response by binding to the IL-1 receptor (77) known to exhibit increased activity in the early stage of inflammatory response (78); chemokine (C-X-C motif) ligand 16 (CXCL16), a chemoattractant belonging to the CXC chemokine family whose expression is induced by inflammatory cytokines, such as IFN- γ and TNF- γ (79); chemokine (C-C motif) ligand 11 (CCL11), an inflammatory mediator belonging to the CC chemokine family that is known as Eotaxin-1; chemokine (C-C motif) ligand 9 known as macrophage inflammatory protein (MIP)-1 γ which is constitutively expressed in macrophages (80). The release of the pro-inflammatory cytokines of Cluster 1 postburn are hypothesized to trigger and enhance the inflammatory response and to mediate catabolic effects (81). A list of representative genes is depicted in Table 1.

Cluster 2 (**Figure 3.2**) exhibits characteristics of a persistent down regulation following burn injury, beginning at about 2hr post-burn, compared to the temporal differential expression under sham (ANOVA, $p < 0.01$), thus indicating suppression of expression in burn. A Student t-test ($p < 0.01$) reveals that the most significant suppression occurred at 8 h and 16 h post-burn. The functional characterization of Cluster 2 revealed down-regulation of genes involved primarily in unsaturated fatty acid biosynthesis, fatty acid metabolism, synthesis of ketone bodies and lipid metabolism and transport, consistent with earlier indications. The decrease in fatty acid biosynthesis as well as increase in fatty acid oxidation during the first 24h suggests that fatty acid is utilized in the liver during the first 24h postburn as the early energy source (50). Thus, down-regulation of fatty acid biosynthesis associated enzymes is possibly implying an enhanced energy

demand. Prior studies elucidating the circadian rhythmicity of gene expression in rat indicated that fatty acid biosynthesis is up-regulated in the late afternoon and early evening hours (82). Our sham results, consistent with this observation, indicate the possibility of circadian rhythmicity in Cluster 2 with a return to base-line values within 24 hr. This observation leads us to speculate the possibility of circadian disruption following burn injury. Furthermore, genes related to cell-cell junctions are also identified in Cluster 2 including: ABLIM3, a molecular component of adherence junctions (AJs) and possibly a novel component of adherens junctions with actin-binding activity (83); alkaline ceramidase 2 encoded by *Acer2* playing an important role in regulating $\beta 1$ maturation and cell adhesion mediated by $\beta 1$ integrins (84); cadherin 17 is a $\text{Ca}(2+)$ -dependent cell-cell adhesion molecule expressed in liver and intestine which plays a role in the morphological organization of liver and intestine (85). The products of those genes are associated with the integrity of the barrier function of hepatocytes linings. Given the many studies revealing that intestinal permeability is increased in burn patient shortly after the injury possibly due to the junction integrity alterations (86), the suppression of cell-cell junctions and membrane structural integrity may be indication of the liver damage caused by burn injury. Representative genes are depicted in Table 1.

Cluster 3 (Figure 3.2) captures a dynamics response which, under burn, exhibits deviation from sham around 8 hr post injury. Beyond this point, the burn responses remain effectively suppressed (ANOVA, $p < 0.01$). Functional annotation of Cluster 3 reveals gene products involved in complement and coagulation cascade, N-Glycan biosynthesis, ribosome and Jak-STAT signaling as well as involved in transcription/translation, protein synthesis/folding and targeting. All the above constitute processes critical in the production of acute phase proteins (APP) which are diffusible anti-inflammatory mediators (87). Furthermore, the anti-inflammatory response is induced by the suppressor of cytokine signaling proteins (SOCS) activated by Jak/STAT signaling pathway. Thus these gene encompassing Cluster 3 point to the activation of anti-

inflammatory mechanisms resulting in an increase in the synthesis of the acute phase proteins and important anti-inflammatory cytokines. APPs produced by the liver is a prominent characteristic of the acute phase response following thermal injury, which is believed to be critical for the adaptation of the body to stress (9). In addition, the transcription of APPs is activated in the late phase starting around 8 h post-burn, consistent with previous observations that the level of amyloid A, a APP, is not increased until the concentration of IL-6, a late phase cytokine, increases (88). The requirement of the energy and amino acids (AA) to produce large amount of APP in liver are satisfied by the increased flux of amino acids from the periphery to the liver, especially from the accelerated breakdown of muscle proteins (89). The alterations in nitrogen and protein metabolism represent a major threat for the organism, as it leads to a debilitating loss of lean body mass (90). Thus, a sustained or exaggerated acute phase response has been shown to be an indicator of a potentially life threatening uncontrolled and prolonged action of pro-inflammatory cytokines leading to multiple-organ failure.

Critical cytokines in this cluster are well known anti-inflammatory cytokines such as IL-13 and IL-4. IL-13 inhibits the ability of host immune cells to destroy intracellular pathogens by recruiting a large number of Th2 cells while IL-4 induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. IL-4 promotes the activation of macrophages into repair macrophages which is coupled with secretion of IL-10 and TGF-beta that result in the diminution of pathological inflammation. Anti-inflammatory cytokines, such as IL-4, are released later on in an attempt to counter-regulate the effects of the pro-inflammatory cytokines (91). Following the burn injury, a state of immunosuppression occurs whose intensity and duration is closely related to morbidity and mortality in burn patients (92). The inflammatory response after burn injury may play a role in the induction of adaptive immunosuppression. Both *in vivo* and *in vitro* studies manifest the altered adaptive immunity after burn which have shown that there is a decreased production of Th1- type cytokines (IL-2 and IFN- γ) and an increased production of Th2- type

cytokines (IL-4 and IL-10) (93). In the current study, the gene expression of Th2- type cytokines, IL-4 and IL-13, is enhanced starting from 8 h post burn, which may imply the onset of the host immunosuppression. Our results reveal that the gene expression of critical proteins (IRAK1, LBP, and TRAF3) in the TLR4 signaling pathway is upregulated at 8 h post burn injury (**Figure 3.2**, cluster 3). The TLR4 signaling pathway is critical for Gram-negative bacterial infections. It is well known that patients with severe burn injury are exceedingly susceptible to bacterial infections. Not only bacterial infection from the injured area but also bacterial translocation from the gut cause septic complications in the hosts. Mesenteric lymph nodes and liver indeed contain bacteria after burn injury in mice (33). It is generally accented that the decreased resistance to infection and enhanced secondary inflammatory response following serious injury is associated with abnormalities of both natural and adaptive immunity. Fang et al. (94) observed that thermal injury can markedly up-regulate lipopolysaccharide-binding protein (LBP) gene expression in various organs. LBP, a soluble acute-phase protein, binds to bacterial LPS to facilitate the immune response. Excessive LBP mRNA expression may be associated with enhanced synthesis and release of TNF- α stimulated by burn induced-endotoxin. Paterson et al. demonstrated that burn injury significantly increased TLR2- and TLR4-induced IL-1, IL-6, and TNF- α production by liver cells as early as 1 day after injury and they were found to be persistent for at least 7 days (95). Thus, the alteration of the TLR4 signaling pathway may imply that burn injury primes the innate immune system for enhanced TLR4-mediated responses to subsequent infection and provides evidence to suggest that an augmented Toll-like receptor signaling pathway might contribute to the development of increased systemic inflammation following severe burn injury.

Finally, a number of bile acid production related genes were also identified in this cluster. Bile acids are end products of cholesterol and the major driving force for bile formation, and the major excretory products of cholesterol. Bile acid production is expected to increase following burn injury (50). The main function of bile acids is to promote the formation of micelles, which

facilitate fat digesting and absorption. Therefore, the enhanced production of bile acids may also reflect the demand of the energy from food intake. In fact, nutritional therapy is commonly used with burn patients (96) in an attempt to compensate for burn injury-induced metabolic abnormalities although it is limited given that it does not address the underlying mechanisms that are responsible for hypermetabolic and catabolic induction. Although nutritional supplements partially alleviate the hyper-catabolic condition, they seldom can cannot reverse or completely restore the nitrogen balance. Representative genes are listed in Table 1.

Cluster 4 encompasses genes which are downregulated long after the injury is induced (16 h post burn) (**Figure 3.2**, cluster 4). The probesets of this cluster in both sham and burn groups exhibit an early down-regulation. Although the control group to recover their expression within 24 h, persistent downregulation is observed in the burn group. The maximum deviation between the sham-burn and burn groups occurs at 24 h postburn (two sample t-test, $p < 0.01$). The genes in this cluster are involved in the insulin signaling pathway, glycine, serine and threonine metabolism, and galactose metabolism. Consistent with prior observations, our data point to the possibility of impaired insulin signaling (97). Insulin is an anabolic hormone which promotes the storage of substrates in liver by stimulating lipogenesis, glycogen and protein synthesis (98). 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. Furthermore, the expression of genes associated with amino acid metabolism are known to be under circadian regulation in rat liver (82). Consistent with this observation, the insulin and amino acid metabolism-related genes in the sham-burn group also exhibited characteristics reminiscent of daily oscillation reaching a nadir at the interface of the light and dark phases. However, this daily oscillation was disrupted and suppressed maximally 24 h postburn, as demonstrated by the dynamic gene expression profile of the burn group pointing again to the possibility of circadian rhythms disruption. Representative genes are listed in Table 1.

3.3.2. Assessing And Interpreting The Differences In Transcriptional Dynamics Between Sham And Burn

The richness of our data and the fact that we analyze in tandem the dynamics of sham and burn injuries allowed to identify not only the differentially expressed responses but also the critical turning points where deviations induced by the burn injury manifest themselves. We identify, therefore, that the release of pro-inflammatory cytokines (Cluster 1) is almost instantaneous, whereas the synthesis of APP is delayed (Cluster 3), fatty acid biosynthesis (Cluster 2) precedes impairment of insulin signaling (Cluster 4). In a manner analogous to (99) we hypothesize that the time dependence among the profiles, may imply putative causal relations, which are succinctly summarized in the putative network structure of **Figure 3.3**, where arrows indicate possibly activation and/or induction, and circles indicate inhibition. The relations are derived based on the time lag elucidated from the temporal dynamics of individual responses.

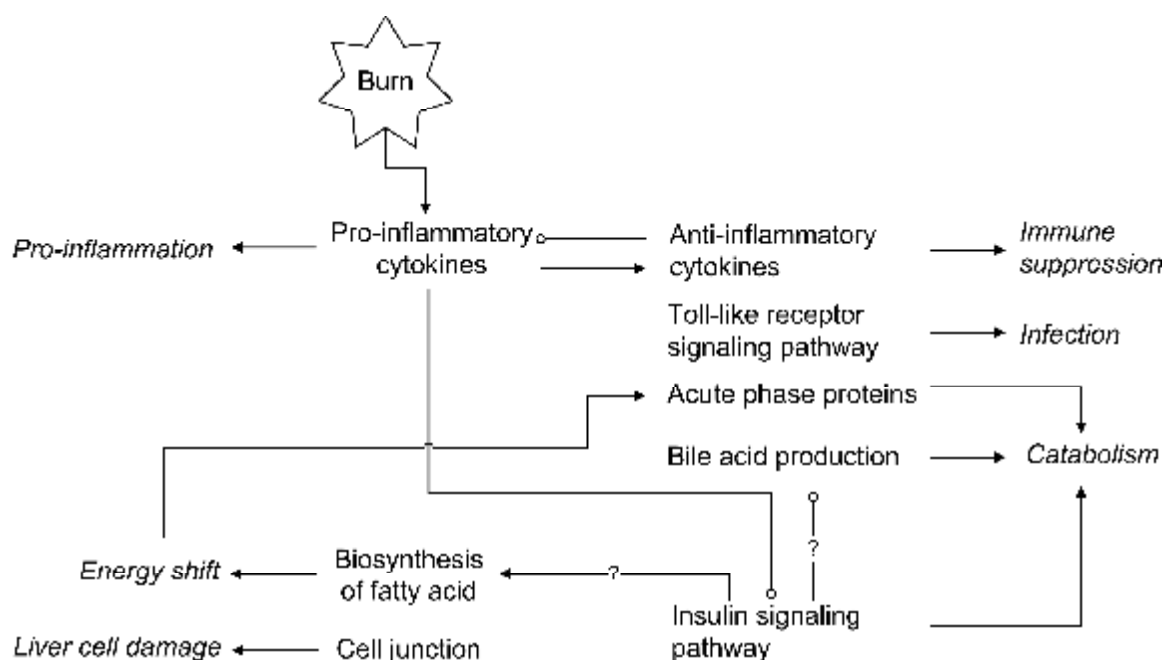


Figure 3.3 Proposed network of changes in the liver following burn injury

Italics represent outcomes following burn-induced gene expression alterations. Arrows indicate activation and/or induction and circles indicate inhibition.

The early upregulation of pro-inflammatory cytokines and chemokines, and their corresponding receptors in Cluster 1 indicates the activation of the immune system and a pro-inflammatory response, whereas the suppression of fatty acid biosynthesis associated genes in Cluster 2 implies an enhanced energy demand. In Cluster 3, the downregulation of the genes functioning as cell-cell junctions and providing membrane structural integrity indicate possible damage caused by the injury. Later activation of the expression of well-known anti-inflammatory cytokines may suggest the upcoming immune suppression. The activation of the Toll-like receptor signaling pathway, also in the Cluster 3, is possibly indicative of a priming effect to a subsequent secondary stimulus, i.e., infection. The most significant feature of Cluster 3 is the enhanced production of positive APPs, which is correlated to hyper-catabolism in muscle. In the same cluster, the enhanced expression of bile acid synthesis related genes may also be an indication of enhanced energy demand from nutrition supply. Finally, the late downregulation of the insulin signaling pathway-associated genes in Cluster 4 leads to the catabolism and insulin resistance. The dynamic picture which is assembled is indicative of the fact that once a pro-inflammatory response has been mounted there is a subsequent release of signals stimulating an anti-inflammatory response that inhibits the pro-inflammatory response, which drives the system back to homeostasis. The burn-induced response in Cluster 4, representing insulin-mediated metabolism, was characterized by an early and persistent downregulation. While prior work (100), indicated the possibility of an early downregulation the anabolic response in liver, our results indicate that the down-regulation is in fact delayed in time, given that the nature progression of the sham responses also points to an early down-regulation, although it recovers, pointing to the possibility that the burn-specific down-regulation occurs only later in time. Therefore, we argue

that the onset of insulin resistance and the putative associated catabolic response (which is regulated by insulin) is not as immediate as previously thought (100). A delayed response is in fact more consistent with the observed dynamics of cytokine and chemokine activation, which presumably drive the molecular mechanisms leading to insulin resistance (101), because impaired insulin signaling should occur after and not before the release of cytokines. In addition, insulin is known to suppress bile acid synthesis in cultured rat hepatocytes by down-regulating the key enzymes in the synthesis of bile acids from cholesterol. Therefore, the impaired insulin signaling can also explain the increased bile acid excretion observed in humans with untreated diabetes mellitus and in experimental animals with insulin deficiency (102). However, in our study, the insulin signaling pathway was suppressed around 16 h postburn, which was later than the enhanced production of bile acids. Thus, our results suggest that the increased bile acid production in inflammation is more likely caused by mechanisms other than the impaired insulin signaling pathway. Moreover, insulin is a well-known critical anabolic hormone which promotes the storage of substrates in liver such as lipids (98). However, since the decreased synthesis of fatty acids (~2 h postburn) occurs earlier than the impaired insulin signaling (~16 h postburn), the suppression of fatty acid biosynthesis herein may not be caused by the insulin resistance either.

We observed that a significant number of positive APP genes were up-regulated, which requires an increased energy utilization. Thus, the biosynthesis of unsaturated fatty acid starts to be suppressed around 2 h post burn which may imply the preservation of the energy sources for the synthesis of positive APP activated later - around 8h post burn. This suggests that FA is utilized in the liver during the first 16 h after the burn injury. However, new energy sources and AA pool to produce positive APP are required after the exhaustion of available energy and AAs in the liver. It is well known that burn injury results in accelerated breakdown of muscle proteins which increase the AA concentrations in the circulation thus AA uptake-rates in the liver (89). We observed that the impaired insulin signaling pathway occurs later, starting around 16h postburn

consistent with previous reports indicating that muscle protein breakdown is exacerbated in burn-injured patients as a result of insulin resistance (103). Thus, our results suggest the possibility that the impaired insulin signaling pathway may further intensify the catabolic response which has already been driven by the uncontrolled positive APP production. In addition, due to the suppression of fatty acid biosynthesis, it seems that the fatty acid oxidation might serve as the main source of energy in the liver following the burn injury.

3.4. Conclusions

We have shown a short-term liver gene expression profiling in response to thermal injury. This analysis characterizing the dynamic patterns of both burn and sham groups elucidated that temporal changes in the expression levels after the injury are mainly associated with the pro-inflammatory response, fatty acid biosynthesis, the anti-inflammatory response, and insulin-regulated metabolic responses. The network of dynamic changes in gene expression observed in this study revealed the possible links between the diverse burn-induced responses. Based on our results, the pro-inflammatory response is activated immediately around 2 h following burn treatment which triggers the anti-inflammatory response starting around 8 h postburn. The biosynthesis of unsaturated fatty acid starts to be suppressed around 2 h which may imply the preservation of the energy sources for the synthesis of APPs whose genes were activated later around 8 h post burn. In addition, the impaired insulin signaling pathway, starting from around 16 h postburn and putatively as a result of the alterations in inflammatory gene expression, is expected to further strengthen the catabolic response. A Suppression of fatty acid synthesis and enhanced production of bile acids were also observed, but were not likely due to the impaired insulin signaling because of the discrepancy in the dynamics of these responses. In conclusion, simultaneous analysis of both burn and sham-burn groups' expression profiles enables to characterize the dynamic patterns of both groups. Our results reveal critical gene expression

pattern changes triggered by burn injury which reflects host physiological and biological alterations and provides a more comprehensive understanding of the pathophysiology of the disease state.

CHAPTER 4

4. Dynamics of Short Term Gene Expression Profiling In Liver Following CLP

4.1.Introduction

Sepsis remains a major clinical challenge for physicians in the United States (Angus, Linde-Zwirble et al. 2001). The incidence of sepsis is increasing, despite the fact that fatality rates of severe sepsis cases between 1993 and 2003 have decreased from 46% to 38%, possibly as a result of better treatments(104). Despite improved clinical outcomes, sepsis often resists treatment and successful clinical trials of novel drugs are rare. The difficulty in developing new and more effective medications for sepsis is due, in part, to our incomplete understanding of the underlying pathophysiology of the disease.

In order to decipher and investigate septic responses, a number of animal models have been proposed and developed. Among them, the most prevalent is cecal ligation and puncture (CLP) in rodents (53, 105). The basic premise is that sepsis is driven by microbial infection originating within the abdominal cavity while bacteria subsequently translocate into the blood compartment, eventually triggering a systemic inflammatory response. More specifically, the experiment involves midline laparotomy, exteriorization of the caecum, ligation of the caecum distal to the ileocecal valve and punctures of the ligated caecum(105). 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 states at the whole body level. Given the fact that the liver plays a major role in hypermetabolism and produces acute phase proteins during systemic inflammation, the characterization of the hepatic response to these challenges can be very helpful to monitor the dynamics of the induction and resolution of the inflammatory response, as well as to investigate the underlying molecular mechanisms and the impact of therapeutic approaches on the septic state.

Genome-wide microarray technology has been already applied to reveal transcriptional changes in liver following the CLP treatment (58, 63, 65). However, these studies either focus on a single time point (24 h post-CLP), (63, 65) therefore missing the critical early response, or do not take the time scale into account (58), i.e. the inherent ordering and spacing provided by the time points are ignored. In addition, a Sham CLP (SCLP) group is usually included to serve as control for the CLP, which consists of animals treated identically without receiving cecal ligation and puncture. Although this enables isolating the specific effects of the infection associated with the CLP procedure, the fact that SCLP is in fact a surgically-induced injury that also impacts on the gene expression pattern of the liver is often ignored. Therefore, alteration of the gene expression following CLP is the combination of the effects of two different stresses, i.e. injury and infection.

Herein, our goal was to compare the effects of SCLP or CLP with healthy animals on hepatic gene expression in order to identify the “net” response induced by injury, sepsis, and both. For this purpose, we generated a rich time series of DNA microarray data from liver samples isolated during the first 24 h after each respective injury, and utilized bioinformatics tools to compare the responses to a time dependent sham control in order to identify the dynamic patterns unique to SCLP and CLP. Because these patterns are temporally coordinated, we are also able to identify putative transcription factors that regulate these processes in a time dependant manner, and contrast these regulatory elements between the two injury models.

4.2. Materials and Methods

4.2.1. Animal Model

Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing between 150 and 200 g were used. 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.

The infection was induced by applying CLP treatment. Rats were first anesthetized, and then the analgesic buprenorphine (0.01 to 0.05 mg/kg) and Bupivacaine (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 through and through for 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. SCLP consist of animals treated identically without receiving cecal ligation and puncture. Rats were single caged after the treatments and given standard rat chow and water ad libitum until sacrifice. Unoperated animals (Sham) serve as controls. Animals are sacrificed (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 flash frozen for offline 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 probe sets.

4.2.2. Data Analysis

Analysis of CLP, SCLP and sham gene expression data includes normalization, filtering, combining the datasets and clustering which is depicted in **Figure 4.1**. 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 (106). 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” (74). 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.

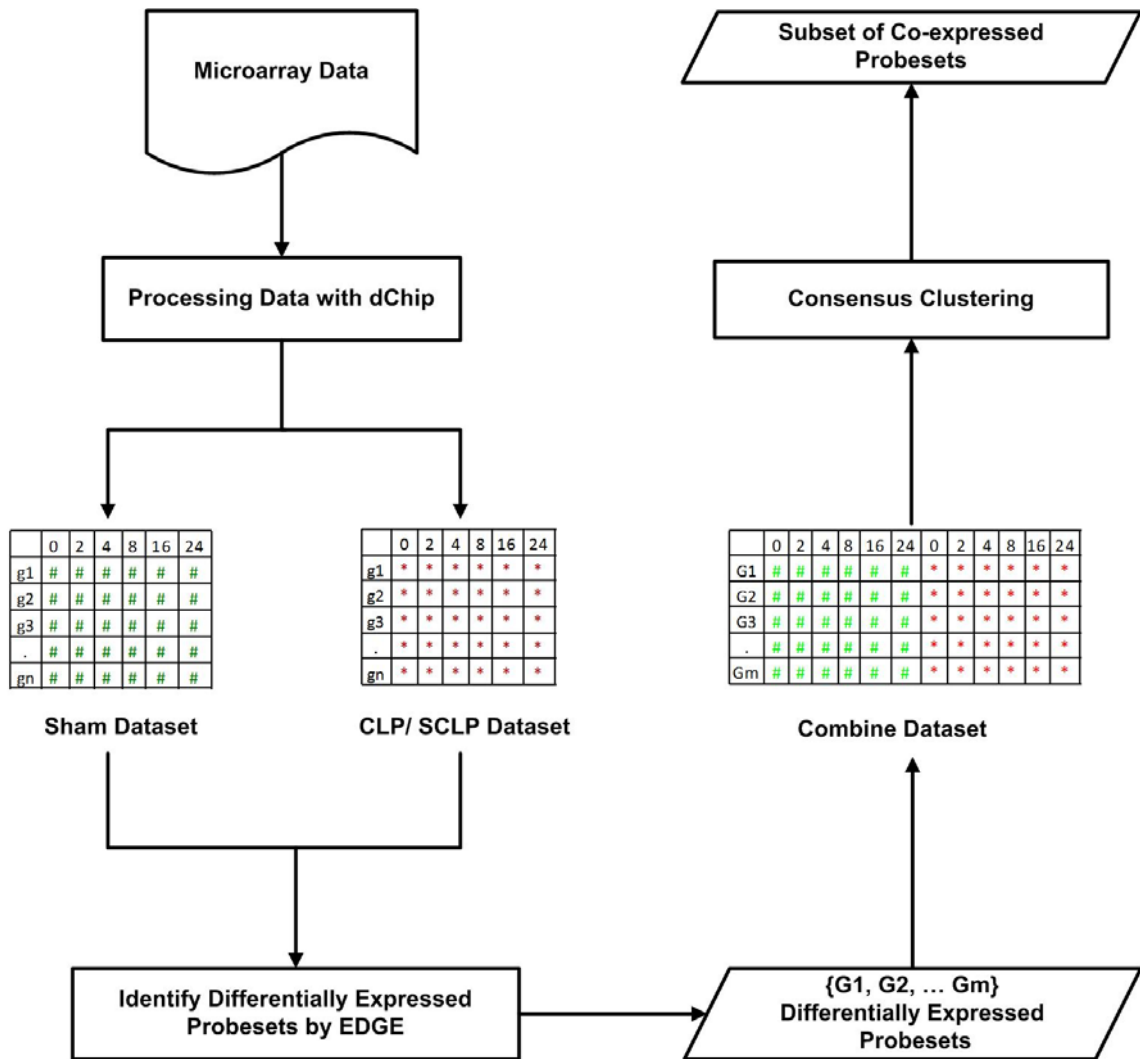


Figure 4.1 Schematic overview of the microarray data analysis

Microarray data was preprocessed by using dChip. Then, two data sets corresponding to sham and CLP/ SCLP groups respectively, were analyzed to identify the differentially expressed probesets by using EDGE with ‘between classes’ option under the statistical threshold $q < 0.001$, $p < 0.001$. Finally, the data sets corresponding to those differentially expressed probesets in sham and CLP/ SCLP groups were combined to form one single matrix, which was then clustered using the approach of “consensus clustering” with threshold $p < 0.01$.

4.2.3. Promoter Extraction And Computational Prediction Of Putative Transcriptional Regulators

Promoters of genes including all transcript-relevant alternative promoters are extracted from a rich database of promoter information with a default length (500bp upstream and 100bp downstream of the transcription start site) if there is no experimentally defined length suggested by Genomatix (107). In order to accelerate the process of identifying putative transcriptional regulators, promoters are pre-processed as in (108). Specifically, MatInspector (109) is applied to scan for Position Weight Matrix (PWM) matches on those promoter sequences using optimal parameters from MatBase which ensures that the minimum number of matches found in non-regulatory sequences i.e. the false positive matches is minimized (107). Each promoter of a gene is then re-modelled to become a list of transcription factor binding sites (TFBSs) ordered by their local positions on the promoter sequences and represented by corresponding TF names along with their binding orientations. The conversion supports for fast search the presence of a TFBS or a cis Regulatory Modules (CRM) on promoter sequences. In order to predict putative transcriptional regulators, we utilize the context-specific CRM search technique to identify over-represented CRMs in the promoter set of a gene battery (108). Each gene battery contains a certain set of genes that are hypothetically co-regulated, i.e. co-expressed and co-functional in this study. With the hypothesis that common functions activated across multiple tissues may play important roles in response to CS treatment, we applied our previous tool developed in (108) to identify transcription factors relevant to CS transcriptional responses. In brief, we computationally define a CRM as a list of non-overlapping TFBSs ordered by their positions on the promoter sequence and characterized with their corresponding binding strand orientation. The procedure will first identify all potential TFBSs that are common in the corresponding promoter set and then search for all possible combinations of all commonly found TFBSs above using the breadth first search technique. Due to the fact that a CRM can be present on promoters of many genes in the

background set, we estimate the statistical significance of commonly identified CRMs for each gene battery vs. the background set to select those that are significantly overrepresented. Subsequently, selected CRMs are decomposed to obtain a list of TFs that are associated with corresponding TFBSs present on CRMs.

4.3.Results

4.3.1. Identification And Functional Characterization Of SCLP Regulated Expression Patterns

Hepatic gene expression levels were measured at 0, 2, 4, 8, 16 and 24 h in the livers of rats following sham, CLP and SCLP treatment. By considering the time-dependent variations in the gene expression profiles of the sham group, differentially expressed SCLP responsive genes that showed altered short term dynamic profiles when compared to sham were identified. In total 1722 probe sets in the SCLP group exhibit altered gene expression patterns over time compared to the corresponding sham control group. Consensus clustering further determined 6 statistically significant clusters composed of 191, 389, 193, 52, 123 and 73 probe sets respectively. The average expression patterns of the 6 clusters are depicted in **Figure 4.2** (right panel) while a heat map of all probe sets is shown in the left panel. IPA pathway analysis as well as single gene ontology analysis was used to further elaborate on the functional annotations of SCLP injury responsive genes.

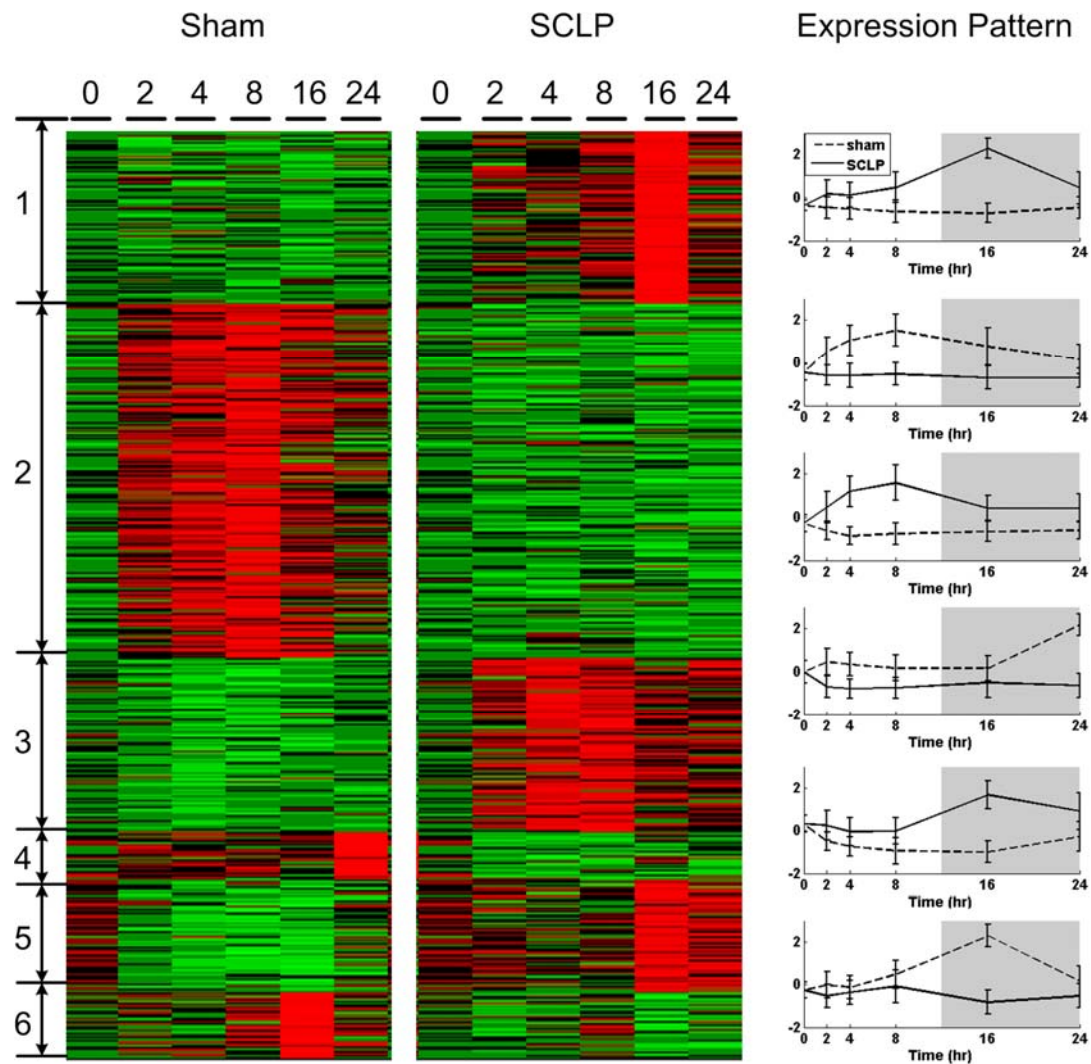


Figure 4.2 Gene expression profiles of rat livers in responses to sham to SCLP injury

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 heatmap.

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

- 1) In cluster 1, SCLP induced a significant activation of a response peaking 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*). Overall, this cluster is mainly related to the fatty acid, amino acid, and glucose metabolism.

- 2) The second SCLP induced-response cluster exhibits a persistent suppression during the entire 24h post treatment. Genes in this cluster are enriched in pathways which include the protein ubiquitination pathway (*Usp7*, *Psmb2*, *Ube2n*, *Hspa9*, *Psm4*, *Psme2*, *Psm14*, *Psm1*, *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 of the SCLP-induced response are highly activated early on starting from 0 h to reach a maximum at 8h following injury. Pathway analysis indicates that this cluster is mainly relevant to pro-inflammation, and more specifically in IL-6 signaling (*Il6st*, *Il1rl1*, *Il6r*, *Lbp*), role of Jak family kinases in IL-6- type cytokine signaling (*Il6st*, *Il6r*), acute phase response signaling (*Il6st*, *Il1rl1*, *Il6r*). Besides, 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* (12). 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 (110). 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 (19). 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(111). 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 (112). 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*), 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 (113-115). 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) (116). 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 and 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 of the normal structure and function of the cell.

4.3.2. Identification Of Putative Regulators Of SCLP-Specific Transcriptional Responses

The genes that were identified as belonging to critical functional annotations of the SCLP gene expression pattern were subjected to putative transcription factor identification using promoter regions. In order to understand the underlying regulatory dynamics that drive the system, pathways were selected that, due to their differential expression post SCLP/CLP when compared to sham, appear to be critical to the initiation, and maintenance of a systemic pro inflammatory response. This response, which functionally encompasses broad networks such as cytokine signaling, acute phase protein production, and oxidative stress, is represented by IL-6 signaling, acute phase response signaling, the coagulation system, the complement system, and the metabolism of xenobiotics by cytochrome p450. A full list of predicted transcription factors is presented in Table 4. 1.

Table 4.1 Predicted TFs relevant to the transcriptional regulation of some activated functions in SCLP injury

No.	Gene batteries		Genes	TFs*
	Patt.	Functions		
SCLP				
1	3	IL6 signaling	il1rl1, il6r, il6st, lbp	MYBL, HOMF, CREB, NKXH, NFKB, NFAT, ETSF, HBOX, HOXF, SP1F, BRNF, GATA, SORY, EREF, LHXF
2	5	Acute phase response signaling	c1r, c4b, c5, pik3r2, pik3r6, rras	STAT, HOXF, CLOX, ETSF, YY1F, ZBPF
3	5	Coagulation system	f8, f9, kng1, thbd	ETSF, IRFF, AP4R, BRNF
4	5	Complement system	c1r, c4b, c5, masp1	NOLF, AP2F, NR2F, HOXF, LEFF, SORY, FKHD, OCT1, COMP, GKLF, CLOX, MYOD, RXRF, SP1F, STAT
5	4	Metabolism of xenobiotics by cytochrome P450	cyp2d6, gstm1, gstm2, mgst1	NEUR, PAX6, SORY, ETSF, PLAG, ABDB, EGRF, INSM, MZF1, SRFF
5	3	Metabolism of xenobiotics by cytochrome P450	adh7, cyp51a1, dhfr4, gstm1	LEFF, AP4R, E2FF, ETSF, CREB, MYBL, WHNF, SP1F, PAX2, HESF, AP1R, EGRF, HEAT, SORY

1. The functional pathway corresponding to IL-6 signaling was identified to be present in cluster 3 of the SCLP gene expression data, which is functionally characterized as the early pro inflammatory response. In particular, *MYBL*, *CREB*, *ETSF*, *HOXF*, *SP1F*, *BRNF* and

SORY were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.

2. The functional pathway corresponding to acute phase response signaling was identified to be present in cluster 3 of the SCLP gene expression data, which is functionally characterized as the early pro inflammatory response. In particular, *MYBL*, *CREB*, *ETSF*, and *SP1F* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
3. The functional pathway corresponding to the coagulation system was identified to be present in cluster 5 of the SCLP gene expression data, which is functionally characterized as the late pro inflammatory response. In particular, *ETSF* and *BRNF* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
4. The functional pathway corresponding to the complement system was identified to be present in cluster 5 of the SCLP gene expression data, which is functionally characterized as the late pro inflammatory response. In particular, *HOXF*, *SORY* and *SP1F* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
5. The functional pathway corresponding to the metabolism of xenobiotics by cytochrome p450 was identified to be present in cluster 4 of the SCLP gene expression data. In particular, *SORY* and *ETSF* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.

4.3.3. 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 the sham group, differentially expressed CLP responsive genes that showed altered short term dynamic profiles were identified. 2039 probe sets are identified to be differentially expressed over time between the sham and CLP conditions. Then, 6 statistically significant clusters composed of 437, 295, 171, 154, 91 and 73 probe sets respectively are obtained by applying the consensus clustering method. The average expression patterns of the 6 clusters are depicted in **Figure 4.3** (right panel) while a heat map of all probe sets 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.

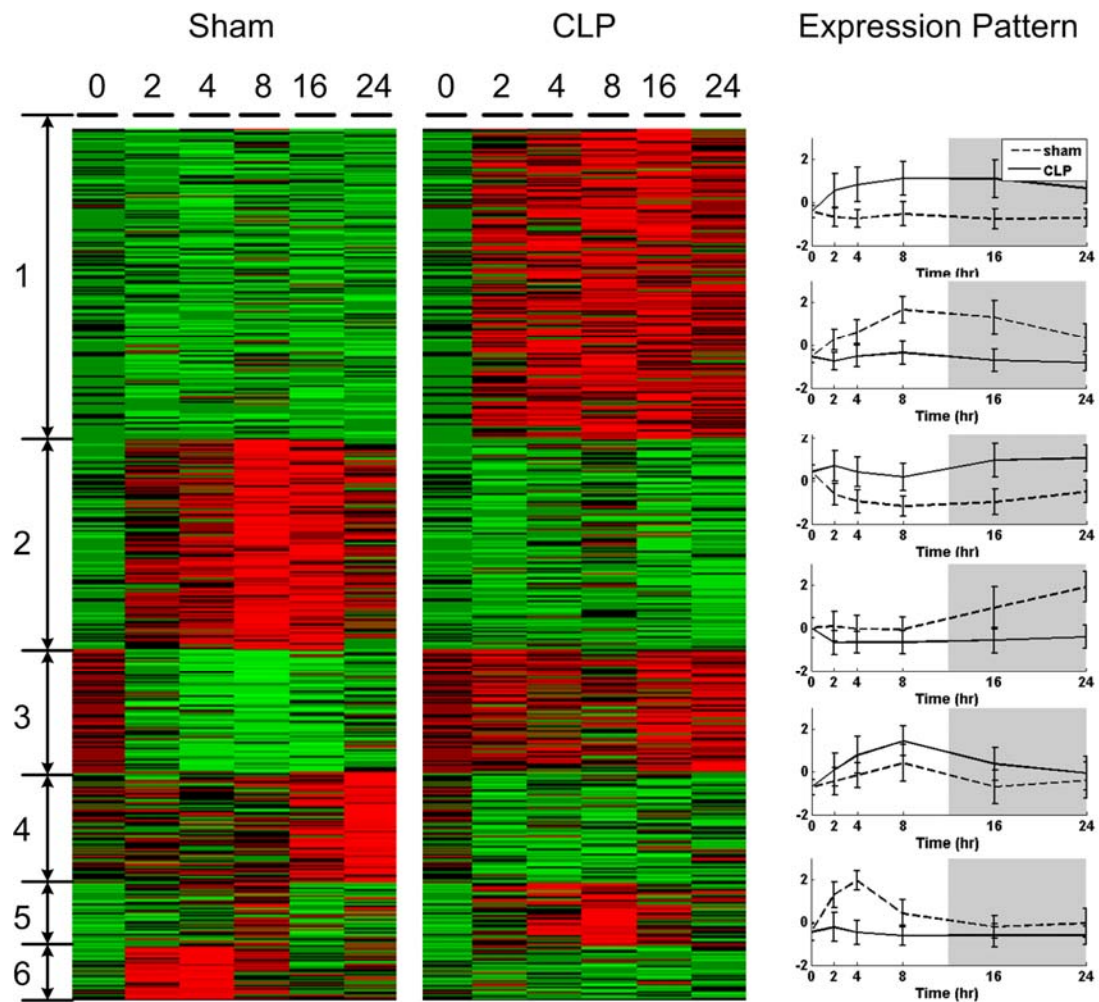


Figure 4.3 Gene expression profiles of rat liver in response to sham or CLP injury

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 heatmap.

Right Panel, the expression patterns are shown by plotting the average normalized (z-score) expression values of 437, 295, 171, 154, 91 and 73 probe sets in 6 clusters in sham and CLP groups (displayed as the means $\pm$ SEM).

- 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 *Rpn2*).

The acute phase response signaling, role of jak family kinase in IL-6 type cytokine signaling and IL-6 signaling were also observed in the previously described SCLP-induced responses, and are all indicators of the activation of pro inflammatory pathways. In addition, T helper cells include two types, T_H1 and T_H2. Importantly T_H1 cells are responsible for phagocyte- dependent protective host response as well as producing pro inflammatory cytokines including IFN- γ and IL-2 (117). 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 (118). 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 (119). Thus, combined with the complement and coagulation systems, 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 the inflammatory response. Out of 36 genes, a group of them participates in the activation of leukocytes (*Adam9*, *Blk*, *C5*, *Cfh*, *Ddost*, *F5*, *Hla-Dqb1*, *Ifnb1*, *Il6*, *Kng1*, *Lbp*, *Mgat5*,

Nfkb1z, *Prf1*, *Ptprj*, *Serping1*, and *Vcan*), inflammation (*Bdkrb2*, *C5*, *Cxcl2*, *Hpx*, *Inf1b1*, *Il11*, *Il13ra1*, *Il6*, *Il6r*, *Il6st*, *Knq1*, *Lbp*, and *Prf1*), infiltration by neutrophils (*C5*, *CD36*, *Cfh*, *Cxcl2*, *Il6*, and *Il6r*), infiltration of granulocytes (*C5*, *Cd36*, *Cfh*, *Cxcl2*, *Il6*, *Il6r*, and *Knq1*), activation of monocytes (*Adam9*, *Blk*, *C5*, *Ddost*, *Hla-Dqb1*, *Inf1b1*, *Il6*, *Lbp*, *Mgat5*, *Nfkb1z*, *Prf1*, and *Ptprj*), activation of phagocytes (*Adam9*, *C5*, *Cfh*, *F5*, *Inf1b1*, *Il6*, *Knq1*, *Lbp*, and *Serping1*). 5 and 12 of them are associated with infection mechanism (*Inf1b1*, *Il6*, *Nfkb1z*, *Prf1*, and *Il11*) and antigen presentation (*C5*, *C8a*, *Cfh*, *Cfhr1*, *Knq1*, *Masp1*, *Adam9*, *Inf1b1*, *Lbp*, *F5*, *Il6*, and *Serping1*), respectively.

- 2) In cluster 2, the CLP induced response exhibits a constant expression pattern throughout the 24h post treatment period, indicating in fact a suppression comparing to the control. Genes in this major temporary class are critical in the protein ubiquitination pathway (*Rbx1*, *Psmb5*, *Psmc7*, *Ub2n*, *Hspa9*, *Hspd1*, *Hsp90b1*, *HSP90AB1*, *PSMC6*, *PSMB2*, *PSMA4*, *HSP90AA1*, *PSMD14*), fatty acid elongation in mitochondria (*Acaa2*, *Hadha*). Besides these signaling pathways, annotation of genes reveals that this cluster also includes genes involved in catabolism of protein (*Kiaa0368*, *Loc643751*, *Psmc14*, *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 a disruption of cellular organization proteins, which may indicate cellular damage in the liver as a result of systemic inflammation.

- 3) Compared to the sham, 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 levels during the dark phase in the 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 (120). Thus, our result is consistent with the sepsis induced hypermetabolism. Besides these pathways, individual gene ontology analysis indicates that lipid-related pathways are enriched in this cluster including metabolism of lipid (*Acadsb*, *Acs3*, *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, 32, and 10 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 are mainly involved in mitochondrial function (*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 are 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 (121). Insulin is an anabolic hormone which promotes the storage of substrates in liver by stimulating lipogenesis, glycogen and protein synthesis (98). 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 timing of UV-sensitive cellular processes to occur at night to avoid UV-induced damage (122). 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 more elevated response compared to the sham group 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 (123).

- 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 may probably suggest the damage of the injury to normal cellular function and structure.

4.3.4. Identification Of Putative Regulators Of CLP-Specific Transcriptional Responses

The promoters of genes belonging to the pathways earlier indicated were also analyzed based on the clusters identified to be representative of the CLP response, and the pathways selected were the same pathways used in the SCLP analysis. These pathways also showed a significant difference in dynamics between SCLP and CLP: the cytokine based pathways and acute phase protein production show up regulation early and late respectively in SCLP, while they are co expressed and up regulated in CLP. The pathway associated with signs of oxidative stress was up in CLP, but down in SCLP. Therefore the exploration of the underlying transcriptional dynamics of these pathways is able to capture both the characterization of the underlying transcriptional regulation of an injury, and identify unique regulatory elements in each injury. A full list of predicted transcription factors is presented in Table 4.2.

Table 4.2 Predicted TFs relevant to the transcriptional regulation of some activated functions in CLP injury

No.	Gene batteries		Genes	TFs*
	Patt.	Functions		
CLP				
1	1	IL6 signaling	a2m, hspb3, il6, il6r, il6st, lbp	EREF, HESF, SORY, TBPf, NFKB, EKLF, GATA, LHXF, NKXH, IRFF, MYBL, HOMF, FKHD, BRNF, CEBP
2	1	Acute phase response signaling	a2m, c4bpa, c5, c9, cfb, cp, hpx, il6, il6r, il6st, itih4, lbp, serping1	STAT, HOXF, RXRF, AP4R, DMRT, ETSF, FKHD, HNF1, IRFF, MYOD, NR2F, SORY, BRNF, CEBP, E2FF
3	1	Coagulation system	a2m, bdkrb2, f5, kng1, serpina5, serpincl	ABDB, DLXF, DMRT, HOMF, HOXF, MYOD, MYT1, NF1F, PDX1, STAT
4	1	Complement system	c4bpa, c5, c8a, c9, cfh, serping1	STAT, CAAT, AP4R, ETSF, BCL6, CLOX, HNF1, NEUR, RREB, RXRF
5	3	Metabolism of xenobiotics by cytochrome P450	adh7, cyp51a1, dhrrs4, gstm1	LEFF, AP4R, E2FF, ETSF, CREB, MYBL, WHNF, SP1F, PAX2, HESF, AP1R, EGRF, HEAT, SORY

1. The functional pathway corresponding to IL-6 signaling was identified to be present in cluster 1 of the CLP gene expression data, which is functionally characterized as the early pro inflammatory response. In particular, *HESF*, *IRFF*, *MYBL*, *HOMF*, *FKHD*, *BRNF*, *CEBP* and *SORY* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
2. The functional pathway corresponding to acute phase response signaling was identified to be present in cluster 1 of the CLP gene expression data, which is functionally characterized as the early pro inflammatory response. In particular, *STAT*, *HOXF*, *RXRF*, *AP4R*, *DMRT*, *ETSF*, *FKHD*, *HNF1*, *IRFF*, *MYOD*, *SORY*, *BRNF*, *CEBP* and *E2FF* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
3. The functional pathway corresponding to the coagulation system was identified to be present in cluster 1 of the CLP gene expression data, which is functionally characterized as the late pro inflammatory response. In particular, *DMRT*, *HOMF*, *HOXF*, *MYOD*, and *STAT* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.
4. The functional pathway corresponding to the complement system was identified to be present in cluster 1 of the CLP gene expression data, which is functionally characterized as the late pro inflammatory response. In particular, *STAT*, *AP4R*, *ETSF*, *HNF1*, and *RXRF* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.

5. The functional pathway corresponding to the metabolism of xenobiotics by cytochrome p450 was identified to be present in cluster 3 of the CLP gene expression data. In particular, *SORY*, *AP4R*, *E2FF*, *ETSF*, *MYBL* and *HESF* were the transcription factors that were the most significant in the gene batteries, with a frequency of at least two occurrences per 5 gene batteries.

4.4. Discussion

4.4.1. SCLP Dynamics: Aseptic Inflammation from Surgery

SCLP treatment imposes a surgical 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 over 40% following trauma (124). 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 the ubiquitin-proteasome pathway is activated in muscle tissue which leads to muscle wasting in septic patients (125). Thus, the requirement of amino acids (AA) to produce large amount of APPs in liver may be satisfied by the increased flux of amino acids from the periphery tissue to the liver, presumably from the accelerated breakdown of muscle proteins. Furthermore, the enhanced production of the genes involved in metabolism, which includes fatty acid metabolism, amino acid metabolism, and glucose metabolism, in cluster 1 and cluster 5 may be an indication of the hypermetabolism (123) following injury. The fuel for these changes would be likely be energy and substrate sources from peripheral tissues, including muscle. The suppression of the genes involved in cellular assembly and organization in cluster 2 and cluster 6 indicates the presence of cellular damage in the liver, which may be caused by oxidative stress. Finally, the data suggest that 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 resulting in reduced therapeutic or detoxification effect (126). It has been 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 (127). However, due to the fact that the maximum suppression of the P450 expression (~ 24h post SCLP) occurs later than the maximum enhanced production of the acute phase (~16h post SCLP), further work is needed to confirm that the down regulation of the synthesis of P450 genes is a consequence of the shift in transcriptional focus toward acute phase proteins.

4.1.2. CLP Dynamics: Systemic Response to Surgically Induced Sepsis

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 proinflammatory 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, one report states that protein synthesis in liver tissue was increased by 164% following trauma and sepsis (124). 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 thought to recreate(53). Inflammation induced downregulation of the insulin signaling pathway in cluster 4 down regulates mechanisms that promote energy storage in the 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, coupled with acute phase protein production,

is expected to increase energy output by the liver in order to meet the increased demand for protein synthesis. The substrates that fuel the increased energetic output and protein production are likely generated by peripheral tissue and further exacerbate the catabolism in muscle. Besides the transcriptional alteration in inflammation and metabolism, genes expression related to other functions also change expression patterns compared 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 (128). 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, which in the context of the systemic inflammatory response has been previously associated with oxidative stress(129). The decreased mitochondrial function 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 (130). Interestingly, mitochondrial dysfunction and the insulin signaling pathway are in the same cluster sharing the same expression patterns, indicating possible coregulation of these pathways. Following CLP injury, the downregulation of the production of the energy produced by oxidative phosphorylation may alter the balance of important metabolites, including the ATP/cAMP ratio. In order to avoid this, the low energy production state may be compensated by production of the quick energy through increased degradation of fatty acids, amino acids, and sugars, compensating for decreased mitochondrial function with an increased volume of substrate. 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 different facets of metabolism.

4.1.3. Comparison Between SCLP and CLP- Induced Response

Injuries such as trauma, surgery, and infection produce immune, hormonal and metabolic responses. Both injuries induce the activation of pro-inflammatory response characterized by the enhanced synthesis of acute-phase proteins, increased metabolic rate and oxidative stress induced damage. In addition, the signaling elements found in both SCLP and CLP appear to characterize the inflammatory response, which acts as the driving force for the rest of the other cellular alterations, in particular the increased energy and substrate demand which is compensated for by the observed metabolic changes. Due to the central role the liver plays in many physiological processes, its metabolic and inflammatory changes will impact peripheral tissues, which results in muscle degradation as a mechanism to scavenge substrate for acute phase protein production. A succinct summary of the differences in functional groups between CLP and SCLP, along with a visual representation of the pro inflammatory modules involved can be found in **Figure 4.4**.

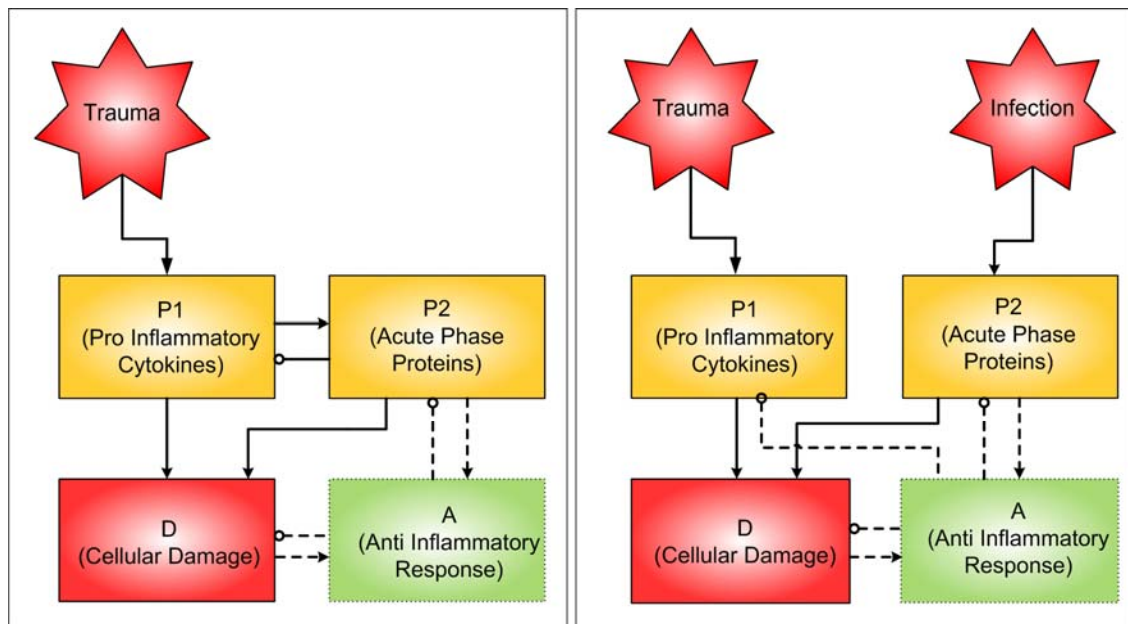


Figure 4.4 Comparison between the SCLP- and CLP- induced responses

Solid arrows represent inferred connections from time scale data, and dashed arrows represent responses expected but not observed due to the 24 hour time course limitation.

Though both types of injury exhibit a systemic inflammatory response, there are significant differences in both the gene ontology and dynamics between clusters of comparable function. Following the injury, no matter whether the initial stimulus is SCLP or CLP, signaling mediators translocate to the liver and trigger the first pro-inflammatory response (P1) which is manifested by the release of pro-inflammatory cytokines, chemokines and expression of cytokine receptors. Activation of synthesis of acute-phase proteins in both injury models is expected, since it is a common consequence of diverse injuries ranging from infection, trauma, surgery burns, tissue infarction, and various immunologically mediated inflammatory conditions (131). However, the altered dynamics between the aseptic and septic traumas indicate that this process is under different regulatory controls in each system. Pro-inflammation (P1 and P2) will cause damage (D) to the cells represented by the DNA damage, cellular assembly and organization damage and the

energy production failure. Thus, the signs of stress that appear in both conditions are consistent with the upregulation of pro inflammatory mediators that are observed in both. In addition, previously, it has previously been assumed that the synthesis of acute-phase proteins is beneficial to the host (131). However, from a broader context, the synthesis of the acute phase protein does have a strong adverse influence on the peripheral tissues due to their pro inflammatory effects when circulated through the host(132). Thus, the pro-inflammatory response (P1, P2), which serves as the protection guard to trauma and infection, drives a series of subsequent metabolic and cellular changes, which if left unchecked, can result in severe damage (D) to the host. The level of damage caused by these changes is variable based on the injury, and is potentially the result of the altered dynamics present in the CLP injury, compared to the SCLP model.

Bacterial removal movement is observed only following CLP injury, which is reasonable considering that the aim of the CLP treatment is to establish an infection with mixed bacteria in the host's circulation system. This anti bacterial response is clustered with other pro inflammatory mediators, and contains activators of T cells and NK cells, representing a deviation in the immune response from aseptic inflammation in SCLP to an anti bacterial state in CLP. In addition, the down regulation of the insulin signaling pathway (133), mitochondrial dysfunction (134) and DNA damage observed following trauma in other studies which are present in the CLP-induced response but absent in the SCLP-induced response may be a result of the increased severity of injury caused by the increased severity of the host's response to active bacteria in the surgical site.

More notably, some functions are in common in both conditions but exhibiting different dynamics post treatment. The pro-inflammatory response triggered by SCLP exists in two distinct phases: it exhibits an early upregulation and peak at 8h in cluster 3 (P1) and then following this, a second wave of pro inflammatory proteins (P2) is further activated, and consists of acute phase protein synthesis of complement and coagulation mediators, which reaches its maximum at 16h

post SCLP in cluster 5. Thus, there is a clear distinction between the two aspects of pro-inflammation following SCLP injury, namely the cytokines and other signals, and the acute phase response proteins involved in the complement and coagulation systems. However, all of the pro-inflammatory genes share the same expression pattern in CLP-induced response, which is early and persistently upregulated within 24h post CLP treatment. In this cluster, P2 is co activated simultaneously with P1, and the response is maintained over the entire time course, potentially indicating a higher volume of acute phase protein production compared to SCLP. This may suggest that CLP treatment is a more severe injury which triggers a much more rapid and persistent response. The presence of more severe signs of oxidative stress and cellular damage in CLP is consistent with the stronger, more sustained upregulation of pro inflammatory mediators in that condition. It also suggests that pro inflammation is more complicated than a single dynamic response, and is comprised of various, independent transcriptional modules that can be recruited in a combinatorial fashion for an enhanced response against a variety of different stimuli. In this case, P1 represents an inflammatory response to surgical trauma, and in the absence of infection leads to the induction of P2. However, with a bacterial stimulus, P1 can be augmented by P2 to create a more severe and persistent response. In addition, the xenobiotics biodegradation pathway is significantly downregulated in SCLP cluster 4 indicating the dampened detoxification ability in liver. However; the same pathway exhibiting an upregulation in CLP cluster 3 is hypothesized to play a role in the clearance of the poisonous bacterial component. The differences in regulation in the xenobiotics pathway may be explained by the fact that many compounds that have been oxidized by free radicals are degraded by these xenobiotic proteins(135), and that CLP shows increased signs of oxidative stress in other clusters. The lack of these signs in SCLP may indicate that there is no need for xenobiotics degradation in that response, and thus it is down regulated in favor of the transcription of acute phase proteins. Thus, the pathways present in both conditions showing different expression dynamics may indicate

altered regulatory modes in response to injuries that lead to different facets of inflammation being displayed that are optimally suited to deal with the type of trauma involved.

4.1.4. Dynamics Of Induced Transcriptional Responses

The systemic responses of the two injury models have been previously presented as separate gene clusters that each have a domain of characterized functions, however, the physiological processes that they represent do not operate in isolation. Thus, to address this, networks have been constructed by utilizing the observed timescales of the gene clusters and the known ontologies of the involved genes for both SCLP and CLP. In a manner analogous to (99) we hypothesize that the time dependence among the profiles, may imply putative causal relations, which are succinctly summarized in the putative network structures, where arrows indicate possibly activation and/or induction, and circles indicate inhibition. The relations are derived based on the time lag elucidated from the temporal dynamics of individual responses. These networks can be found in **Figure 4.5** and **Figure 4.6** and represent the functional relationships that translate pro inflammatory signals and acute phase mediators into characteristic systemic effects, such as hypermetabolism, muscle catabolism, oxidative stress, and bacterial removal. Because cytokines, chemokines, and their receptors are known to initiate many of the cascades associated with the inflammatory response, these mediators are considered to be the first response of the system, which triggers the activation of innate immunity, as well as increased metabolism. Although both networks contain pro inflammatory cytokines and acute phase proteins, the dynamics of their activation are significantly different, with acute phase protein production being stimulated by the injury in the CLP network, as opposed to the cytokines in the SCLP network. Some other notable differences in the networks are the absence of bacterial removal components from the SCLP network, which have been previously discussed as the result of the added septic stimulus in CLP. Additionally, the lack of insulin in the SCLP network may represent a less severe hyper metabolic

response, as the insulin pathway is known to repress many metabolic functions(133). This is reinforced by the presence of multiple indicators of oxidative stress in the CLP network, which include mitochondrial dysfunction(134) and DNA damage. Overall these networks are comprised of the functional dynamics of the CLP and SCLP injury models as observed from the gene expression data, and represent the integration of large scale pathways to form a systemic response.

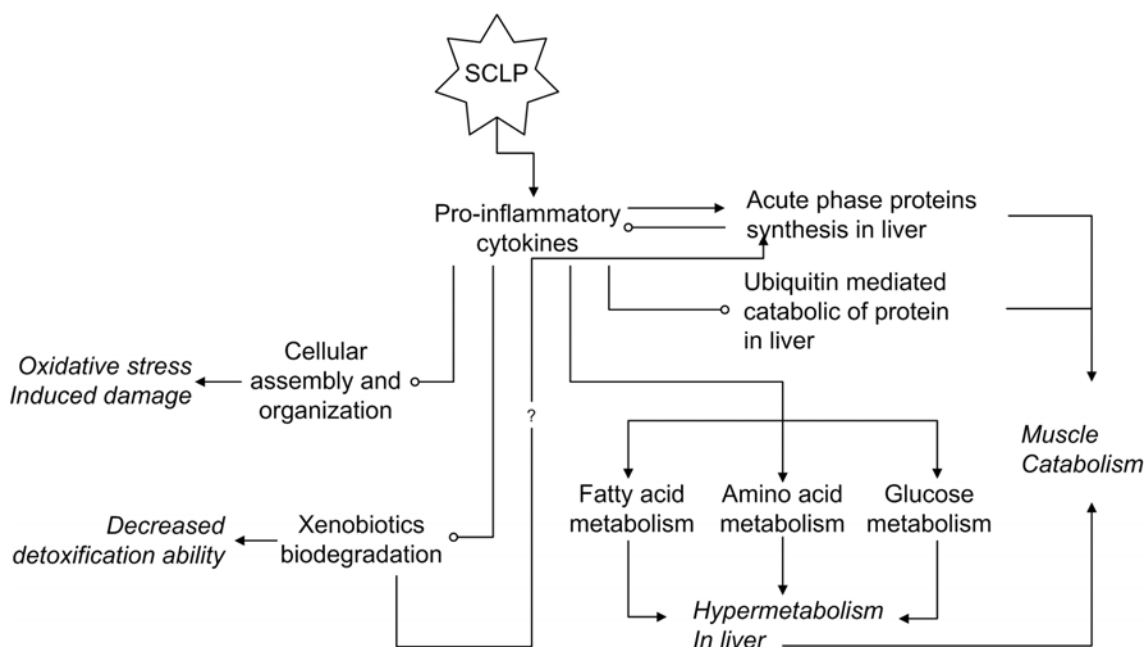


Figure 4.5 Proposed network of changes in the liver following SCLP injury

Italics represent outcomes following burn-induced gene expression alterations. Arrows indicate activation and/or induction, and circles indicate inhibition.

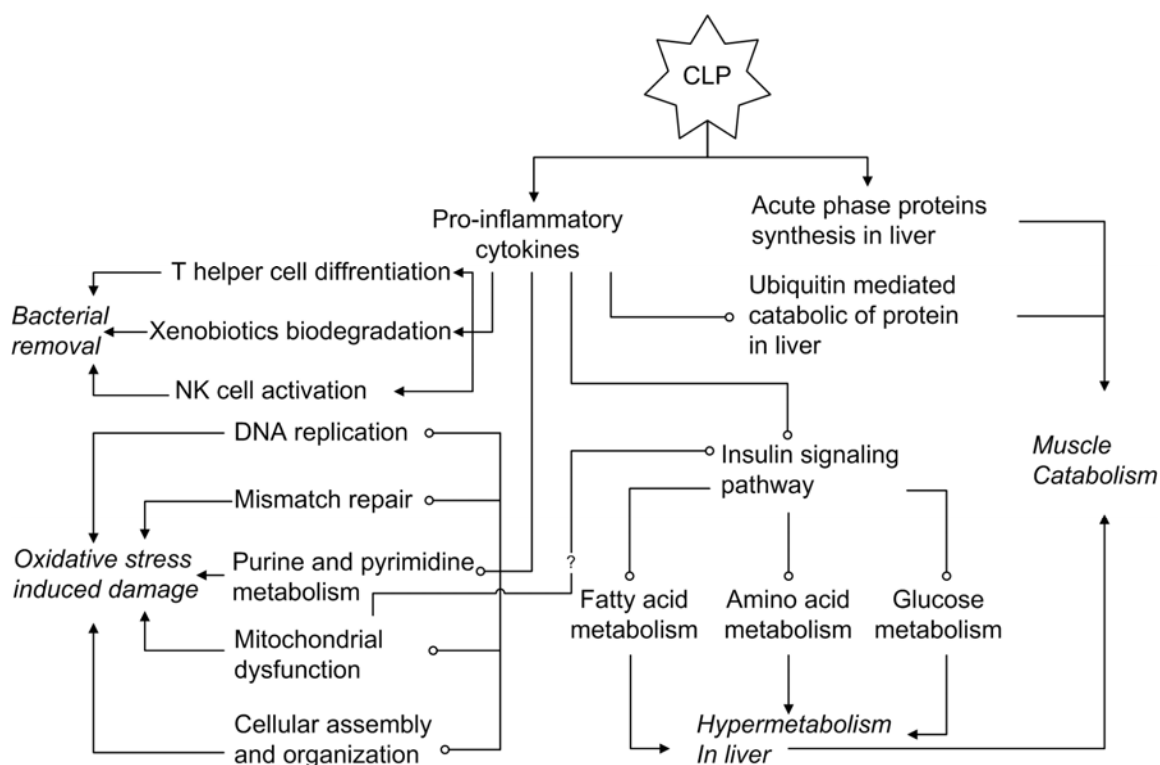


Figure 4.6 Proposed network of changes in the liver following CLP injury

Italics represent outcomes following burn-induced gene expression alterations. Arrows indicate activation and/or induction, and circles indicate inhibition.

4.1.5. Transcriptional Regulation In SCLP And CLP Conditions

The transcriptional responses associated with injury have been previously characterized and described in terms of their functional annotations. These transcriptional changes represent, at a cellular level, the alterations in behavior in response to an inflammatory stimulus. However, by using gene promoter regions, we have been able to identify putative transcription factors which govern the expression of those genes. These transcription factor families represent the underlying regulatory mechanisms which govern the evolving cellular response over time, and are important in understanding the level in which the responses to different injuries differ. Previously, we have

suggested that SCLP and CLP represent two different forms of inflammatory stimulus, which have significantly different pro inflammatory dynamics. In particular, the behavior of pro inflammatory cytokine pathways was characterized as P1 (early pro inflammatory response), and was significantly up regulated during the early time points of SCLP, but consistently up regulated across the CLP time course. The coagulation and complement cascade proteins which represent an acute phase protein based inflammatory response was characterized as P2 (late pro inflammatory response), and was significantly up regulated during the late time points of SCLP, but was consistently up regulated in the same cluster as P1 in CLP. This result was further analyzed in the context of putative transcription factor identification, which attempts to elucidate the differences in regulatory architecture that give rise to different pro inflammatory responses.

In **Figure 4.7a**, a graphical comparison of the transcription factors involved in IL-6 signaling (an important functional group found in P1) shows that while there is significant overlap between the two injuries, many transcription factors are unique to each injury. This indicates that the injuries do not simply vary in dynamics, but that the SCLP regulatory architecture deviates significantly from the CLP architecture. The presence of well known transcription factor families such as CREB, HOXF, SP1F and ETSF in SCLP, that are absent in CLP may indicate that these factors are important in promoting an IL-6 based inflammatory response that does not activate immune cells. Similarly, transcription factor families such as EKLF, HESF, and CEBP are present in CLP only, indicating that they may play a role in the activation of an anti bacterial immune response. Well known transcription factor families, such as NFkB, are present in both injuries. This may indicate that these transcription factors represent a basal inflammatory response, whose specificity and dynamics are modulated by the unique transcription factor families.

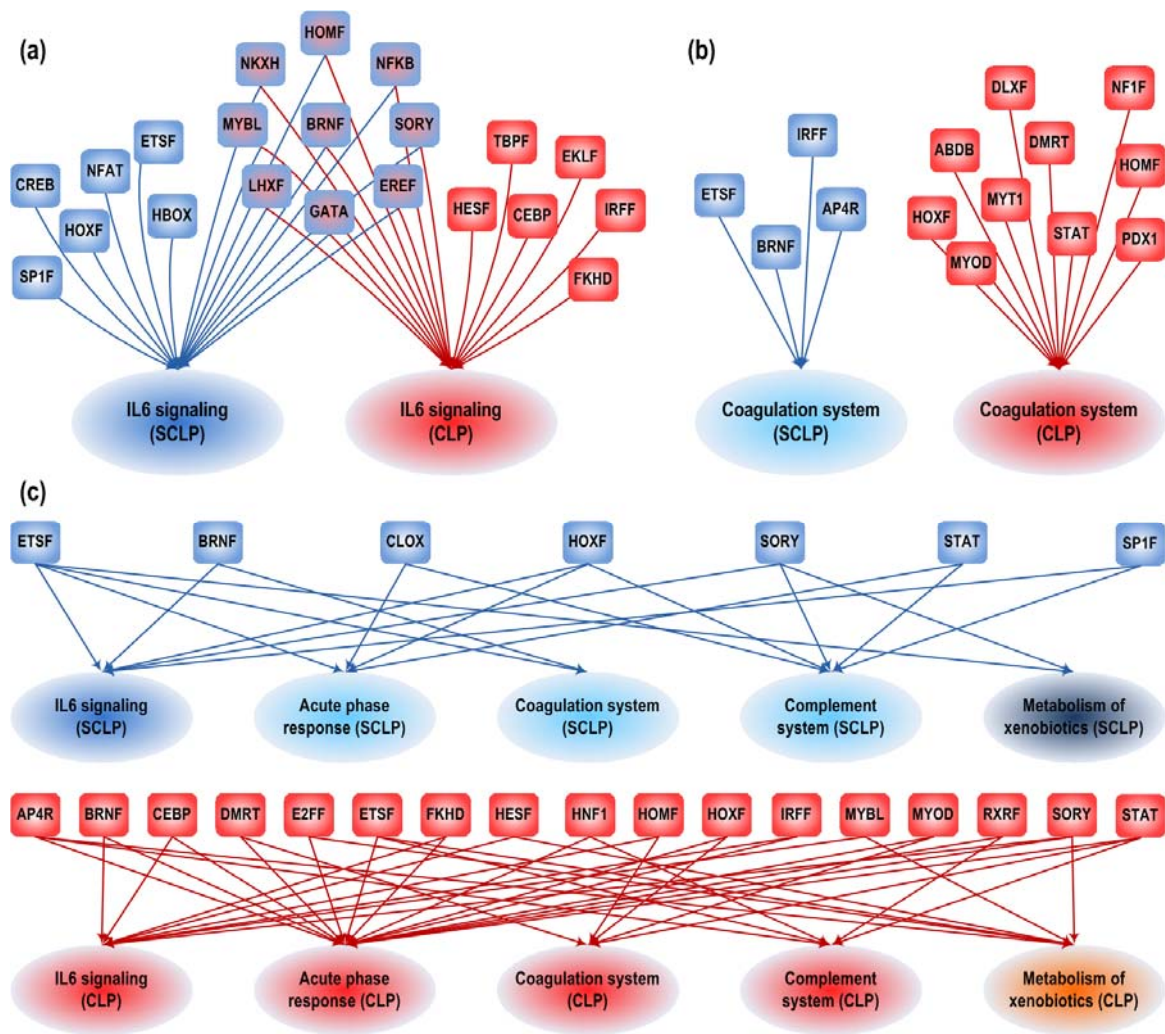


Figure 4.7 Injury- specific transcriptional regulation of inflammatory- relevant functions

Illustration of types of sharing common transcriptional regulators under two injuries through (a) IL6 signaling and (b) coagulation system; (c) Putative transcriptional regulation across five inflammatory-relevant functions in SCLP and CLP injury.

Figure 4.7b shows a graphical comparison of the transcription factor families involved in the coagulation system, which is an important functional group in P2. Unlike the IL-6 signaling pathway, there is no overlap between transcription factor families in SCLP and CLP. This indicates that the regulation of this function is entirely different between injury models, and suggests that while these two transcriptional responses appear to be functionally similar based

upon gene ontology, the underlying regulation that drives them is completely different. ETSF and BRNF are transcription factor families that are found in both IL-6 signaling and the coagulation system functional annotations in SCLP, despite the fact that they regulate the early response (P1) in IL-6 signaling, and the late response (P2) in the coagulation system. This indicates that the target specificity of these transcription factors is changing, perhaps due to the presence of co regulators, or altered dynamics. Well known transcription factor families such as STAT, NF1F, and HOXF are found in the coagulation system genes in CLP, but are not found in the IL-6 signaling pathway transcription factor families. Only HOMF is found in both functional groups in the CLP response, which indicates that despite the fact that the coagulation system and IL-6 signaling (P1 and P2) are co expressed in the same cluster, they are governed by different transcription factors, and thus regulatory changes to one system may occur independently of the other. Thus the response that is observed in CLP does not represent a cluster of co regulated genes, but rather multiple regulatory modules which ensure that these genes are co expressed, presumably to take advantage of the synergy inherent in simultaneous immune activation and anti bacterial acute phase protein production. This response has been characterized as P1 and P2, owing to the fact that in SCLP the two responses are split, and the putative transcription factor identification confirms this: the transcription factors that govern P1 responses are not the same as those that govern P2, making the observed transcriptional response the sum of two individual inflammatory responses that have been coupled for this specific injury.

In **Figure 4.7c**, the transcription factors which are common between at least two out of the five functional pathways are shown in SCLP and CLP. This figure shows transcription factors whose common regulatory features govern more than one process within the cell's transcriptional dynamics. The CLP functional groups contain significantly more transcription factors than the SCLP functional groups, which allow each transcription factor family associated to be associated with fewer functions in CLP. Though SCLP has fewer transcription factors, they are spread over

multiple functions. For example, BRNF is involved in IL-6 signaling (cluster 3), and the coagulation system (cluster 5). Since both of these clusters have significantly different expression patterns, it is impossible that the expression pattern of the ETSF transcription factor is able to match both of them simultaneously, and thus there must be further underlying regulation that modulates the effect that this transcription factor has. These alternate forms of regulation could potentially be transcriptional regulators, or histone modifications, that affect the transcriptional availability of the DNA and factor binding capacity(136). In contrast, the BRNF transcription factor found in the CLP analysis is involved in IL-6 signaling (cluster 1) and the acute phase response (cluster 1), and since both functions belong in the same cluster, the genes involved all have similar transcriptional dynamics. Thus, in CLP, it is more likely that BRNF is expressed in similar patterns to those observed in the gene clusters themselves, without the need for alternative regulation. Overall, the transcription factor analysis provides further evidence for the hypothesis that the pro inflammatory response observed in response to sepsis is not a singular event (P), but rather the sum of modular inflammatory responses (P1, P2) whose dynamics and ontology can be altered based in the type of injury sustained. This is observed in the gene clusters of SCLP, where the inflammatory response was directly split into an early and late phase, and can also be observed in the lack of overlap between transcription factors involved in the P1 and P2 responses in sepsis. Furthermore, the transcription factor differences between common functions in CLP and SCLP suggest that there may be different pro inflammatory regulatory architectures that can be applied to functional pathways in order to further control injury response.

4.5.Conclusions

The genes which had their expression patterns significantly altered following SCLP or CLP treatment compared to sham over time are identified and clustered respectively. Then groups of co-expressed genes in both SCLP and CLP are obtained by consensus clustering and individually

analyzed by pathway enrichment. Our results indicate that both CLP and SCLP induce the activation of a pro-inflammatory response that encompasses enhanced synthesis of acute-phase proteins, increased metabolism and tissue damage, though 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 factor or regulatory mechanism. These functional results were then integrated into networks which visually represent the process of systemic inflammation in both injuries. In order to further explore the underlying transcriptional dynamics that give rise to the different responses in these injuries, putative transcription factors were identified for select functions and compared between CLP and SCLP. Although there are some transcription factors that are common to both types of injuries, there are many others that are unique. In particular, there is no overlap between the transcription factors involved in the regulation of the complement cascade between the two injuries, suggesting that although the function is superficially similar, the regulatory dynamics are completely different, and may play an important role in defining different ways in which these acute phase proteins can be applied during inflammation for host defense. The representation of the responses as P1 and P2 reflects the underlying regulatory differences that are present, which are shown by the presence of unique and numerically plentiful CLP based transcription factors that drive the differences in the ontology and dynamics of the response compared to SCLP. This study

demonstrates how gene microarray techniques can be used to comprehensively study and compare gene expression profiles in rat surgeries for both aseptic and sepsis models, providing a molecular framework for future study on the pathophysiology of systemic inflammation. 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. This study may aid to find new research objectives and gene therapy strategies for surgery and infection induced inflammation.

CHAPTER 5

5. DYNAMICS OF LONG TERM GENE EXPRESSION PROFILING IN LIVER FOLLOWING CLP

5.1.Introduction

Although patient outcomes following sepsis have been improving over the past several years, the disease still poses a significant challenge in hospitals, and remains a major drain on time and care costs for patients (137). Despite the fact that better treatment options have decreased the fatality rates of severe sepsis cases, the incidence of the illness is increasing, and disturbing symptoms are being observed (104),(138). Though treatment options are improving, the lack of fundamental understanding of the pathophysiology of the disease prevents the discovery of drugs and techniques that can significantly relieve the burdens of treatment. A major difficulty in the treatment of sepsis is the fact that it develops in patients over an extended period of time, and is characterized by a slow wasting of body mass. This aspect of the disease represents a major clinical concern(139), and yet has not been characterized: this study aims at modeling sepsis in rats and studying the long term response with the goal of characterizing the impact that the disease has on the host over a prolonged time period.

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(140). However, recent studies have proposed the concept of a compensatory anti inflammatory response (141), 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(142), known as immunoparalysis, provides evidence that 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: the loss of this balance in turn determines the severity of the illness(143). Thus, our previous studies (144, 145), which capture the short term dynamics of the acute phase response, only represents one half of the story, and the long term response that follows it is equally significant in characterizing the pathophysiology of the condition.

A number of animal models have been proposed and developed in order to explore the response of mammals to sepsis, the most prominent among them being cecal ligation and puncture (CLP) in rodents(53, 105). The basic proposed mechanism for sepsis is that the phenomenon is driven by microbial infection caused by bacteria that translocate from the intestine into the peritoneal cavity, which are then picked up by mesenteric lymph nodes(146). Signals from these lymph nodes travel first to the lungs, and then to other organs, triggering a systemic inflammatory response via the innate immune system that can eventually lead to hypermetabolism in the liver, and eventually MODS. CLP is considered to be the gold standard for creating this condition in animals(147), which is accomplished by mimicking this effect by surgically performing a midline laparotomy, followed by the exteriorization of the caecum, ligation of the caecum distal to the ileocecal valve and punctures of the ligated caecum(105). The control for this procedure, known as Sham Cecal Ligation and Puncture (SCLP), involves the midline laparotomy and exteriorization of the caecum, but lacks ligation and puncture. Comparison of SCLP to CLP allows for the effects of the bacterial flora within the abdominal cavity to be observed in isolation from the inflammatory response from the procedure itself. Typical rodent responses to CLP include bacteremia, hypothermia, hypotension, and whole body hypermetabolic and catabolic

states(148). Though severe forms of CLP can be fatal to the animals, in this study, care was taken to ensure that the procedure had a 100% survival rate, in order to ensure that the animals survived long enough to provide long term data (and that variability in more severe cases were not overlooked due to animal death). The 100% survival rate of the animals has the unfortunate side effect of creating an injury that does not quite mimic clinical outcomes (where patient mortality is a factor), but instead allows for the identification of key points of activity in the response that appear to lead the host to a healthy resolution. These time points not only represent the healthy response to injury within the liver, but also represent the opportunity for potential dysfunction in that response, which may have been obscured by markers of the early onset of MODS that leads to mortality in the rat. Because hypermetabolism and acute phase protein production are critical responses during systemic inflammation, and because the liver is the primary organ responsible for both whole body metabolism and blood protein synthesis(149), the characterization of hepatic responses to injury is critical to the understanding of the dynamics and ontology of transcriptional changes following the induction of the inflammatory response, and in monitoring its effective resolution.

Genome-wide microarray technology has been already applied to characterize changes in transcriptional dynamics in the liver following CLP (58, 63, 65). However, by focusing on a single time point (24 hours post-CLP) (63, 65), or by analyzing gene ontologies without considering the time scale of gene expression change(58), these studies do not capture the dynamics of the system, and therefore cannot answer questions regarding the onset, duration and resolution of the response. By measuring sequential time points over a long period of study, it is possible to capture the dynamic activity of genes which continue to affect the animal following exposure to the injury. These genes represent deviations from the healthy state of the animal, and thus, the type, length and severity of their change is equally important as their functional characteristics.

While we recently examined short term responses to CLP (144) and burn (145), our goal in this study is to characterize the long term hepatic gene expression dynamics of rats subject to CLP in order to characterize the impact of sepsis upon liver function over an 8 day time period. Because the dangers represented by sepsis in a clinical setting are present in both the long and short term(139), it is critical to characterize the response of the liver over periods that extend beyond 24 hours. Many previous studies have investigated the dynamics of the acute phase response following CLP(150-152), and its impact on survival. However, long term studies following the injury have been limited to survival rates(153), or effects of surviving the trauma and sepsis, such as cognitive impairment(154). By analyzing the ontology and dynamics of gene expression following CLP over 8 days, it is possible to observe the underlying mechanics that drive the long term response, which can potentially identify both the characteristics and dynamics of the compensatory anti inflammatory response that is expected to manifest following the acute phase response early in the timescale. Though it is possible that the host's response to the infection could persist for weeks, we only consider the first 8 days for the purpose of identifying the dynamics of the host response to infection once the acute pro inflammatory phase has passed. This response is critical, as it has been previously shown that clinical drugs that positively impact patient outcome all share the ability to ameliorate the long term anti inflammatory response within patients, as opposed to the short term acute phase response(155). Our findings indicate that both CLP and SCLP show dynamics consistent with this two wave model of sepsis, however, each condition has unique dynamics that indicate fundamental differences in the response. Furthermore, the gene ontologies suggest a link to oxidative stress over the long term that may be able to be explored for clinical benefit.

5.2. Materials and Methods

5.2.1. Animal Model

Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing between 150 and 200 g were used. 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.

The infection was induced by applying CLP treatment. Rats were first anesthetized, and then the analgesic buprenorphine (0.01 to 0.05 mg/kg) and Bupivacaine (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 for 4 times (not through and through) 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. SCLP consist of animals treated identically without receiving cecal ligation and puncture. Rats were single caged after the treatments and given standard rat chow and water ad libitum until sacrifice. Animals were sacrificed at 9am in each group on days 0, 1, 2, 5 and 8 post injury and liver tissues were collected and flash frozen for offline 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 probe sets.

5.2.2. Data Analysis

Analysis of CLP and SCLP gene expression data includes normalization, filtering, combining the datasets and clustering which is depicted in **Figure 5.1**. 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 (106). 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” (74). 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. We then transformed the data into principal components, which were plotted in a 2 dimensional plot showing the first and second principal components (PC1 and PC2), which account for 65% of the total data.

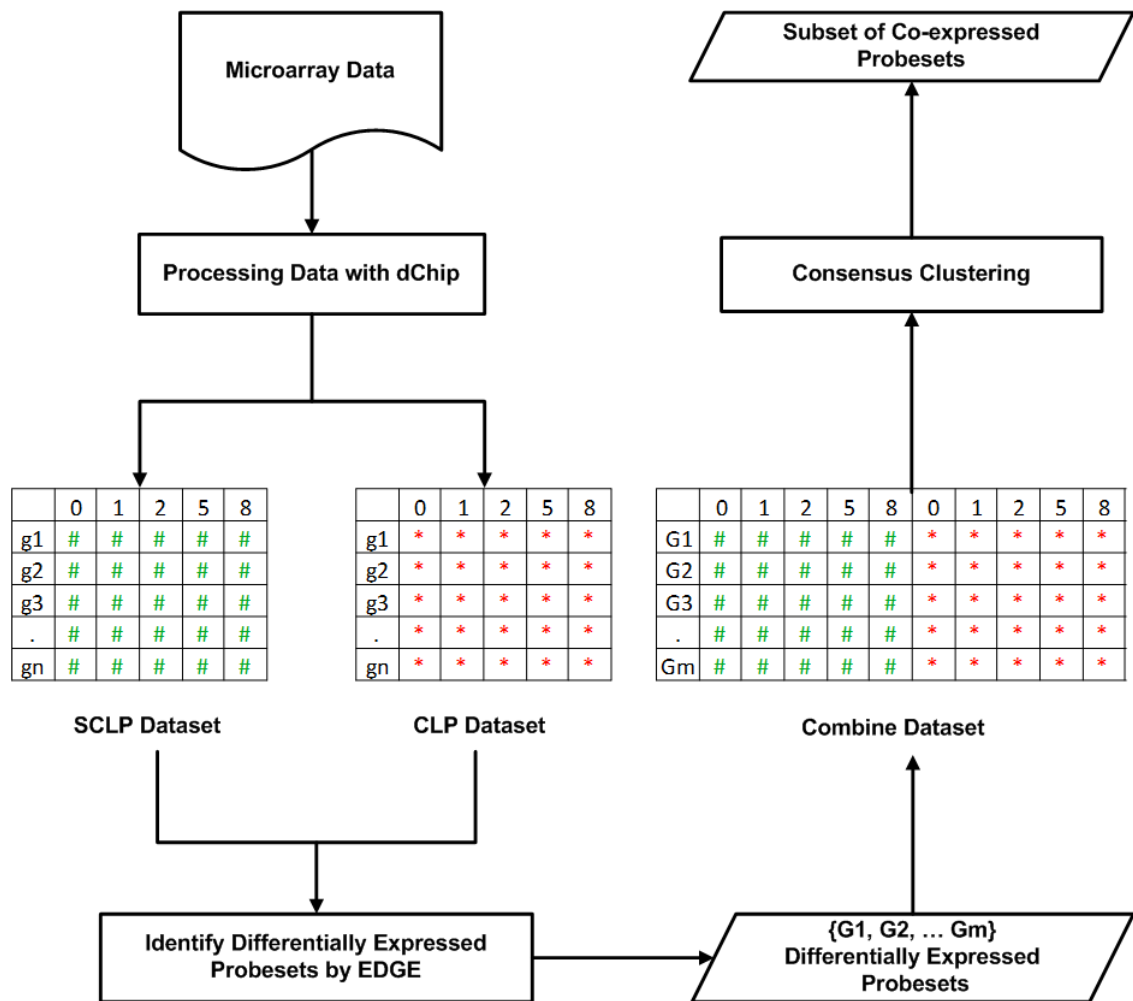


Figure 5.1 Schematic overview of the microarray data analysis

Microarray data was preprocessed by using dChip. Then, two data sets corresponding to SCLP and CLP groups respectively, were analyzed to identify the differentially expressed probesets by using EDGE with ‘between classes’ option under the statistical threshold $q < 0.001$, $p < 0.001$. Finally, the data sets corresponding to those differentially expressed probesets in SCLP and CLP groups were combined to form one single matrix, which was then clustered using the approach of “consensus clustering” with threshold $p < 0.01$.

5.3.Results

5.3.1. Identification of CLP Related Patterns and Characterization of Cluster Function

Hepatic gene expression levels were measured at 0, 1, 2, 5 and 8 days 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 above. 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, which allowed for the identification of three major motifs within the differentially expressed gene set, with the clusters containing 152, 200, and 56 probe sets respectively. The genes contained in each of the clusters, as well as the average cluster patterns, are shown in **Figure 5.2**, 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, it was possible to characterize the function and dynamics of each cluster. A summary of the differences in each can be found in **Table 5.1**, while a detailed description of the dynamics of each cluster, and the genes used to characterize their function can be found below:

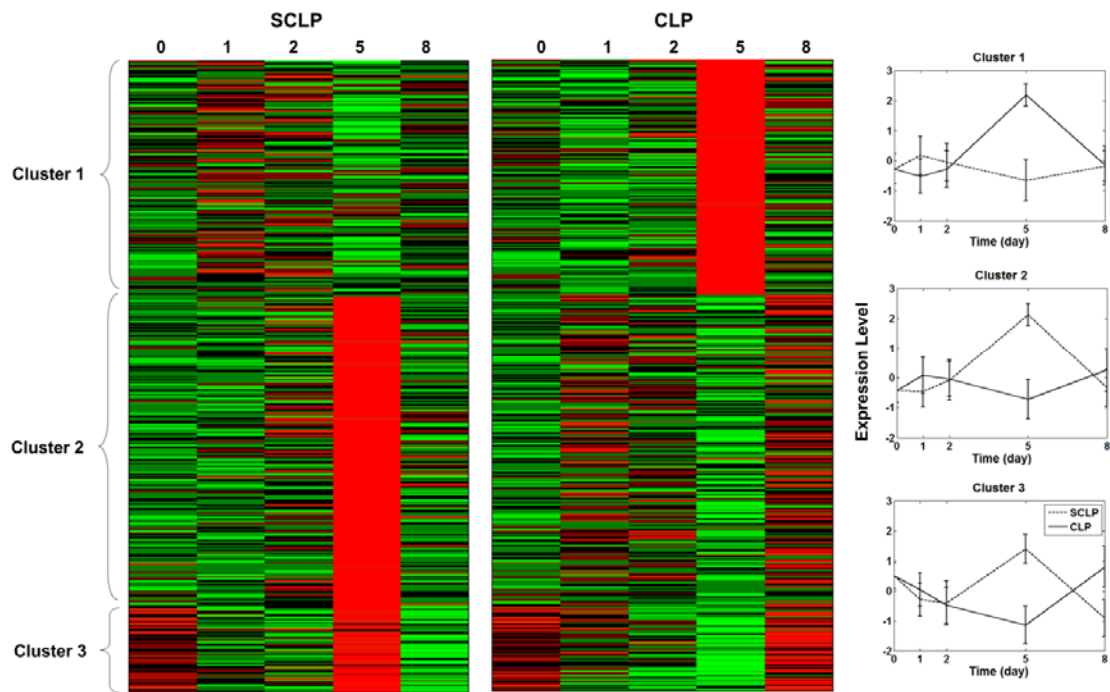


Figure 5.2 Gene expression profiles of rat livers in response to sham or SCLP injury

Left Panel, expressions of 152, 200 and 56 probesets in 3 clusters in CLP rats compared to SCLP rats before (day 0) and at days 1, 2, 5 and 8 days post injury

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

Table 5.1 Comparison of functional transcription clusters following CLP and SCLP

	Cluster 1	Cluster 2	Cluster 3
# of Probes	152	200	56
CLP Dynamics	Up early, suppressed late	Suppressed early, up late	Suppressed
SCLP Dynamics	Suppressed early, up late	Up early, suppressed late	Suppressed early, up late
Characterized Function	Anti Inflammatory, Anti oxidative	Pro inflammatory, Oxidative	Pro inflammatory
Specific Pathways	Pentose Phosphate Pathway (2)	NF-KB Signaling (3)	Mitochondrial Fatty Acid/Amino Acid Metabolism (2)
	Insulin Signaling (3)	Cell Cycle Regulation (2)	DNA Repair (1)
	MapK Signaling (2)	HIF-1 α Signaling (2)	Toll Like Receptor Signaling (2)
	Fatty Acid Metabolism (1)	Amino Acid Degradation (4)	Nitrogen Metabolism (1)
	Calcium Signaling (4)	ERK Signaling Pathway (3)	Anti Oxidant Production (3)
	Innate Immunity (2)	Cell Cycle/Gap Junction Regulation (3)	

- 7) Cluster 1 is comprised of 152 probes which were clustered into a single motif, shown in **Figure 5.2**. 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 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.

- 8) Cluster 2 is comprised of 200 probes which were clustered into a single motif, shown in **Figure 5.2**. 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 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-KB signaling (*Ntrk2, Bmpr2, Tnf*), Cell cycle regulation (*Hdac7, Hdac10*), HIF-1a 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.
- 9) Cluster 3 is comprised of 56 probes which were clustered into a single motif, shown in **Figure 5.2**. 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 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(156), 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(157). 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.

5.3.2. Characterization of Response Dynamics

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 5.3** 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 5.3** 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.

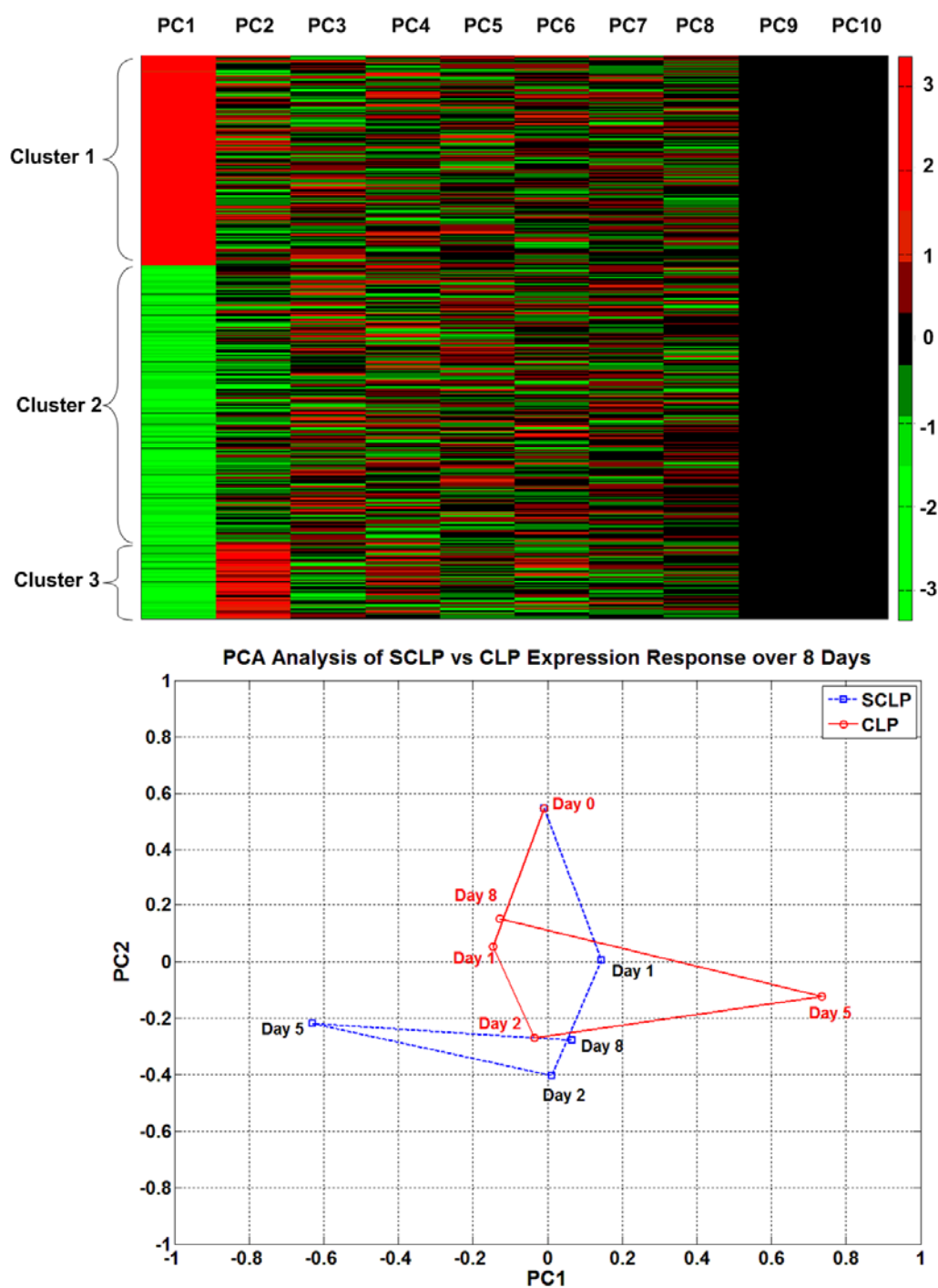


Figure 5.3 Principal component analysis of the clustered genes in SCLP and CLP

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 65.0078% of the information dataset, where red represents CLP, and blue represents SCLP.

5.4.Discussion

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. Although critical aspects of the individual responses reflect common elements of the response to an inflammatory stimulus (surgery), there are significant differences that manifest in the three clusters that indicate that both injuries have unique dynamics over the long term related to anti inflammatory and pro inflammatory genes, coupled with oxidative stress biomarkers.

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(158). Insulin acts as an anabolic hormone, stimulating lipogenesis, glycogen and protein synthesis in the cell in order to promote the storage of excess metabolites(98). 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 compensate 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 (159), 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(160). 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(161), and thus the early suppression of proteins related to calcium signaling but not sepsis (such as *Tpm1*, which encodes a critical actin skeletal protein(162)) 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-KB signaling pathway, the cell cycle, HIF-1A and the degradation of various amino acids. Interestingly, the NF-KB 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 signaling pathway, which include *Tnf* among others, act in an antagonistic manner with those in Cluster 1, where the Cluster 2 genes promote the activation of an inflammatory response, while the Cluster 1 genes divert the signal toward an anti-inflammatory response. NF-KB signaling has been well characterized as a pro-inflammatory response(163), which indicates a stronger early response in CLP compared to the lesser 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(164), 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-1a signaling within Cluster 2 is a phenomenon which links the presence of stronger pro-inflammatory signals to markers of oxidative stress. HIF-1a is a transcription factor family which is well known for its response to oxidative stress(165). Since septic conditions have been shown to produce increased levels of reactive oxygen species in the liver(166), this transcription factor is potentially 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. Protein synthesis in the liver has been reported to increase by over 160% following CLP (124), therefore the maintenance of amino acid supplies are critical to support increased production. Since CLP appears to be up-regulating hyper-metabolic genes that degrade certain selenoamino acids, along with lysine, the corresponding degradation of peripheral tissue must be greater 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(53). 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(167). 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-1a, 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(168), 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(169). 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 TLR 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(170). 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.

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 a primarily anti inflammatory response which includes anti oxidant activity and macromolecule biosynthesis, its early suppression indicates the onset of an acute phase response, which resolves by Day 2, whereby 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(142), 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(171). 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-KB signaling pathway, HIF-1A 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 laparotomy incision 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 partially 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 lower panel of **Figure 5.3**). 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 5.3** 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(172): 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(141), 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-1a and glutathione transferase) that feedback mechanisms exist which modulate the oxidative stress levels in order to drive the switch to an anti inflammatory mechanism. Oxidative stress has been shown previously to affect gene expression and alter regulatory dynamics(173), 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(174), 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 our previous short term study(144), we were able to identify functions that were characteristic of the acute phase response to both CLP and SCLP. Interestingly, 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. 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, we were 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.

5.5. Conclusions

In this study, the long term response of the rat following CLP induced sepsis was studied over a period of 8 days following injury. By identifying differentially expressed genes, and using consensus clustering methods, three major profiles were isolated 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 1 and 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. By taking advantage of the fact that the pro and anti inflammatory responses following bacterial infection appear to be linked to the production of reactive oxygen species, and that these dynamics have distinct cross over points, it may be possible to design therapeutic interventions that target these thresholds and maintain an anti inflammatory response.

CHAPTER 6

6. PRIMING EFFECTS OF BURN INJURY ON CECAL LIGATION AND PUNCTURE IN RATS

6.1.Introduction

The mammalian response following many different injuries revolves around the innate immune system, which triggers an inflammatory response to both resolve any foreign pathogens within the host and promote wound healing. It has been well established in clinical settings that multiple organ failure is a major cause of death in many hospital admissions(39), and that incidents of sepsis that occur after the initial injury are a major contributing factor(175). Animal models that utilize burn injuries followed by sepsis have been used to mimic this clinical phenomenon, since burn injuries are known to lead to immunosuppression that increases susceptibility to polymicrobial sepsis(176, 177). The burn injury causes significant disruption of the host's defense to sepsis, including drastic depletion of CD4+ T-cells(178), disruption of wound healing coagulation cascades(179), and massively increased vasodilation following sepsis(180). Furthermore, peritoneal mast cell function does not determine the severity of the response(181), indicating that the pathophysiology associated with sepsis occurs on a systemic level. Mechanistically, it is believed that burn induces an inflammatory response, which is then followed by an anti inflammatory, wound healing response that is dominant when the septic insult occurs(141). It has been hypothesized that the imbalance in these responses that shifts the host toward an anti inflammatory state is what leads to pathophysiological sepsis(142), however, in order to address the underlying pathophysiology, it is necessary to map out the ways in which the burn injury interacts with the septic response on a systemic level.

According to the ‘two hit’ theory (39), major trauma (such as burn injury) increase the production of proinflammatory mediators, primarily 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. It is hypothesized 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(129). The liver represents an intersection of innate immune responses and metabolic shifts(8), making it a useful organ to characterize the systemic response of an immune compromised host. Metabolic analysis is critical towards understanding the nature of the double hit response, as severe hyper metabolism has long been clinically associated with the severity of the septic response(182). Rodents can therefore be subject to burn injuries in order to elicit an inflammatory response, and then further perturbed using cecal ligation and puncture(147) (CLP) to directly compare the effects of burn injury on the host response to infection over the course of a week following injury. Though previous studies have used CLP to induce a septic inflammatory response within rodents(148), a comparison of the states of double hit animals to their single injury counterparts allows for an analysis of the ways in which injuries interact in *in vivo* systems to produce unique and often more severe responses.

Due to the complexity and diversity of the phenotypical responses that occur within the liver following injury, it is necessary to employ tools that are capable of assessing the systemic response in an efficient manner. Functional genomics allows for the characterization of a wide subset of transcriptional responses, which can elucidate changes in multiple pathways, ranging from fatty acid metabolism to innate immune protein synthesis. Since the liver is simultaneously managing pro inflammatory signaling functions and metabolic changes, it is critical to the understanding of the response that these responses be characterized in parallel. A technique known as SAM (Significant Analysis of Microarrays) was utilized(183) to determine a set of

significantly varying genes following injury. This method uses multiple gene specific t-tests to verify the significance of the fold change of the double hit injury relative to sepsis alone, followed by a false discovery rate test, which aims to exclude genes whose variations are the result of random chance. However, SAM can only identify significantly altered genes, but cannot assess whether an up regulated gene is no longer being suppressed, or is instead seeing a spike in transcription. In order to assess the dynamics of the system, consensus clustering was used on genes that were identified using EDGE to be differentially expressed in either BCLP or CLP. Ingenuity Pathway Analysis is then used to functionally characterize the resulting genes, in order to identify relevant pathways that change during a double hit stimulus.

This study aims to characterize the effect of burn priming on critical liver functions in a septic injury, by comparing the dynamics and ontologies of transcriptional genes that were altered between the two conditions. As the liver is a metabolically active organ that also participates in immune responses, our ontologies focused on critical functions in both of these areas in order to reconcile metabolic and immune dysfunctions that occur in a clinical setting.

6.2.Materials and Methods

6.2.1. Animal Model

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.

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 (66). This model was chosen because it has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant alterations on feeding patterns (52). 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 (69). 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.

Septic infection was induced by applying CLP treatment 48 hours post burn treatment. Rats were first anesthetized, and then the analgesic buprenorphine (0.01 to 0.05 mg/kg) and Bupivacaine (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. We took care to not 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 (not through and through) 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.

Rats were single caged after the treatments and given standard rat chow and water ad libitum until sacrifice. Animals are sacrificed (starting at 9am) at different time points (2, 4, 8, 12, 16, 20, 24, 48, 120 and 192 hr post-treatment) 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 probe sets.

6.2.2. RT-PCR Analysis

qPCR gene expression studies were performed using TaqMan gene expression assays. Genes were selected from the 2 hour (*Slc1a4*, *Angptl4*), 4 hour (*Pcolce*, *Nfkb1a*) and 120 hour (*Stam2*, *G6pd*) time points, due to the fact that these particular points have significantly increased altered expression levels in BCLP. Genes were selected that were both up regulated and down regulated at each time point. The level of GAPDH was used as an internal reference, and all fold changes are displayed as comparisons between BCLP and SCLP levels of the gene.

6.2.3. Data Analysis

Genomic expression data analysis was done through pairwise comparisons between CLP and SCLP groups at each time point. DNA chip analyzer (dChip) software (70) was used with invariant-set normalization and perfect match (PM) model to generate expression values. 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(183). This method compares the gene expression of the two response variables, BCLP and CLP. Briefly, by computing a statistic g_i for each gene i , the strength of the relationship between the response variable (BCLP) and the standard (CLP) 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. In order to observe the temporal dynamics associated with the SAM results, the data sets corresponding to BCLP vs. SCLP groups were investigated to identify the temporally and differentially expressed probesets over time for either of the two conditions, or both, by applying EDGE for each gene (106). The significance threshold for this test was set as q-value <0.001 and p-value <0.001. Genes that passed the threshold for differential expression in either BCLP or SCLP were clustered using the approach of “consensus clustering” (74), which provided three data sets: clusters that were differentially expressed in SCLP only, BCLP only, and both SCLP and BCLP. 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 the pathway enrichment function ($p < 0.05$) in Ingenuity Pathway Analysis tools (Ingenuity Systems, Mountain View, CA) as well as analyzing the functions of individual genes extensively. The same analysis was performed for the clusters identified using consensus clustering, and each of the identified motifs was assigned a function: in order to simplify the motifs, representative clusters were selected that had distinct, recurring dynamics, and functional ontologies related to the CLP response. 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 (184).

6.3.Results

6.3.1. Identification Of BCLP Related Patterns And Characterization Of Per Day Changes

Liver gene expression levels were measured 2, 4, 8, 12, 16, 20, 24, 48, 120 and 192 hours post-treatment in the livers of rats following BCLP and SCLP treatment, which in turn was performed 2 days following the burn treatment. Genes that had expression changes in BCLP, when compared to SCLP, were identified at each of those time points using the methods outlined above. It should be noted that at 16, 20, 48, and 168 hours post injury, no probe sets could be significantly identified, indicating that the BCLP condition had statistically identical gene expression to the SCLP condition for those time points. Summarized below are the most relevant pathways related to innate immunity and metabolism in the liver following the double hit injury. **Figure 6.1** graphically displays a timeline of gene activation for both metabolic and innate immune functions: details of individual genes shown there are given below.

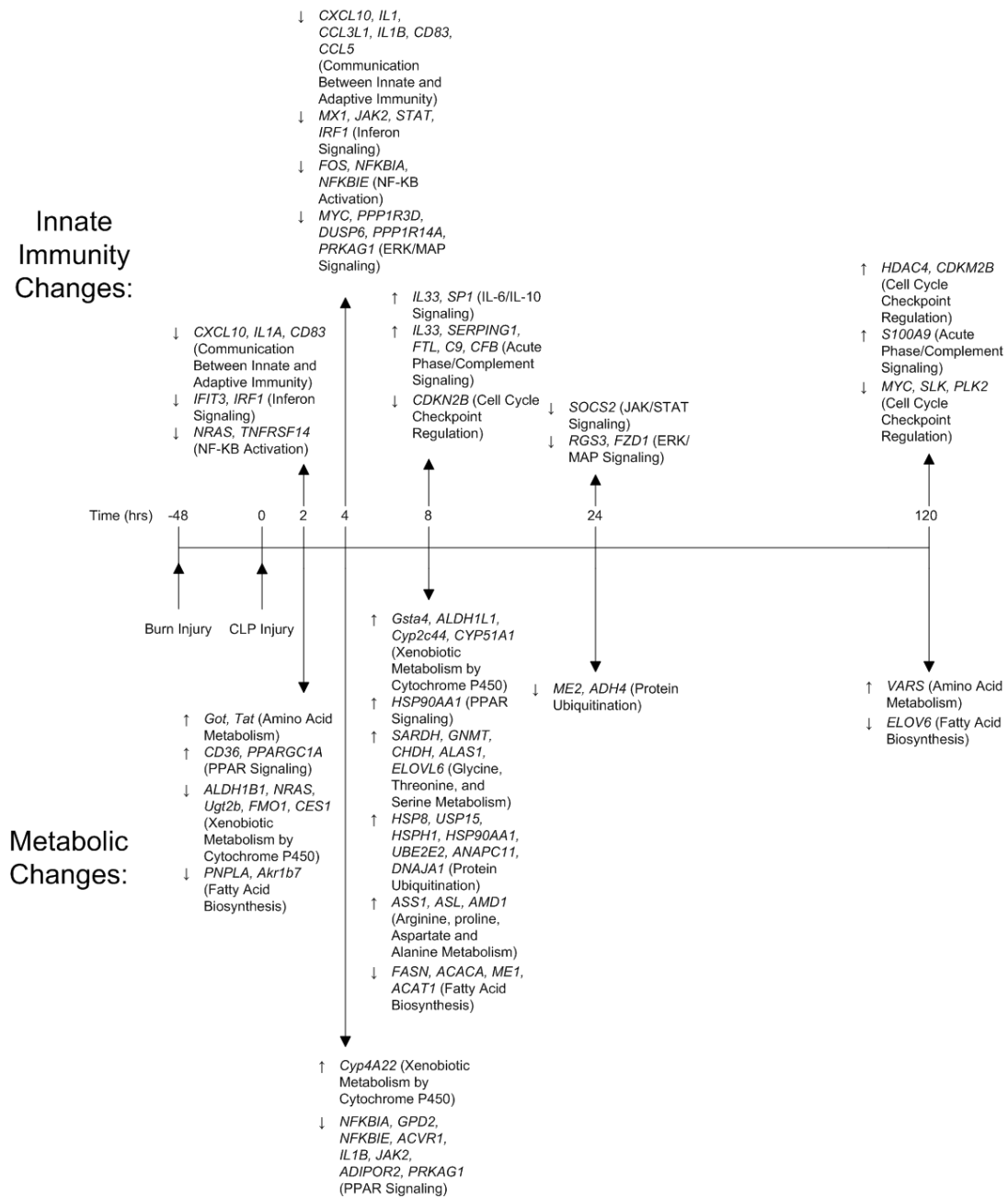


Figure 6.1A time line displaying when significant genes were altered following the double hits injury

Genes above the line are related to innate immune functions, while genes below the line are related to metabolic changes. Up and down arrows represent the respective fold change of the

genes, and the IPA pathways for each group of genes is given in brackets. Time points with no significant gene expression change are omitted from the timeline.

The identified genes from 2 hours post injury consist of 199 probes which passed the SAM cutoff, 45 of which were up regulated and 154 were down regulated. The up regulated genes associated with this time point consist of functions related to Amino Acid metabolism (*Got1, Tat*), Circadian rhythm dynamics (*Per1, Cry2*) and PPAR signaling (*CD36, PPARGC1A*). The down regulated genes at this time point are related to a variety of functions, including Xenobiotic metabolism (*ALDH1B1, NRAS, Ugt2b, FMO1, CES1*), communication between adaptive and innate immunity (*CXCL10, IL1A, CD83*), Interferon Signaling (*IFIT3, IRF1*), NF-KB activation (*NRAS, TNFRSF14*), and glycerolipid metabolism (*PNPLA3, Akr1b7*). The changes due to burn at this time point contain up regulated amino acid degradation and peroxisome production, but down regulated innate immune function and biosynthesis.

The identified genes from 4 hours post injury consist of 435 probes which passed the SAM cutoff, 9 of which were up regulated and 426 were down regulated. There is only one up regulated gene which was identified by pathway analysis, and *Cyp4A22* is associated with metabolism through cytochrome P450. The down regulated genes at this timepoint are related to a variety of functions, including Interferon Signaling (*MX1, JAK2, STAT1, IRF1*), TNFR1 signaling (*FOS, NFKBIA, NFKBIE*), ERK/MAPK signaling (*MYC, PPP1R3D, DUSP6, PPP1R14A, PRKAG1*), IL-17 signaling (*CXCL10, FOS, CCL5, CXCL11*), communication between adaptive and innate immune cells (*CXCL10, IL1, CCL3L1/CCL3L3, IL1B, CD83, CCL5*), IL-6 signaling (*FOS, IL1A, NFKBIA, NFKBIE, IL1B, JAK2*), IL-10 signaling (*FOS, IL1A, NFKBIA, NFKBIE, IL1B*), acute phase response signaling (*FOS, IL1A, SOD2, NFKBIA, NFKBIE, IL1B, JAK2*), PPAR signaling (*NFKBIA, GPD2, NFKBIE, ACVR1, IL1B, JAK2, ADIPOR2, PRKAG1*), glycolysis/gluconeogenesis (*ALDH1B1, HK2, PKLR, PFKM*), pyruvate

metabolism (*ALDH1B1*, *PKLR*, *ACAT1*, *ACACA*, *ME1*), and propanoate metabolism (*ALDH1B1*, *ACAT1*, *ACACA*). The changes due to burn at this time point contain up regulated cytochrome P450 metabolism, but heavily down regulated innate immune signaling, and central carbon metabolism.

The identified genes from 8 hours post injury consist of 97 probes which passed the SAM cutoff, 82 of which were up regulated and 15 were down regulated. The up regulated genes associated with this time point consist of functions related to glycine, threonine, and serine metabolism (*SARDH*, *GNMT*, *CHDH*, *ALAS1*, *ELOVL6*), protein ubiquitination (*HSPA8*, *USP15*, *HSPH1*, *HSP90AA1*, *UBE2E2*, *ANAPC11*, *DNAJA1*), acute phase response signaling including the complement system (*IL33*, *SERPING1*, *FTL*, *C9*, *CFB*), arginine, proline, aspartate and alanine metabolism (*ASS1*, *ASL*, *AMD1*), xenobiotic metabolism (*Gsta4*, *ALDH1L1*, *Cyp2c44*, *CYP51A1*), nicotine and nicotinamide metabolism (*HIPK1*, *SACM1L*), .IL-10 signaling (*IL33*, *SP1*), and PPAR signaling (*HSP90AA1*). The down regulated genes in this time point are associated with ketone body metabolism (*ACAT1*) and anti proliferative cell cycle check point regulation (*CDKN1B*). The changes at this time point contain heavily up regulated immune system signaling, particularly within the innate immune system, as well as amino acid based nitrogen metabolism and potential markers of oxidative stress, along with down regulated cell cycle inhibition (promoting proliferation), and down regulated ketone body metabolism.

The identified genes from 12 hours post injury consist of 21 probes which passed the SAM cutoff, 10 of which were up regulated and 11 were down regulated. The up regulated genes associated with this time point consist of functions related to metabolism of xenobiotics by cytochrome P450 (*ADH1C*, *CYP2B6*), Tyrosine and Tryptophan metabolism (*AOX1*), arginine and proline metabolism (*PRODH*) and RXR activation (*CYP2B6*, *CD36*). The down regulated genes at this time point are related to the Nrf-2 mediated oxidative stress response (*SOD2*, *Gstm3*), JAK/STAT

signaling (*CISH*) and antigen presenting pathways (*HLA-G*). Overall, the changes occurring at this time point are slight, but consist of an up regulation of amino acid degradation and the disposal of bacterial and oxidized products, as well as a down regulation of anti oxidative responses, adaptive immunity, and inhibitors of cytokine signaling.

The identified genes from 24 hours post injury consist of 35 probes which passed the SAM cutoff, all of which were down regulated. All of the genes at this time point were down regulated, and are associated with pyruvate metabolism (*ME2,ADH4*), interconversions between C6 and C5 sugars (*ADH4*), inhibition of cytokine signaling via the JAK/STAT pathway (*SOCS2*), and the down regulation of G-protein function (*RGS3,FZD1*). The changes that occur at this time point as a result of the burn priming are the down regulation of parts of central carbon metabolism, increased cytokine sensitivity, and increases MAPK activation through the down regulation of G-protein inhibitors.

The identified genes from 120 hours post injury consist of 180 probes which passed the SAM cutoff, 107 of which were up regulated and 73 were down regulated. The up regulated genes associated with this time point consist of functions related to cell cycle checkpoint genes (*HDAC4,CDKN2B*), valine, leucine and isoleucine biosynthesis (*VAR5*) and the up regulation of anti bacterial components (*S100A9*). Changes at this time point also include the down regulation of fatty acid biosynthesis (*FASN,ACACA, ME1, ELOV6*), and apoptosis inhibitors (*MYC*), and mitosis (*SLK,PLK2*). The changes occurring at this long range time point are primary associated with mild anti bacterial activity, the biosynthesis of presumably depleted amino acids, the suppression of fatty acid biosynthesis, and the suppression of wound healing.

6.3.2. RT-PCR Confirmation of Expression Levels

Select genes were chosen from the list which passed both the SAMS tests, and the false positive tests, in order to more accurately verify the results from the microarray. Genes were selected from

three time points (2, 4 and 168 hours), one for up regulation and down regulation, and the results are shown in **Table 6.1**. Though the magnitudes of the fold changes differ for some genes between the RT-PCR and the microarray, others show surprising fold change accuracy, and in all cases the trend of up or down regulation remain the same.

Table 6.1 RT-PCR confirmation of Affymetrix gene expression folds change

Gene Symbol	Gene Name	Mean Fold Change	
		Affymetrix	RT-PCR
Slc1a4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2.69	3.47
Angptl4	Angiopoietin-like 4	0.3347	0.36
Pcolce	Procollagen C-endopeptidase enhancer	3.05	6.43
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.49	0.77
Stam2	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	2.49	1.07
G6pd	Glucose-6-phosphate dehydrogenase	0.42	0.54

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.

6.3.3. Identification of Critical Gene Expression Motifs Between BCLP and SCLP Conditions

Genes that were differentially expressed in at least one condition were identified and then clustered into motifs. There were 30 total unique gene expression motifs that were identified.

Clusters were then subject to functional annotation through Ingenuity Pathway Analysis, and were compared to the SAM results, described above. Many of the motifs were extremely similar in dynamics, and thus 5 gene expression motifs were selected that were representative of major patterns found amongst the clusters, and contained gene ontologies closely related to the genes found in the SAM statistical analysis. These expression motifs can be found in **Figure 6.2**, and have been labeled according to their functional ontologies.

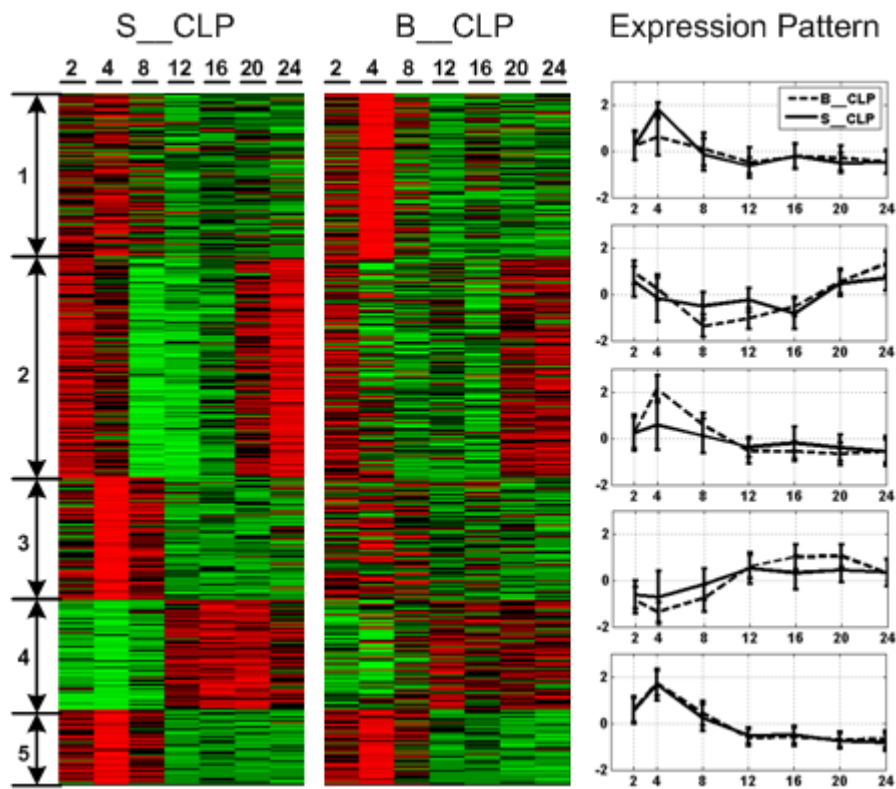


Figure 6.2 Gene expression profiles of rat liver in response to S_CLP and B_CLP injury

Left Panel, expressions of 269, 347, 197, 173, and 120 probesets in 5 clusters in S_CLP rats and B_CLP rats at 2, 4, 8, 12, 16, 20, 24 h post-treatment are exhibited in a heatmap.

Right Panel, the expression patterns are shown by plotting the average normalized (z-score) expression values of 269, 347, 197, 173, and 120 probesets in 5 clusters in S_CLP and B_CLP groups (displayed as the means $\pm$ SEM).

6.4. Discussion

Though the response of the innate immune system to septic threats is thought to always involve a pro inflammatory followed by an anti inflammatory phase to respectively cleanse the host of infection and heal the damage caused by both the bacteria and the immune cells(142), evidence has begun to emerge in recent years that the severity of the inflammatory response is highly dependent on the status of the host at the time of injury(185). Coupled with the fact that septic outcomes radically differ between patients(186), it is necessary to understand the ways in which prior injuries that alter the state of the innate immune system, such as burn(147), can influence the progression of the inflammatory response during sepsis. In this study, we utilized thermal injury to induce an immunosuppressed state(147), and assessed the effects that this prior condition had on a septic insult. Since the CLP injury, even considering burn priming, is non fatal, it is also possible to observe general trends in the trajectories of the recovery of the animals following these two distinct injury types.

In addition to statistical methods for assessing the accuracy of the microarray gene expression results, gene clustering, along with a small scale RT-PCR experiment was conducted. As seen in **Table 6.1**, the trends that are observed from RT-PCR match those from the microarray, and some of the genes even have matching magnitudes of fold change. It is worth noting that this subset of genes includes important metabolic enzymes, as well as inflammatory signaling proteins, indicating that the trends observed for both of these broad functions are the result of real mRNA differences. Furthermore, these genes were selected from time points where the changes in gene expression are numerous between BCLP and SCLP, in order to assess the accuracy of the microarrays at these critical time points. **Figure 6.2** represents critical motifs discovered through clustering techniques: these confirm the significant amount of activity that occurs at the early time points in the first 24 hours following CLP. Furthermore, the activities of the clusters indicate

that though the SAM comparison between BCLP and SCLP is able to identify functionally important genes at specific time points, the fact that certain time points identify no genes does not equate to zero activity within the liver. These motifs display strong temporal activities in response to CLP that show varying degrees of sensitivity to the burn priming event. Despite these positive results, the study is limited by the fact that gene expression is being studied in whole liver samples, as opposed to specific cell types. Though this blends the transcriptional responses from multiple cell types (including hepatocytes and Kupffer cells), the general trends for liver function are still valid, and in particular, represent a mammalian *in vivo* tissue response to injury.

6.4.1. The Effect of Burn Priming on Immune Function

In our previous work (187), we identified key motifs in the liver with respect to the short term response of rodents to cecal ligation and puncture when compared to uninjured animals. Within this study, we identified a predominantly pro inflammatory cluster, which contained multiple cytokines, cytokine signals, complement, and coagulation proteins, all of which were consistently upregulated during the entire 24 hour period. Because our current study used cecal ligation and puncture without a burn injury as a baseline, it was necessary to employ clustering techniques in order to identify the background inflammatory response that may not have been varied by the burn priming injury. This can be seen clearly in **Figure 6.2**, where all of the motifs show identical expression patterns in each condition at time points where SAM does not identify differentially expressed genes, and some even are identical throughout the timecourse, indicating that some expression motifs are entirely unaffected by the priming event.

The burn priming causes a partial disruption of immune functions over the early time course of the injury, which may be an indication of an impaired response that is proportional to the severity of the insult. Over the first 4 hours of the injury, many of the immune regulated genes are suppressed under BCLP compared to SCLP. These genes are primarily involved in cytokine

production, NF-KB signaling, and innate and adaptive communication through chemokines and other motifs. The suppression appears to be significantly stronger at the four hour time point, with critical inflammatory genes including *IL1*, *STAT1*, and *JAK2* being suppressed after thermal priming. Interestingly, the following 4 hours post injury (corresponding to the 8 hour time point) show significant up regulation of the immune system, which is in significant contrast to the first four hours post injury. Within this time point, several cytokines are up regulated, including IL-33, a known Th2 cell attractor(188). Furthermore, this time point includes complement proteins, indicating that the pro inflammatory behavior at this time point extends to the production of anti bacterial acute phase proteins, unlike the suppression of inflammatory mediators observed previously. The gene expression motifs in **Figure 6.2** that best capture this phenomenon are the related to MAP kinase and NF-KB signaling where immune signaling spikes up under the SCLP condition, but is suppressed when the animal is primed with burn injuries and Complement and Gap Junction Protein production, where there is significant early suppression in the SCLP condition that is reversed during burn priming. When compared to the final gene expression motif, which is related to cytokine and growth factor signaling, it appears that many other innate immune related genes remain unaffected by the burn priming, and that these specific functions may be more sensitive to influence from prior injuries. Following the 8 hour time point, very few of the other time points contain relevant immune function, indicating that, during the short term response, the priming effect of burn is most prevalent over the first 8 hours post sepsis, with the response returning back to the non primed baseline over the remainder of the 24 hour period.

When compared to our previous short term study, which shows that CLP alone maintains significant, and consistent, up regulation of immune mediators throughout the timescale, the BCLP condition shows significant differences in a subset of the genes, which appear to become deregulated from the baseline CLP response, but eventually return back to that baseline, and eventually to a recovery profile. This may indicate that unlike our previous study, wherein

different inflammatory stimuli created entirely different expression patterns, prior injury does not create an entirely novel response, but rather impairs the liver from following an appropriate response path, which in this case results in the dysregulation of a subset of the inflammatory response. The early suppression of immune mediators is likely the result of the anti inflammatory state of the host following burn(4), which impairs the animal's ability to recognize bacterial mediators and mount an effective response. However, because this response does not address the initial stimulus, namely the CLP injury, mediators which alert the host to foreign threats will continue to build up, until the system can finally overcome the anti inflammatory state imposed by burn and initiate a full anti bacterial response. This delay in the first four hours, which allows for endotoxin and other bacterial mediators to potentially build up, likely causes the increase in acute phase protein production and cytokine activity at the 8 hour mark. The return back to the CLP baseline observed for later short term time points indicates that the overreaction is short lived, and that once the system recognizes the threat, it proceeds with unprimed anti bacterial expression. It is worth noting that the expression motifs indicate that the genes that were altered by the burn priming event represent a subset of the total inflammatory activity, which can be seen directly through motifs that are clearly differentially expressed, but not different between the BCLP and SCLP conditions. A stronger injury would necessitate a more intense response, which may cause a larger fraction of the genes that traditionally respond to the CLP condition to become altered, making it significantly more difficult to realign into a recovery profile. Though our study uses rats with a 100% survival rate, this surge of inflammatory mediators at the 8 hour time point may indicate a critical time point at which the immunosuppressive effects of burn injuries can result in much more severe septic responses.

6.4.2. The Effect of Burn Priming on Metabolism

Our previous work on the short term response of animals following CLP indicates that the sepsis that is induced is associated with large scale metabolic shifts toward hypermetabolism and a negative nitrogen balance(187). The metabolic changes that are identified as a result of burn priming are changes to the already hyper metabolic state of the liver, as opposed to new metabolic directives in their own right. This can be seen in **Figure 6.2**, where the motifs related to Coagulation Protein Production, Amino Acid Degradation, and Xenobiotic Metabolism indicate a changing profile in both BCLP and SCLP, although BCLP appears to deviate at specific time points. Burn priming in the liver appears to disrupt metabolic functions more severely than innate immune functions, which may indicate that the metabolic disruption is causing a partial disruption of the innate immune response. Over the first 4 hours following the CLP injury in the burn primed animals, significant changes to the metabolic response are observed when compared to the unprimed injury. Amino acid metabolism is significantly up regulated at 2 hours, and since the degradation of amino acids is the primary mechanism by which the liver enters a negative nitrogen balance through the urea cycle(189), this implies that the liver enters a heightened state of hyper metabolism following the priming of the burn injury, even while the immune response has been suppressed. The motif that corresponds functionally to Coagulation Protein production and Amino Acid degradation shows significant up regulation over the first 4 hours that reflects this, and further indicates that while the SCLP condition does not change expression at all, the increase in amino acid degradation observed at this early point represents a spike in expression behavior which could be an indication of a metabolic shift toward amino acids as a source of fuel. Simultaneously, on hour 2, genes related to the biosynthesis of lipids, and to xenobiotic metabolism have been suppressed: the suppression of lipid synthesis is likely due to a catabolic shift within the liver, but our previous characterizations of the liver have linked xenobiotic metabolism through cytochrome P450 to inflammation, both as a method of degrading bacterial

products and for metabolizing products of oxidative stress. The motif that is functionally related to Xenobiotic Metabolism also shows a down regulation of metabolic function over the first four hours, though it indicates that both functions are suppressing the activity of these enzymes, but the burn priming is causing an increased suppression of the genes. Since inflammation has also been suppressed at this time point, similar regulators may be governing both the expression of cytokines and phosphoproteins as xenobiotic enzymes. At 4 hours, only a single gene is up regulated, which is related to xenobiotic metabolism through cytochrome p450, which may serve as a warning for the pro inflammatory response to come.

After 8 hours, amino acid metabolism and xenobiotic metabolism continues to be increased, while NRF2 mediated oxidative responses are down regulated. This suggests that the system is at a higher level of oxidation post burn at this time point than it was in just the CLP, at a period when inflammatory markers are also much more severe. This provides further evidence that levels of high reactive oxygen species, along with xenobiotic metabolism to degrade any oxidized proteins, are linked to the pro inflammatory response. A similar increase in xenobiotic metabolism is observed at the 12 hour time point, along with a slight increase in amino acid metabolism, which corresponds to a slight increase in immune function. This increase can be clearly seen in the expression motif that contained many xenobiotic metabolism enzymes, wherein the SCLP condition continues to suppress the functions of these genes over the 8 and 12 hour time points, while the burn primed condition has mitigated this suppression in order to generate a relative up regulation of these functions. Though the 24 hour mark shows a brief increase in central carbon metabolism, the metabolic response appears to have resolved. It is worth noting that hypermetabolism manifests itself in a variety of ways, even when the BCLP host is under relative immune suppression: this indicates that the regulation that underlies a shift toward the degradation of amino acids for energy is not under the same controls as the intracellular pro inflammatory signaling machinery, and may be more sensitive to prior injuries.

Furthermore, xenobiotic metabolism appears to be very sensitive to the effects of the prior burn injury, and remains completely unsuppressed over the first 12 hours of injury in the burn condition. Since xenobiotic metabolism has been linked to oxidative stress functions(190), in addition to its primary role in degrading foreign agents and oxidized proteins, these genes may indicate an increase in oxidative stress in the system caused by further exacerbation of the inflammatory condition. Though the animals are able to recover, which can be seen transcriptionally in the return of the burn primed profiles to their CLP baselines, this potential redox imbalance may suggest a motive for the observed metabolic shifts towards amino acid degradation. Overall, metabolic functions appear to be disrupted to a larger degree than those found in innate immunity: this can be observed in the number of genes that have been disrupted in metabolic functions, in addition to the fact that there were no expression motifs that were functionally related to metabolism that were identical between the BCLP and SCLP conditions. Since the liver does not synthesize its acute phase proteins in isolation from its metabolic pathways, this metabolic disruption may be a driving force behind the partial disruption of the innate immune functions that were previously observed. Amino acid synthesis, which represents a well known symptom of hypermetabolism, appears to be heavily affected by the burn priming, which may be a source of fatal dysfunction in the liver in injuries primed more severely by burn.

6.4.3. The Effect of Burn Priming on the Long Term Response

In our previous work, which describes the long term response of the host to CLP, when compared to an aseptic injury (JSR, 2012, in press), we observe that the septic injury creates an entirely different dynamic than the aseptic wound, but both appear to have their maximum deviation from the baseline at 5 days post injury. Though the burn priming observed in this study does not appear to significantly alter the basic functions of genes expressed (manifesting a pro inflammatory response instead of an anti

inflammatory one, for instance), it does show a consistent peak at the 5 day mark, which represents the maximum effect of the burn priming on the septic system. This peak consists primarily of metabolic and wound repair: the genes observed are focused in the suppression of wound repair following burn priming, along with 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 the burn priming's preference for amino acid degradation at the expense of lipid degradation in the short term response. The burn primed injury must compensate for the relatively larger loss of amino acids, hence the increased synthesis, which may be an indication of a switch to a recovery response aiming at restoring nutrient stores, rather than an anti bacterial response. This finding is interesting for a variety of reasons, firstly because it is further evidence that at around 5 days post injury, a second, strong response manifests whose timing is consistent, and shows dependence both on the type of injury, and on the presence of prior insults. Note that in our previous study, many anti inflammatory genes were found to be expressed at the 5 day mark: these genes do not deviate significantly under the burn priming, indicating that in the burn primed injury is also under immunosuppressive and anti inflammatory dynamics. Another interesting conclusion worth noting is that on the second day, the response appears to have fully resolved, but in Day 5, new transcriptional changes are observed. Thus, there is a memory associated with the system that can trigger alternative expression levels at critical time points, creating unique responses from combinations of injuries. Finally, the fact that the long term response under burn requires significantly more amino acid

synthesis than the unprimed CLP response implies that the hypermetabolism observed under burn priming is more severe than without the priming.

6.5. Conclusion

Using high throughput functional genomics techniques, we are able to show that by priming animals with thermal injury, it is possible to disrupt the inflammatory response following infection by suppressing immune mediators, and altering the dynamics of acute phase protein production. The prior injury does not affect the fundamental inflammatory response of the host, but it does exacerbate the symptoms of the traditional CLP response through partial disruption of inflammatory signaling and exacerbated hypermetabolism through aggressive amino acid catabolism. These disruptions, while not fatal in this study, represent partial deviations from a response that soon correct themselves, which, if exacerbated, could lead to negative outcomes. This response also manifests at 5 days post injury, which we previously identified as a critical resolution point in the long term response: this indicates that transcriptional resolution does not indicate full resolution, and that the 5 day mark may be critical in the host's recovery to injury. Finally, metabolic genes appear to be more sensitive to disruption than their innate immunity counterparts, and may represent a driving force in this dysfunction in the liver. Interventions in the areas of amino acid catabolism and increased oxidative stress may be able to relieve the metabolic disruption, and in turn alleviate the partial innate immune disruption.

CHAPTER 7

7. CONCLUDING REMARKS AND FUTURE DIRECTIONS

7.1.CONCLUSIONS

In current dissertation, the gene expression in liver of the rats without injury, post short term burn injury, short term CLP injury, long term CLP injury, and both burn and CLP injuries are measured by using the microarray technology in multiple time points. All the data sets are analyzed by using the proper statistical methods to investigate the alterations in gene expression spectrum of liver tissue in order to elucidate the hepatic transcriptional responses to burn injury, sepsis and burn & subsequent sepsis. The results indicate that the dynamic analysis by simultaneous analyzing of gene expression profiles for treatment and sham control groups provided a more accurate estimation of the activation time, expression patterns, and characteristics of a certain injury-induced response based on which the cause-effect relationship among responses were revealed.

7.1.1. Short term burn injury

Severe trauma, including burns, triggers a systemic response that significantly impacts on the liver, which plays a key role in the metabolic and immune responses aimed at restoring homeostasis. We characterized the response within the first 24 h in a standard animal model of burn injury using a time series of microarray gene expression data.

Rats were subjected to a full thickness dorsal scald burn injury covering 20% of their total body surface area while under general anesthesia. Animals were saline resuscitated and sacrificed at defined time points (0, 2, 4, 8, 16, and 24 h). Liver tissues were explanted and analyzed for their gene expression profiles using microarray technology. Sham controls consisted of animals

handled similarly but not burned. After identifying differentially expressed probesets between sham and burn conditions over time, the concatenated data sets corresponding to these differentially expressed probesets in burn and sham groups were combined and analyzed using a “consensus clustering” approach.

The clustering method of expression data identified 621 burn-responsive probesets in 4 different co-expressed clusters. Functional characterization revealed that these 4 clusters are mainly associated with pro-inflammatory response, anti-inflammatory response, lipid biosynthesis, and insulin-regulated metabolism. Cluster 1 pro-inflammatory response is rapidly up-regulated (within the first 2 h) following burn injury, while Cluster 2 anti-inflammatory response is activated later on (around 8 h post burn). Cluster 3 lipid biosynthesis is downregulated rapidly following burn, possibly indicating a shift in the utilization of energy sources to produce acute phase proteins which serve the anti-inflammatory response. Cluster 4 insulin-regulated metabolism was down-regulated late in the observation window (around 16 h postburn), which suggests a potential mechanism to explain the onset of hypermetabolism, a delayed but well-known response that is characteristic of severe burns and trauma with potential adverse outcome.

7.1.2. Short Term CLP and SCLP injury

Sepsis remains a major clinical challenge in intensive care units. The difficulty in developing new and more effective treatments for sepsis exemplifies our incomplete understanding of the underlying pathophysiology of it. One of the more widely used rodent models for studying polymicrobial sepsis is cecal ligation and puncture (CLP). While a number of CLP studies investigated the ensuing systemic inflammatory response, they usually focus on a single time point post CLP and therefore fail to describe the dynamics of the response. Furthermore, previous studies mostly use surgery without infection (herein referred to as Sham CLP, SCLP) as a control for the CLP model; however SCLP represents an aseptic injurious event that also stimulates a

systemic inflammatory response. Thus, there is a need to better understand the dynamics and expression patterns of both injury- and sepsis- induced gene expression alterations to identify potential regulatory targets. In this direction, we characterized the response of the liver within the first 24 h in a rat model of SCLP and CLP using a time series of microarray gene expression data. Rats were randomly divided into three groups, sham, SCLP and CLP. Rats in SCLP group are subjected to laparotomy, cecal ligation and puncture while those in CLP group are subjected to the similar procedures without cecal ligation and puncture. Animals were saline resuscitated and sacrificed at defined time points (0, 2, 4, 8, 16, and 24 h). Liver tissues were explanted and analyzed for their gene expression profiles using microarray technology. Unoperated animals (Sham) serve as negative controls. After identifying differentially expressed probesets between sham and SCLP or CLP conditions over time, the concatenated data sets corresponding to these differentially expressed probesets in sham and SCLP or CLP groups were combined and analyzed using a “consensus clustering” approach. Promoters of genes that share common characteristics were extracted, and compared with gene batteries comprised of co expressed genes in order to identify putative transcription factors which could be responsible for the co regulation of those genes.

The SCLP/CLP genes whose expression patterns significantly changed compared to sham over time were identified, clustered, and finally analyzed for pathway enrichment. Our results indicate that both CLP and SCLP triggered the activation of a pro-inflammatory response, enhanced synthesis of acute-phase proteins, increased metabolism and tissue damage markers. Genes triggered by CLP which can be directly linked to bacteria removal functions were absent in SCLP injury. In addition, genes relevant to oxidative stress induced damage were unique to CLP injury, which may be due to the increased severity of CLP injury vs. SCLP injury. Pathway enrichment identified pathways with similar functionality but different dynamics in the two injury models, indicating that the functions controlled by those pathways are under the influence of different

transcription factors and regulatory mechanisms. Putatively identified transcription factors, notably including CREB, NF-KB and STAT, were obtained through analysis of the promoter regions in the SCLP/CLP genes. Our results show that while transcription factors such as NF-KB, HOMA, and GATA were common in both injuries for the IL-6 signaling pathway, there were many other transcription factors associated with that pathway which were unique to CLP, including FKHD, HESF and IRFF. There were 17 transcription factors that were identified as important in at least 2 pathways in the CLP injury, but only 7 transcription factors with that property in the SCLP injury. This also supports the hypothesis of unique regulatory modules that govern the pathways present in both the CLP and SCLP response.

By using microarrays to assess multiple genes in a high throughput manner, we demonstrate that an inflammatory response involving different dynamics and different genes is triggered by SCLP and CLP. From our analysis of the CLP data, the key characteristics of sepsis are a pro inflammatory response which drives hypermetabolism, immune cell activation, and damage from oxidative stress. This contrasts with SCLP, which triggers a modified inflammatory response leading to no immune cell activation, decreased detoxification potential, and hyper metabolism. Many of the identified transcription factors that drive the CLP-induced response are not found in the SCLP group, suggesting that SCLP and CLP induce different types of inflammatory responses via different regulatory pathways.

7.1.3. Long Term CLP injury

Despite the fact that the treatment options for septic patients have been significantly improved, the pathophysiological changes caused by various septic cases have not been well understood. One commonly observed clinical phenomenon is the onset of a polymicrobial infection caused by bacteria that originate in the intestine but enter the peritoneum via translocation from the gut. This triggers a systemic inflammatory response via the innate immune system, which needs to be well

characterized. Cecal ligation and puncture (CLP) is considered to be the gold standard animal model by establishing infection with mixed bacterial flora and necrotic tissue to induce an inflammatory response. The aim of this study is to analyze the long term gene expression dynamics in the rats subject to CLP in order to characterize the impact of sepsis upon liver function over an 8 day time period.

Rats received CLP or its control, sham CLP and then they were sacrificed at 9am on days 0 (no treatment), 1, 2, 5 and 8 post injury to collect liver samples for microarray analysis. Differentially expressed probesets in CLP vs. SCLP (*q-value* <0.001 and *p-value*<0.001) were combined to form one single matrix, which was then clustered using the approach of “consensus clustering” to identify subsets of transcripts with coherent expression patterns. Finally, the gene expression patterns of the clusters were further transformed into principal components which account for 65% of the total data.

Three major clusters were obtained. The first cluster which is mainly related to genes of anti inflammatory response and anti oxidative properties is suppressed early in the CLP condition and later up regulated 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 hyper metabolism 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 and pro inflammatory response, however the SCLP response in Cluster 3 shows persistent down regulation.

The characterization of long term hepatic responses to injury is critical to understand the dynamics of transcriptional changes following the induction of the inflammatory response, and in monitoring its effective resolution. These results showed that each condition has unique dynamics

that indicate fundamental differences in the response. Furthermore, the gene ontologies suggest a link to oxidative stress over the long term that may be able to be explored for clinical treatments.

7.1.4. Double Hits injuries

Systemic insults in mammalian systems produce a response which acts in distinct pro-inflammatory and anti-inflammatory phases. This response can be highly destructive, particularly when multiple injuries occur in succession. It is thought that immunosuppressive effects from the anti inflammatory phase of the initial insult can render the host more prone to a secondary insult. We hypothesize that burn followed by sepsis will disrupt the immune and metabolic response due to this immunosuppressive effect, and we aim to characterize these disruptions in the burn primed response in rat models.

Rodents were burned using a 20% total surface area (TSA) burn injury, and then underwent cecal ligation and puncture two days later to induce sepsis. Animals were sacrificed at various time points, and whole liver samples were analyzed using Affymetrix Rat Genome 230 2.0 Arrays. Statistical Analysis of Microarrays (SAM), combined with false discovery analysis was used to identify genes with significant differences in fold changes at each time point. Clustering techniques were employed to observe temporal changes between the conditions. Ingenuity Pathway Analysis (IPA) was used to functionally annotate genes, and RT-PCR was used to confirm microarray trends.

957 genes met the criteria outlined above, from each time point. Significant gene expression changes were observed at 2, 4, 8, 12, 24 and 120 hours post injury. 253 genes were up regulated over the time course, while 704 genes were down regulated. Trends were confirmed by the RT-PCR, which qualitatively agreed with microarray results. 30 motifs emerged from the clustering, 5 of which were chosen as representative of the entire set. IPA identified 18 relevant pathways, split evenly between innate immunity and metabolism. Immune function showed partial

disruption at early time points, with significant suppression of signaling and increased acute phase protein production. Metabolic functions such as xenobiotic metabolism and amino acid metabolism were sensitive to disruption, and showed significant up regulation. Long term expression showed significant differences at Day 5, which appears to be a critical point for recovery in mammalian systems.

Burn priming prior to cecal ligation and puncture disrupts the transcriptional response in the liver to septic injury in the rat by altering the onset of anti bacterial functions in the liver, and enhancing hyper metabolic conditions through aggressive amino acid degradation at critical time points.

7.2.Future Directions

The dynamic analysis of the gene expression in rat liver in current research reveal information that is important for the understanding of the transcriptional change of the liver following burn injury and its associated bacterial infection from global perspective as well as in a timely manner. Besides the study in current research, other relevant aspects could be taken into consideration in future directions.

7.2.1. The Effect Of Circadian Rhythm

The circadian clock is one of the most critical biological regulators for all living entities controlling behavioral, physiological and biochemical processes. Acting like a multifunctional timer with a roughly 24-h cycle (191), the circadian clock offers fitness advantages by endowing organisms with anticipatory mechanisms predicting periodic events, as opposed to responding in a reactive way to external signals (192). In addition to primary circadian "clock" located in the suprachiasmatic nucleus (SCN) (193), independent circadian rhythms are also found in many

organs and cells outside the SCN, such as the oesophagus, lungs, liver, pancreas, spleen, thymus, and the skin (194).

Genome-wide transcriptome-mapping studies revealed that the fraction of diurnally accumulating liver transcripts amounts to 1-10%, depending on the stringency of algorithms used for the analyses of microarray hybridization data (195-199). The identified genes exhibiting circadian rhythm in the rat liver participate in broad functions including regulation of transcription (195, 196), cell signaling (196, 198), energy metabolism (82, 195, 197), amino acid metabolism (82, 195, 198), lipid metabolism (82, 195, 197), carbohydrate transport and metabolism (195, 197), cholesterol metabolism (197), DNA replication (82, 195), protein synthesis (82, 195), signal transduction mechanisms (195), immune response (198).

Evidences suggest the possibility that both the circadian rhythm and immune functions are the regulators and manifestation of the homeostasis and are demonstrated to be closely associated to each other. A number of prior studies have placed significant emphasis on the fact that the immune system is under the control of the circadian rhythm. It is reported that the cytokines gene expression level will fluctuate with the light- dark cycle in the brain and peripheral tissues (200), and NK cells (201) in rats. Moreover, it is further explored that the inflammatory immune responses are also under control of the circadian clock displayed. In Keller et al' s study, the isolated spleen cells stimulated with bacterial endotoxin at different circadian times display circadian rhythms in TNF- α and IL-6 production (202). One interesting study indicates that both T cell IL-2 and adherent cell TNF- α production were altered by time of burn injury. TNF- α secretion was significantly increased in burn vs. sham adherent cells when injury took place in the morning (203).

Due to the significant differences of the responses following the different intervention time of the day, it is necessary and critical to identify the gene expression following the burn injury at

different time point. In current studies, the burn injury is introduced at 9 am, in future 9pm may be selected to be the intervention time. Thus, the aim of future study is to gain a better understanding of the response under the influence of the circadian rhythm in rat liver.

7.2.2. Mathematical Modeling

Our greater knowledge today of the diverse results of the thermal insult must find some way of being put to practical use. What is needed is an interpretation which encompasses most, if not all, phenomena in one hypothesis. The difficulty is the causal connectivity of the findings, since a clear distinction has not always been made between the early shock phase and the later sequelae of organ failure. The long list of abnormal immune effects found in burn injury induces the predictable consequence that there is a long list of treatments to deal with each type. Surgical intervention techniques like fluid resuscitation and plasma exchange, early excision, nutrition and ventilaton, have all helped improve some immune parameters as well as the general condition of the patients to some degree. However, the innumerable consequences, involving cytokines and products of lipid oxidation, represent the action of chaos, or fractal generation; the lack of control of the cytokine cascade represents activity beyond feedback control. It becomes logical that one has to postulate combination therapy to deal with numerous branches of a fractal pattern. However immune failure in burn injury, like all immune functions, develops on a time-dependent scale and each fractal branch stems from a simpler order which came before it. In short, dealing with an effect is never as effective as dealing with an earlier cause of that effect, which itself may be an effect of yet an earlier cause.

Mathematical modeling offers the opportunity of studying the dynamics of the interacting elements of a complex system while it provides a systematic framework for integrating research work from many disciplines (204). *In silico* models are quantitative representations of the dynamic properties of quantitative data. Modeling enables the systematic integration of massive

amounts of relevant information shedding invaluable insight into the progression of the disease state and into possible therapeutic interventions (205). Mathematical models as previously stated being the *in silico* representations of the non-linear signal flow offer the opportunity of studying the complex behavior of a biological system not through its isolated components but through their dynamic integration. As such, the computational integration of the interacting elements that constitute the unified inflammatory response is supposed to capture the inherent complexity of this response understanding how they behave collectively over time: making it critical enabler to both predict and evaluate various immunomodulatory strategies that perturb system's homeostasis. In essence, such a prediction is impossible to be performed without developing mathematical models given that *in silico* models can establish a causal inference relationship through the manipulation of its dynamic elements. It is widely accepted that it is difficult to unravel such threads experimentally encouraging the hypothesis-driven development of computational disease models. The development of such mathematical models provides a mechanistic understanding about the probable effects of various perturbations on a system facilitating the making decision process with regard to putative targets and timing of intervention; henceforth facilitating the design of clinical trials(206).

The dynamics of inflammation are highly complex and make appealing model-based approaches that explore simultaneously multiple hypotheses for deciphering complex modes of action and the possibility for proposing combination therapies (207). In addition to this, quantitative models aim at exploring the putative effects of perturbations on a biological system identifying areas amenable to therapeutic intervention (205). Thus, the potential for studying such complex phenomena in a model-based manner opens the possibility for generating and exploring simultaneously multiple hypotheses. Therefore, the fundamental hypothesis is that a reduced mathematical modeling of the inflammatory response enables the prediction of the behavior of the entire system through its constituent parts. Such an approach is necessary in system-level

disease process, like systemic inflammation (208) and allows us to model the host defense response through its key inflammatory components. To address this, it is important to explore the potential of a mathematical model in that it can describe the indirect effect which is exerted by the inflammatory stimulus burn or infection on the transcriptional response level. Based on the model, we can enhance the understanding the relationship between the input and the responses, and make a prediction which may be used for practical clinical application.

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