PATTERNS OF GENE EXPRESSION AMONG MURINE MODELS OF HEMORRHAGIC SHOCK/TRAUMA AND SEPSIS

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Running Title: Murine transcriptomic comparisons

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Abstract

Controversy remains whether the leukocyte genomic response to trauma or sepsis is dependent upon the initiating stimulus. Previous work illustrated poor correlations between historical models of murine trauma and sepsis (i.e. trauma-hemorrhage and lipopolysaccharide injection, respectively). The aim of this study is to examine the early genomic response in improved murine models of sepsis (cecal ligation and puncture; CLP) and trauma (polytrauma; PT) with and without pneumonia (PT+Pp). Groups of naive, CLP, PT, and PT+Pp mice were sacrificed at two hours, one or three days. Total leukocytes were isolated for genome-wide expression analysis and genes that were found to differ from control (FDR adjusted p<0.001) were assessed for fold-change differences. Spearman correlations were also performed. For all time points combined (CLP, PT, PT+Pp), there were 10,426 total genes that were found to significantly differ from naive controls. At two hours, the transcriptomic changes between CLP and PT showed a positive correlation ($r_s$) of 0.446 (p<0.0001), but were less positive thereafter. Correlations were significantly improved when limiting the analysis to common genes whose expression differed by a 1.5 fold-change. Both pathway and upstream analyses revealed the activation of genes known to be associated with PAMP and DAMP signaling, and early activation patterns of expression were very similar between polytrauma and sepsis at the earliest time points. This study demonstrates that the early leukocyte genomic response to sepsis and trauma are very similar in mice.

Keywords: mouse, transcriptomics, correlations, polytrauma, cecal ligation and puncture
Introduction

Despite improvements and advances in clinical management, sepsis and trauma continue to remain substantial burdens to both hospitals and society (24, 32, 42). Sepsis, as a whole, is the leading cause of death within non-cardiac intensive care units and mortality rates remain around 20-30% (23). Mortality rates for septic shock can be as high as 50% (30). Trauma also remains a significant problem, and to date, pharmacotherapeutic agents targeting host immunity have had little effect in regards to modifying outcome (37).

Since Matzinger first published the ‘danger hypothesis’, much research has focused on identifying pathways that lead to a host inflammatory response (29). Specific pattern recognition receptor (PRR) pathways rely on both damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) to signal the presence of tissue damage or microbial infection, respectively. However, both endogenous and exogenous ‘alarmins’ appear to signal through many of the same PRRs, including toll-like receptors (TLRs) (19, 31). Due to these apparent similarities, significant effort has been dedicated to understanding these common pathways that lead to an activation of early inflammatory and innate immune responses.

Recently, murine models of sepsis and trauma have received scrutiny for their failure to fully recapitulate comparable human responses at the level of the leukocyte transcriptome (10, 38). Interestingly, the genomic responses to these various stressors were very similar in humans, but not in mice (38). One explanation is that the current murine models are poor representations of the human condition (9, 10). In support of that conclusion, we have
reported that increasing injury severity in a murine trauma model produced a modest but significant improvement in correlation between patterns of human and murine gene expression (16).

An alternative explanation is that the timing of the host response varies significantly between humans and mice, and among different injury models in mice. In this report, we sought to compare the murine blood leukocyte gene expression responses to various inflammatory states including polytrauma, sepsis, and polytrauma followed by pneumonia. We specifically asked whether each form of inflammation would lead to a unique leukocyte genomic response, or whether there existed a core genomic response common to different inflammatory challenges produced by either microbial invasion or tissue injury and hypovolemic shock. In addition, we sought to examine whether microbial invasion after trauma induced a response more similar the initial genomic expression pattern after severe sepsis or severe injury.

Materials and Methods

Mice. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida. Specific pathogen-free male C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6–7 weeks and allowed to acclimatize for one week before being used for experimental procedures. Mice were maintained on standard rodent food and water ad libitum. For each experiment, 4-5 mice were used per group for each time point.
Cecal ligation and puncture (CLP). For induction of polymicrobial sepsis, CLP was performed under isoflurane anesthesia, as described previously (6, 8). Briefly, the cecum was exposed after a midline laparotomy, ligated with 2-0 silk suture one cm from the tip, and punctured through and through with a 25-gauge needle. The cecum was returned to the abdomen, and the incision was closed using surgical clips. After the procedure, the mice were administered 0.05–0.20 mg/kg buprenorphine every 12 hours for 48 hours, and returned to their respective cages. This murine model induces an LD_{10-30} within the first seven days.

Polytrauma (PT). Groups of mice were anesthetized using inhalational isoflurane and restrained in the supine position. Mice underwent 90 minutes of hemorrhagic shock followed by long bone fracture and cecectomy as previously described (15). The combined level of injury produces an equivalent injury severity score in humans of 18. After injury, mice were housed in groups and all mice were administered buprenorphine (0.2 mg/kg body weight) prior to arousal from anesthesia and every 12 hours afterward until sacrifice. Of note, this model is non-lethal, and the animals were able to maintain the ability to ambulate, groom, feed and drink.

PT followed by Pseudomonal pneumonia (Pp). Pneumonia following polytrauma was induced using *P. aeruginosa* (PAK) as described previously (8) one day after polytrauma. PAK was grown overnight, transferred to fresh medium, and grown to midlog phase. The bacterial density was measured at OD 600\(\lambda\) (DU 640 Spectrophotometer, Beckman Coulter, Inc., CA) and washed with saline. Under isoflurane anesthesia, these mice received intranasal instillation of \(1 \times 10^7\) bacteria, delivered in 50 µl. This murine infection model has an LD_{10-20} over seven days after polytrauma; it is a non-lethal model when administered to healthy animals (34).
Transcriptomics. Blood was collected by intracardiac puncture using one ml syringes containing 100 μl 169 mM EDTA at two hours, one or three days after CLP or polytrauma, and one day after Pseudomonas pneumonia in polytrauma mice. Red blood cells were lysed using Buffer EL™ (Qiagen, Valencia, CA). The supernatant was decanted after centrifugation, and the cell pellet was homogenized in RLT™ buffer (Qiagen) supplemented with 2-mercaptoethanol and passed through the homogenizer (Qiagen). Subsequently, total RNA was isolated using RNeasy™ kit (Qiagen, Valencia, CA), and the quality and quantity were assessed using an Agilent Bioanalyzer 2000™. Nucleic acids were labeled using the 3′ IVT Express™ Kit, and 15 μg labeled cRNA was hybridized to mouse genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Arrays were hybridized for 16 h at 45°C. Following hybridization, arrays were stained and washed using a FS450 Affymetrix Fluidics Station and Affymetrix FlexFS 450-0004 protocol. Arrays were then scanned in an Affymetrix GeneChip scanner 7G Plus. Genome-wide expression was performed on total blood (circulating) leukocytes (9, 33). All array data was submitted to the Gene Expression Omnibus (GEO) genomics data repository; GEO accession number GSE69245.

Statistics. Blood leukocyte genome-wide expression patterns were compared between healthy mice and mice experiencing either CLP sepsis, polytrauma, or polytrauma and pneumonia, using a false discovery adjusted F test (p < 0.001) with BRB Tools™. The datasets were analyzed for individual gene expression differences (magnitude of fold change of the significant genes), as well as for individual pathways (Gene Ontology™ and Biocarta™) using the distance from reference (DFR), (p < 0.05) (5, 43), and functional pathway differences (Z-score, <-2, >2) using Ingenuity Pathway Analysis™. A Z-score of less than -2 or greater than 2 represents a significant change at a 95% confidence interval (3). The distance from reference (DFR) calculation derives
a single metric representing the overall differences in gene expression, and is calculated as the natural log of the sum of the differences in gene expression (between healthy and experimental animals) for each probe set divided by the pooled variance for that individual probe set. This allows each specimen’s overall genomic response to be represented by a single natural log transformed value. DFR is only useful to determine the magnitude of genomic expression change from baseline, and does not describe its direction.

Spearman correlations were calculated to assess the correlation between changes in gene expression in the various forms of injury and time points. All correlations were carried out on individual genes, not individual probe sets (16).

**Results**

*Polytrauma and sepsis induce similar lymphocyte dominant responses.* Complete blood count (CBC) analyses at one day revealed no significant difference in the differential leukocyte count (WBC) between polytrauma with and without pneumonia (*Figure 1a*). At one day after CLP, the observed leukocyte count was composed predominantly of lymphocytes, similar to that seen after PT, but to a significantly lesser extent (*Figure 1b*). As expected though, the percentage of neutrophils was significantly increased in all injury models at one day after severe injury or infection.

*Total leukocyte microarray genomic analysis reveals similar genomic responses in the early time points after polytrauma and CLP sepsis.* The expression of 16,728 probe sets representing 10,426 unique genes significantly differed between healthy controls (at p < 0.001) (*Figure 2*) and any of the injury/sepsis models at any of the time periods. The overall Spearman rank
correlation ($r_s$) for the entire leukocyte genome that changed after CLP sepsis versus polytrauma (10,426 genes) regardless of timing was only 0.075 (p insignificant). When changes in gene expression after polytrauma and CLP sepsis were compared at individual time points, a positive correlation was seen at two hours ($r_s= 0.446$) (p<0.0001), while no correlation was seen at one day ($r_s=0.087$) and a highly significant inverse correlation was seen at three days ($r_s= -0.529$) (p<0.0001) (Figure 3). However, when we compared genome-wide expression at the time of maximal genomic up-regulation for both CLP sepsis (one day) and polytrauma (two hours), the overall Spearman ($r_s$) correlation was 0.755 (p<0.0001). Similarly, when genome-wide expression was compared between CLP and polytrauma with pneumonia at one day, the Spearman correlation ($r_s$) was 0.423 (p<0.0001).

We then filtered the genes whose expression was found to be 1.5 fold different from healthy, control mice (p<0.001). At two hours, there were a total of 1400 genes after polytrauma and 615 genes after CLP which had significant gene expression changes that were also altered >1.5 fold from baseline expression (p<0.001). Of these significant genes, there were 260 commonly altered genes common between the two models (Figure 4A). Not surprisingly, the Spearman rank correlation ($r_s$) for these 260 genes was 0.931 (p<0.001) (Figure 4B). When we examined the overall 1400 genes that changed in polytrauma by at least 1.5 fold and compared the change in expression to the same genes in CLP, whether significant or not, we observed a Spearman correlation ($r_s$) of 0.751 (p<0.001) (Figure 4B). Subsequently, we analyzed the 615 genes that were changed in CLP and compared them to the same genes in PT, and the Spearman correlation ($r_s$) was 0.801 (p<0.001) (Figure 4B).
We then evaluated the top 100 responsive genes, whether upregulated or downregulated from baseline controls, from both CLP sepsis and polytrauma for similarities at two hours after injury or infection. The changes from baseline control mouse expression ranged from 2 to 280 fold, and we identified that a large proportion of these genes were involved in the early inflammatory response. Interestingly, the top 100 upregulated genes two hours after CLP sepsis were similarly upregulated two hours after polytrauma. The Spearman correlation \( (r_s) \) for these 100 genes at two hours was 0.901 \( (p<0.001) \) (Figure 5A and B). The same 100 genes continued to be upregulated by CLP at one day; but after polytrauma, several of the genes had either returned to baseline or their expression reduced (Figure 5A). We also examined the top 50 up-regulated and top 50 down-regulated genes after polytrauma and compared these to the genomic expression patterns of the same genes two hours after CLP sepsis - their correlation \( (r_s) \) was 0.865 \( (p<0.001) \).

The DFR calculation provides an overall metric of the deviation in gene expression (5, 43). It is a natural log value that variance normalizes the contribution of highly expressed genes to overall gene expression resulting in a more equitable averaging of the contribution of each gene to the total metric. Overall DFRs from the 10,426 genes that differed revealed that the magnitude of leukocyte gene expression change for CLP peaked at one day (12.05) and was greater than the change in gene expression after polytrauma which occurred at both two hours and one day. However, polytrauma shows a rebound effect at three days that actually reveals the highest degree of gene expression change by overall DFR, but represents an overall down-regulation of early activation genes. As a reference, the DFR in healthy controls was 8.95 (Figure 6).
Ingenuity Pathway Analysis® confirmed that the regulation of the genomic responses after CLP sepsis and polytrauma were most similar at the early time points. Using upstream regulation analysis for the total leukocyte population, the requirement for nuclear factor kappa B (NFκB) activation showed nearly an identical response after sepsis and severe hemorrhagic shock with trauma (Figure 7). Other early response pathways that were similarly activated in both polytrauma and CLP sepsis at two hours were pathways dependent on extracellular regulated kinase (ERK), response gene 88 (MYD88), CCAAT/enhancer-binding protein alpha (CEBPA), toll-like receptors (TLRs), and epidermal growth factor receptor (EGFR) expression.

Surprisingly, we identified through upstream analysis that microRNA (miR)-223 was predicted to be activated in both polytrauma and CLP sepsis at the early time points (Z score 4.43 and 3.76, respectively). MicroRNA-223 is highly expressed in myeloid cells and has been shown to negatively regulate progenitor proliferation (21, 35). It has also been demonstrated to suppress differentiation of myeloid-derived suppressor cells (MDSC) from bone marrow (26). This is highly relevant since expansion of MDSCs is a common response to trauma, sepsis and burns (7).

We were able to identify molecules, predicted by the transcriptomic patterns of the mice after the insult, that were uniquely recognized to be activated or inhibited in either CLP or PT at two hours; an early time point during the innate immune response. Using IPA® Causal Analysis we determined that Cullin 4B (CUL4B) was predicted to be inhibited at two hours after polytrauma, but its expression was not significantly affected in the early CLP sepsis response (Z score -2.093 and 0.246, respectively). Interestingly, CUL4B is a negative regulator of MDSC
function (36) and has also been identified as a promoter of proliferation and inhibitor of apoptosis in cancer cell (4). In addition, we found that miR-483-3p, also an inhibitor of proliferation with pro-apoptotic activity (2), was predicted to be inhibited early in polytrauma but its expression was not significantly affected in CLP sepsis (Z score -2.449 and 0.707 at the two hour time points, respectively). Furthermore, regulator of g-protein signalling-14 (RGS14) was predicted to be activated early in CLP sepsis, but its expression was not significantly affected in polytrauma (Z score 2.234 and -0.364, respectively). RGS14 is involved in \( \alpha \text{M}\beta 2 \) integrin activation, which is important for bidirectional signaling involved in phagocytosis (25); thus, its activation in CLP sepsis and not in trauma would seem appropriate. Lastly, bone marrow stromal cell antigen 2 (BST2) was predicted to be activated early in CLP sepsis but inhibited in polytrauma (Z score 3.883 and -4.982, respectively). The protein encoded by this gene has been demonstrated to mediate antigen delivery to plasmacytoid dendritic cells which subsequently induces initial T-cell priming (27). Thus, downregulation of BST2 expression in polytrauma might be protective by limiting presentation of self-antigens expressed in tissue injury.

*Genes known to be involved with PRRs display similar responses.* Individual fold changes for genes known to be involved with PAMP and DAMP recognition also demonstrated similar degrees of expression changes. These genes include heat shock proteins (HSP) and toll-like receptors (TLR), which are almost universally upregulated at two hours after both sepsis and polytrauma (Table 1). Similar changes were also identified one day after pneumonia in polytrauma animals. However, PRR gene expression began returning to baseline levels within three days after polytrauma, which was dissimilar to sepsis (Table 1).
Discussion

The discovery of pattern recognition receptors has led to a greater understanding of how the host recognizes microbial pathogens and tissue injury, and has opened the possibility of novel therapeutic immunomodulation (20). Murine models remain the bedrock for preclinical mechanistic and therapeutic studies. Recent studies examining the host genomic responses to injury and sepsis have increased the scrutiny of a murine model’s capacity to recapitulate the human processes (10). Recently, we reported that the leukocyte genomic response to human models of injury showed significant overlap while comparable murine models demonstrated little to no correlation among themselves (38). This overlap in the human response is thought to be due to the initiation of the systemic inflammatory response syndrome (SIRS), which is common to both microbial infection and tissue injury.

In contrast, the failure of murine models to recapitulate the human response may well be due to the nature of the injury models and time periods selected (10, 16). Although the response to a 30% burn injury, administration of endotoxin and hemorrhagic shock with a laparotomy were transcriptomically dissimilar, it can be argued that these murine models are suboptimal representations of human infection and injury (10). For example, endotoxin administration is not equivalent to septic polymicrobial infection (6, 15). In fact, the method of bacterial infection can have huge implications on the murine transcriptomic response (18). Additionally, although a steadfast component of trauma research for decades, the murine model of hemorrhagic shock/laparotomy can certainly be improved to increase its similarity to human severe trauma (6, 15).
This study asked a different but fundamental question regarding how the murine model responds to two different inflammatory challenges of similar severity: polymicrobial sepsis produced by a CLP, and polytrauma with hemorrhagic shock. One model represents the host response to an overwhelming infectious cascade, whereas the other represents significant tissue injury and hypovolemic shock in the absence of microbial infection. Although the CLP sepsis model is associated with 10-20% mortality, whereas the polytrauma is not, polytrauma mice become susceptible to nonlethal challenges with *Pseudomonas* pneumonia. Based on the overall hypothesis that PRR’s can recognize both microbial pathogens and endogenous alarmins resulting from tissue injury, we postulated that the early genomic responses should be more similar than disparate. Furthermore, we proposed that not only should there be common genomic responses, but upstream promoter analyses dependent upon PRR signaling should also reveal significant overlap. We also hypothesized that the genomic responses would grow more dissimilar with time, as the CLP represents an ongoing infectious process, while polytrauma is nonfatal and animals rapidly recover.

Using a CLP model of sepsis and an improved model of trauma, we demonstrated that there truly are dramatic leukocyte transcriptomic changes after severe murine infection or injury, with nearly 40% of the host genome expression significantly changing. This is similar to what has been revealed after human trauma, a true ‘genomic storm’ (29). Looking at the entire leukocyte transcriptome that changed (10,426 genes), the correlations in gene expression changes remained poor when timing of the response is not considered ($r_s=0.075$). These findings are consistent with the changes in genome-wide expression seen between burns, hemorrhagic shock and endotoxemia, as we reported (38). However, the failure to see strong
correlations appears to be due primarily to the difference in the timing of the genomic response. Rather, if one focused on the individual time points, the genome-wide associations between severe trauma and sepsis were most similar at two hours ($r_s = 0.446$), and then declined dramatically over time until there was an inverse correlation at three days ($r_s = -0.529$).

The overall differences in the kinetics of gene expression are best revealed by the DFR, which peaked in polymicrobial sepsis at one day. In polytrauma, changes in gene expression show a significant overall up-regulation at two hours but by three days there is actually a rebound effect in which we see our overall highest level of gene downregulation. However, in CLP, there is actually a prolonged up regulation of inflammatory genes. This likely reflects the ongoing presence of nonviable tissue (ligated cecum) and unresolved infection in this model.

Such numbers can be overanalyzed since in any cell type only approximately 50% of the genome is expressed (22). In blood leukocytes in particular, one would purposefully focus on genes involved in activation of protective immunity, inflammation and metabolic activity in response to polytrauma or polymicrobial sepsis. If the genes are filtered to include only those genes that change after injury or sepsis at least 1.5-fold, the number of genes was dramatically reduced (1400 in polytrauma and 615 in sepsis) — in fact, their commonality improved dramatically! This was the approach taken by Takao and colleagues when they re-evaluated our earlier analyses on human and murine gene expression (40). Although the number of genes whose change in expression exceeded 1.5 fold, the Spearman correlation for the 1400 and 615 genes respectively was excellent ($r_s=0.751$ and 0.801, respectively), suggesting that there are subsets of genes whose expression moves in a comparable direction in these two distinct injury and infection models. Further refining the list to the top 100 genes that changed the greatest
magnitude after polytrauma or sepsis revealed even greater concordance between the two injuries \((r_s=0.901)\). Not surprisingly, 88% of these genes were directly involved in early inflammatory and innate immune responses. Such findings demonstrate that there is a core genomic response in murine trauma and polymicrobial sepsis that is shared, although the timing of the response may differ.

Analysis of predicted upstream regulatory control suggested that the two inflammatory models, whether infectious or traumatic, induce a similar set of response elements. The common patterns of gene expression reflect common activation of nuclear factor kappa B (NFkB) and several other early response pathways including myeloid differentiation primary response gene 88 (MYD88), toll-like receptors (TLRs), and epidermal growth factor (EGF). There was also early activation in both models of CCAAT/enhancer-binding protein alpha (CEBPA), which leads to downstream inhibition of peroxisome proliferator-activated receptors (PPAR). PPAR is known to have significant effects on substrate metabolism essential for the cell’s oxidative response (28).

Interestingly, when we compared pneumonia in polytrauma to CLP sepsis at one day there was still a significant correlation at 0.423, albeit less than the correlation between polytrauma and CLP sepsis at one day. This may be due to the model itself in that the infection occurs 24 hours following polytrauma. Trauma clearly altered the baseline changes in expression as well as immune suppression (45), and infection in this setting is likely to induce genomic changes markedly different than would have occurred in a healthy, naïve animal. This may lead to less activation of the immune response following a second stimulus potentially via
myeloid derived suppressor cells (MDSCs) (7). MDSCs, initially described in the cancer literature, consist of a heterogeneous subset of immature myeloid cells whose numbers increase dramatically after inflammation and are potently immunosuppressive while simultaneously contributing to low-grade, chronic inflammation (13).

MicroRNAs (miRNAs), small segments of non-coding RNA, are involved in both pre and post transcriptional gene regulation (1). Several studies performed in the past have demonstrated that various expression levels of these miRNAs can affect myeloid differentiation (12, 21). MicroRNA-223 is a widely studied miRNA that has been shown to be highly expressed in myeloid cells (35). Interestingly, in tumors, it has been shown to decrease differentiation of tumor-induced MDSCs (21). It is possible that these MDSCs, elevated post-trauma and sepsis, are key players in both the chronic inflammation and immunosuppression displayed after severe sepsis and trauma (7).

We have previously demonstrated, that in response to injury, the elderly do not have an exaggerated inflammatory response, nor greater suppression of adaptive immunity genes, but rather a delayed return to baseline (33, 34, 41). Thus, return to homeostasis is a key objective for future immunomodulation therapy of elderly trauma and sepsis patients (33, 34, 41). However, it is clear that juveniles do not return to baseline genomic expression patterns after severe trauma within three days in this study. Without further insults, such as a bacterial pneumonia, there appears to be a complete ‘inversion’ of the transcriptomic expression pattern, completely different from the response to CLP sepsis (Figure 2). Whether this represents an evolutionary benefit to the host, by trying to reduce the likelihood of an
autoimmune response to released damaged cell products or organ injury, or a period of
increased host susceptibility after the initial emergency myelopoiesis response, has yet to be
determined. Unfortunately, animal welfare issues prevent us from determining how long it
truly takes the juvenile and elderly mouse to return to baseline genomic expression patterns,
but preliminary data in elderly humans may indicate that the process may not be rapid (11)
(14).

There are a number of significant limitations to the present study that warrant caution.
First, this study makes no attempt to compare murine to human responses, but focuses entirely
on the similarities and differences in leukocyte gene expression to different inflammatory
stimuli in mice. Second, we are focusing on a single tissue compartment, and that
compartment is heterogeneous and its composition varies with both the polytrauma and
polymicrobial sepsis (Figure 1). Whether these findings can be extrapolated to other tissues
and models of injury is unknown. Third, the findings are conducted in juvenile mice, and
mortality from these conditions, especially sepsis, is most commonly associated with human
patients of increased age (17, 33). We have recently demonstrated that aged animals have a
relatively attenuated early inflammatory response to both trauma and sepsis, and fail to re-
establish homeostasis (33, 34). Furthermore, this study uses C57BL/6 mice, a Th1 dominant
strain (44). It is well known that different inbred mouse strains can have a different response to
injury (39, 44). A Th1 dominant strain has predominant interferon-γ (IFNγ) cytokine production
leading to immune activation via a macrophage dominant response (44), thus favoring the
innate immune response. However, as with all of our prior experiments, we use this strain as it
has proven to provide high reproducibility. In addition, C57BL/6 mice constitute the majority of
knock-out strains and transgenics, thus, providing a platform for genomic manipulation if pursued in future studies.

With these limitations in mind, however, the findings are quite striking in demonstrating that although the overall genome-wide response varies over time between polytrauma and sepsis, there is a core of 1000-2000 genes whose expression changes, and changes in a common pattern, regardless of the initiating inflammatory challenge. The commonality of this response is greatest early after the inflammatory challenge, and the gene expression changes are primarily related to the early inflammatory and innate immunity response. Examination of upstream regulatory elements also reveals a commonality regardless of whether the stimulus was polytrauma or polymicrobial sepsis in many of the early regulatory activators of the immune response.

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Disclosures
The authors declare they have no competing interests as defined by *American Physiological Society*, or other interests that might be perceived to influence the results and discussion reported in this paper.
References


Figure Legends

Figure 1. Complete blood count (CBC) analysis one day post sepsis (CLP), polytrauma (PT), and polytrauma plus pneumonia (PT+Pp). (A) Complete white blood count (WBC) shows decreased WBC in CLP when compared to naïve mice. PT and PT+Pp do not show a significant difference when compared to naïve mice. (B) WBC differential. All models show an increase in neutrophils at one day. CLP: cecal ligation and puncture; PT: polytrauma; PT+Pp: polytrauma + pneumonia. * P<0.05 Tukey’s post-test, ** p<0.01, ***p<0.001 vs. naïve using 2-way ANOVA, Bonferroni posttests.

Figure 2. Heat map illustrating a supervised analysis of gene expression in murine models of sepsis (CLP), polytrauma (PT), and polytrauma plus pneumonia (PT+Pp) at various time points after insult (two hours, one day and three days). The heat map reveals that the closest correlations are at the early time points. Red = up regulation; blue = down regulation; CLP: cecal ligation and puncture; PT: polytrauma.

Figure 3. Spearman rank correlations for all 10426 genes significantly different from control at specific points post insult. Comparisons of (A) all time points combined, (B) two hours post PT versus two hours post CLP, (C) one day post PT versus one day post CLP, and (D) three days post PT versus three days post CLP are displayed. Although the correlations are poor when comparing all 10426 genes, the strongest correlations are seen at the earliest time points. By three days, there is a negative correlation due to the down-regulation of the previously up-regulated genes in PT. CLP: cecal ligation and puncture; PT: polytrauma.
Figure 4. Comparison of genes with significantly differentiated gene expression at 2 hours post insult with a fold change $\geq 1.5$. A. Venn diagram illustrating all genes found to be significantly different from controls. B. Spearman rank correlation graph for all genes found to be significantly different from control two hours post PT versus the corresponding genes two hours post CLP. C. Spearman rank correlation graph for all genes found to be significantly different from control with at least a 1.5 fold change that were shared between PT and CLP at two hours post insult. D. Spearman rank correlations for all genes found to be significantly different from control with at least a 1.5 fold-change for CLP at versus corresponding genes in PT at two hours post insult (whether they met the aforementioned criteria or not). CLP: cecal ligation and puncture; PT: polytrauma.

Figure 5. The correlation between the top 100 up-regulated genes in CLP as compared to the same genes in PT. Correlations between the two models are very strong when considering genes that represent early responders to the inflammatory response. A. Illustration showing the top 100 up-regulated genes for two after CLP and their corresponding changes in various settings and time points. B. Spearman correlation graph showing the same top 100 up-regulated genes in CLP at two hours versus the same genes in PT at two hours. Red = up-regulation; Blue = down-regulation; CLP: cecal ligation and puncture; PT: polytrauma.

Figure 6. Distance from reference (DFR) analysis for all genes in various injury models and time points. DFR is a simplified method of looking at overall level of change in genomic expression and it can be seen in this graph that all injury models create similar levels of change.
in expression. CLP: cecal ligation and puncture, PT: polytrauma; PT+Pp: polytrauma + pneumonia.

Figure 7. Ingenuity pathway analysis (IPA™) illustration showing upstream regulation of the pathway for NFκB. The response to injury in both models at two hours creates a very similar response. Orange = up-regulation; Blue = down-regulation; CLP: cecal ligation and puncture; PT: polytrauma.
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Table 1. The damage associated molecular pattern and pathogen-associated molecular pattern with their pattern-recognition receptor.

CLP: Cecal ligation and puncture; PT: Polytrauma; Pp: Pneumonia
**Fig. 1**

**A.**

**WBC**

![WBC Bars Graph]

- naive
- CLP
- PT
- PT+Pp

p<0.05, one way ANOVA; *p<0.05 Tukey’s post-test

**B.**

**WBC Differential**

![WBC Differential Bars Graph]

- lymphocytes
- neutrophils
- monocytes

**Legend**

- naive
- CLP
- PT
- PT+Pp

***p<0.001***
Fig. 3

**All Time Points**

- PT vs Control fold-change: $r=0.075$
- CLP vs Control fold-change: $r=0.446$

**Day 1**

- PT vs Control fold-change: $r=0.087$
- CLP vs Control fold-change: $r=-0.529$

**Day 3**

- PT vs Control fold-change: $r=0.075$
- CLP vs Control fold-change: $r=0.446$

**2 hr**

- PT vs Control fold-change: $r=0.087$
- CLP vs Control fold-change: $r=0.446$
Fig. 4

A. Juvenile CLP and PT 2hr Analysis Venn Diagram

- PT 2hr vs Control t-test p<0.001 (1400)
- CLP 2hr vs Control t-test p<0.001 (619)

- 1140
- 260
- 355

B. PT unique genes vs CLP

C. Common genes PT vs CLP

D. CLP unique genes vs PT

- r=0.751
- r=0.931
- r=0.801
Fig. 5

A. 2hr PT vs 2hr CLP Top 100 up-regulated genes

B. 2hr PT vs 2hr CLP Top 100 up-regulated genes

r=0.901
DFR Levels for all time points

- CLP
- PT
- PT+Pp
- Control

Time (hr)

DFR
Chemical-chemical interactions, chemical-protein interactions, correlation, protein-protein interactions, RNA-RNA interactions:
non targeting interactions

Activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, protein-DNA interactions, protein-RNA interactions, regulation of binding, transcription

Inhibition, ubiquitination

Inhibits and acts on

Leads to

Processing yields

RNA-RNA interactions; microRNA targeting

Translocation

Reaction

Enzyme catalysis

Direct interaction

Indirect interaction

Fig. 7