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Dynamics of hepatic gene expression profile in a rat cecal ligation and puncture model

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Abstract

Background—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.

Methods—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.

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Results—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, HMEF, 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.

Conclusions—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.

Keywords

sepsis; trauma; gene expression; transcription factor; microarray; inflammation; liver

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(1). 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 (2, 3). 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(2). 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 (4–6). However, these studies either focus on a single time point (24 h post-CLP), (4, 5) therefore missing the critical early response, or do not take the time scale into account (6), 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.

Materials and Methods

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.

Data Analysis

Analysis of CLP, SCLP and sham gene expression data includes normalization, filtering, combining the datasets and clustering which is depicted in Figure 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 (7). 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” (8). 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.

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 (9). In order to accelerate the process of identifying putative transcriptional regulators, promoters are pre-processed as in (10). Specifically, MatInspector (11) 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 (9). 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 (10). 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 (10) 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.

Results

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 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. A complete description for all 1021 probe sets can be found in “Supplementary Materials” which include the cluster number, probe ID, gene name, gene ontology, gene category and expression values at 6 time points for each of the probe set.

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*, *Psma4*, *Psme2*, *Psmd14*, *Psmd1*, *Hspdl*), 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 (13). 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 (14). 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(15). 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 (16). 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(17–19). 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) (20). 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.

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 1.

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*, *SPIF*, *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 *SPIF* 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 *SPIF* 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.

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 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. A complete description for all 1221 probe sets can be found in "Supplementary Materials" which include the cluster number, probe ID, gene name, gene ontology, gene category and expression values at 6 time points for each of the probe set.

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 (21). 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 (22). 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 (23). 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, Ptpnj, Serping1, and Vcan*), inflammation (*Bdkrb2, C5, Cxcl2, Hpx, Ifnb1, Il11, Il13ra1, Il6, Il6r, Il6st, Kng1, Lbp, and Prf1*), infiltration by neutrophils (*C5, CD36, Cfh, Cxcl2, Il6, and Il6r*), infiltration of granulocytes (*C5, Cd36, Cfh, Cxcl2, Il6, Il6r, and Kng1*), activation of monocytes (*Adam9, Blk, C5, Ddost, Hla-Dqb1, Ifnb1, Il6, Lbp, Mgat5, Nfkb1z, Prf1, and Ptpnj*), activation of phagocytes (*Adam9, C5, Cfh, F5, Ifnb1, Il6, Kng1, Lbp, and Serping1*). 5 and 12 of them are associated with infection mechanism (*Ifnb1, Il6, Nfkb1z, Prf1, and Il11*) and antigen presentation (*C5, C8a, Cfh, Cfhr1, Kng1, Masp1, Adam9, Ifnb1, 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, Psmd7, 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, Psmd14, Rbx1, and Ube2n*), folding of protein (*Canx, Hsp90aa1, Hsp90ab1, Hspd1, Pfdn2, St13, and Tcp1*), refolding of protein (*Hsp90aa1, Hspd1, St13*). In addition, genes associated with cellular assembly and organization (*Actr3, Loc643751, Srf, Kif5b, Pfn1, Prpf19, Smn1/Smn2, Mapre1, Eif3a, Rhoa, Tcp1, Map7, Mre11a, Naa50, Hsp90aa1, Immt, Creb1, Abi1, Robo2, Copb2, Ftl, and Serp1*) are present in this cluster. Therefore, the downregulation of this cluster suggests the decrease in the protein degradation coupled with 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 (24). 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 (*Acadslb*, *Acsml3*, *Acssl2*, *Adhl1c*, *Adhl7*, *Cln3*, *Cyp51a1*, *Ebp*, *Ecl2*, *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 (25). Insulin is an anabolic hormone which promotes the storage of substrates in liver by stimulating lipogenesis, glycogen and protein synthesis (26). 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 (27). 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 (28).
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.

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.

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*, *RXR*, *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 *RXR* 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.

Discussion

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 (29). 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 (30). 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 (28) 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 (31). 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 (32). 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.

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 (29). 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(3). 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 (33). 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(34). 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 (35). 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.

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.

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 (36). 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 (36). 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(37). 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 (38), mitochondrial dysfunction (39) 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(40), 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.

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 (41) 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 5 and Figure 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(38). This is reinforced by the presence of multiple indicators of oxidative stress in the CLP network, which include mitochondrial dysfunction(39) 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.

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 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 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 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 c regulators, or histone modifications, that affect the transcriptional availability of the DNA and factor binding capacity(42). 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.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Dombrovskiy VY, Martin AA, Sunderram J, Paz HL. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. *Crit Care Med*. 2007; 35:1244–1250. [PubMed: 17414736]
2. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc*. 2009; 4:31–36. [PubMed: 19131954]
3. Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock--a review of laboratory models and a proposal. *J Surg Res*. 1980; 29:189–201. [PubMed: 6997619]
4. Cobb JP, Mindrinos MN, Miller-Graziano C, Calvano SE, Baker HV, et al. Application of genome-wide expression analysis to human health and disease. *Proc Natl Acad Sci U S A*. 2005; 102:4801–4806. [PubMed: 15781863]
5. Li ZJ, Li YP, Gai HR, Xue YL, Feng XZ. [Research of gene expression profile of liver tissue in rat sepsis model]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue*. 2007; 19:156–159. [PubMed: 17376269]
6. Chinnaiyan AM, Huber-Lang M, Kumar-Sinha C, Barrette TR, Shankar-Sinha S, et al. Molecular signatures of sepsis: multiorgan gene expression profiles of systemic inflammation. *Am J Pathol*. 2001; 159:1199–1209. [PubMed: 11583946]
7. Scheff J, Almon R, DuBois D, Jusko W, Androulakis I. Assessment of Pharmacologic Area Under the Curve When Baselines are Variable. *Pharmaceutical Research*. 2011:1–9.

8. Nguyen TT, Nowakowski RS, Androulakis IP. Unsupervised selection of highly coexpressed and noncoexpressed genes using a consensus clustering approach. *Omics*. 2009; 13:219–237. [PubMed: 19445647]
9. Genomatix. <http://www.genomatix.de>
10. Nguyen TT, Foteinou PT, Calvano SE, Lowry SF, Androulakis IP. Computational identification of transcriptional regulators in human endotoxemia. *PLoS One*. 2011; 6:e18889. [PubMed: 21637747]
11. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, et al. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics*. 2005; 21:2933–2942. [PubMed: 15860560]
12. Bader A, Rinkes IHB, Closs EI, Ryan CM, Toner M, et al. A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes. *Biotechnology Progress*. 1992; 8:219–225. [PubMed: 1368259]
13. Puthier D, Bataille R, Amiot M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. *Eur J Immunol*. 1999; 29:3945–3950. [PubMed: 10602002]
14. Baumann H, Gauldie J. The acute phase response. *Immunol Today*. 1994; 15:74–80. [PubMed: 7512342]
15. Wannemacher RW Jr, Pace JG, Beall RA, Dinterman RE, Petrella VJ, et al. Role of the liver in regulation of ketone body production during sepsis. *J Clin Invest*. 1979; 64:1565–1572. [PubMed: 500825]
16. Pollack GM, Browne JL, Marton J, Haberer LJ. Chronic stress impairs oxidative metabolism and hepatic excretion of model xenobiotic substrates in the rat. *Drug Metab Dispos*. 1991; 19:130–134. [PubMed: 1673385]
17. Haque R, Hwang BY, Appelboom G, Piazza MA, Guo K, et al. Alterations in systemic complement component 3a and 5a levels in patients with cerebral arteriovenous malformations. *J Clin Neurosci*. 18:1235–1239. [PubMed: 21742500]
18. Iyer A, Woodruff TM, Wu MC, Stylianou C, Reid RC, et al. Inhibition of inflammation and fibrosis by a complement C5a receptor antagonist in DOCA-salt hypertensive rats. *J Cardiovasc Pharmacol*.
19. Zeerleder S. C1-inhibitor: more than a serine protease inhibitor. *Semin Thromb Hemost*. 37:362–374. [PubMed: 21805442]
20. Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. *J Cell Sci*. 2004; 117:1281–1283. [PubMed: 15020666]
21. Romagnani S. T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol*. 2000; 85:9–18. quiz 18, 21. [PubMed: 10923599]
22. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, et al. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol*. 1999; 163:4647–4650. [PubMed: 10528160]
23. Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science*. 2001; 291:2364–2369. [PubMed: 11269317]
24. Biolo G, Toigo G, Ciochi B, Situlin R, Iscra F, et al. Metabolic response to injury and sepsis: changes in protein metabolism. *Nutrition*. 1997; 13:52S–57S. [PubMed: 9290110]
25. Brealey D, Singer M. Mitochondrial Dysfunction in Sepsis. *Curr Infect Dis Rep*. 2003; 5:365–371. [PubMed: 13678565]
26. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001; 414:799–806. [PubMed: 11742412]
27. Nikaido SS, Johnson CH. Daily and circadian variation in survival from ultraviolet radiation in *Chlamydomonas reinhardtii*. *Photochem Photobiol*. 2000; 71:758–765. [PubMed: 10857373]
28. Samra JS, Summers LK, Frayn KN. Sepsis and fat metabolism. *Br J Surg*. 1996; 83:1186–1196. [PubMed: 8983604]
29. Hasselgren PO, Jagenburg R, Karlstrom L, Pedersen P, Seeman T. Changes of protein metabolism in liver and skeletal muscle following trauma complicated by sepsis. *J Trauma*. 1984; 24:224–228. [PubMed: 6708141]

30. Mitch WE, Goldberg AL. Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. *N Engl J Med*. 1996; 335:1897–1905. [PubMed: 8948566]
31. Morgan ET. Regulation of cytochrome p450 by inflammatory mediators: why and how? *Drug Metab Dispos*. 2001; 29:207–212. [PubMed: 11181485]
32. Morgan ET. Suppression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. *Mol Pharmacol*. 1989; 36:699–707. [PubMed: 2511427]
33. Almendro V, Carbo N, Busquets S, Figueras M, Tessitore L, et al. Sepsis induces DNA fragmentation in rat skeletal muscle. *Eur Cytokine Netw*. 2003; 14:256–259. [PubMed: 14715419]
34. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. *J Gastroenterol Hepatol*. 2011; 26(Suppl 1):173–179. [PubMed: 21199529]
35. Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R, et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2004; 286:R491–R497.
36. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999; 340:448–454. [PubMed: 9971870]
37. Oikonomopoulou K, Ricklin D, Ward PA, Lambris JD. Interactions between coagulation and complement-their role in inflammation. *Semin Immunopathol*.
38. Thorell A, Efendic S, Gutniak M, Hagglund T, Ljungqvist O. Insulin resistance after abdominal surgery. *Br J Surg*. 1994; 81:59–63. [PubMed: 8313123]
39. Delogu G, Moretti S, Famularo G, Marcellini S, Santini G, et al. Mitochondrial perturbations and oxidant stress in lymphocytes from patients undergoing surgery and general anesthesia. *Arch Surg*. 2001; 136:1190–1196. [PubMed: 11585514]
40. Bak M, Kicinska-Krogulska M, Czerniak P, Michowicz A, Krakowiak A. Toxic liver injuries--a current view on pathogenesis. Part II. *Med Pr*. 62:203–210. [PubMed: 21698879]
41. Qian JD-FM, Lin J, Yu H, Gerstein M. Beyond synexpression relationships: local clustering of time-shifted and inverted gene expression profiles identifies new, biologically relevant interactions. *J Mol Biol*. 2001; 314:1053–1066. [PubMed: 11743722]
42. Medzhitov R, Horng T. Transcriptional control of the inflammatory response. *Nat Rev Immunol*. 2009; 9:692–703. [PubMed: 19859064]

Figure 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 $p < 0.01$.

Figure 2. Gene expression profiles of rat livers in response to sham or 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).

Figure 3. Gene expression profiles of rat livers 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).

Figure 4. Comparison between the SCLP- and CLP –induced responses

Left Panel, SCLP Induced Response; Right Panel, CLP Induced Response

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.

Figure 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 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.

Figure 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.

Table 1

Predicted TFs relevant to the transcriptional regulation of some activated functions in SCLP and CLP injury. Included are TFs associated with overrepresented *cis*-regulatory modules; if the number of associated TFs is 15, only the first 15 most frequently-associated TFs in statistical significant CRMs are reported; those TFs present frequently at least 2 times over 5 gene batteries in each injury are displayed in bold type.

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 , SPIF , 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, SPIF , STAT
5	4	Metabolism of xenobiotics by cytochrome P450	cyp2d6, gstm1, gstm2, mgst1	NEUR, PAX6, SORY , ETSF , PLAG, ABDB, EGRF, INSM, MZF1, SRFF
CLP				
1	1	IL6 signaling	a2m, hspb3, il6, il6r, il6st, lbp	EREF, HESF , SORY , TBP, 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, serpin1	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, dhfr4, gstm1	LEFF, AP4R , E2FF , ETSF , CREB, MYBL , WHNF, SPIF , PAX2, HESF , AP1R, EGRF, HEAT, SORY