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Comparison of the Cytokine and Chemokine Dynamics of the Early Inflammatory Response in Models of Burn Injury and Infection

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Abstract

The inflammatory response, and its subsequent resolution, are the result of a very complex cascade of events originating at the site of injury or infection. When the response is severe and persistent, Systemic Inflammatory Response Syndrome can set in, which is associated with a severely debilitating systemic hypercatabolic state. This complex behavior, mediated by cytokines and chemokines, needs to be further explored to better understand its systems properties and potentially identify multiple targets that could be addressed simultaneously. In this context, short term responses of serum cytokines and chemokines were analyzed in two types of insults: rats receiving a “sterile” cutaneous dorsal burn on 20% of the total body surface area (TBSA); rats receiving a cecum ligation and puncture treatment (CLP) to induce infection. Considering the temporal variability observed in the baseline corresponding to the control group, the concept of area under the curve (AUC) was explored to assess the dynamic responses of cytokines and chemokines. MCP-1, GROK/KC, IL-12, IL-18 and IL-10 were observed in both burn and CLP groups. While IL-10 concentration was only increased in the burn group, Eotaxin was only elevated in CLP group. It was also observed that Leptin and IP-1 concentrations were decreased in both CLP and sham-CLP groups. The link between the circulating protein mediators and putative transcription factors regulating the cytokine/chemokine gene expression was explored by searching the promoter regions of cytokine/chemokine genes in order to characterize and differentiate the inflammatory responses based on the dynamic data. Integrating multiple sources together with the bioinformatics tools identified mediators sensitive to type and extent of injury, and provided putative regulatory mechanisms. This is essential to gain a better understanding for the important regulatory points that can be used to modulate the inflammatory state at molecular level.

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Keywords

Cytokines; Chemokines; Burn injury; Cecum ligation and puncture

1. Introduction

Inflammation is a complex biological response to injuries and infections, involving different inflammatory mediators, such as cytokines and chemokines, as well as nervous system reflexes, and hormones. Precise regulation and coordination of the processes linking them is necessary to mount an appropriate response enabling an eventual return to homeostasis [1]. It is generally accepted that the severity of the inflammatory response and the involvement of the various components can vary depending on type and severity of the injury, infectious agent, as well as genetic and physiological factors (sex, body weight, preexisting conditions including immune system deficiencies).

Burns and infections lead to one or several of the following “primary” events in the inflammatory cascade: denaturation of proteins, loss of plasma membrane integrity, activation of complement proteins, and secretion of local mediators, such as histamine, nitric oxide, and reactive oxygen species [2–4]. This is quickly followed by the production of pro-inflammatory cytokines (such as IL-1, TNF- α , IL-18, IL-6, and IL-12), and chemokines (such as Eotaxin, G-CSF, GM-CSF, GRO/KC, MCP-1, MIP-1, Rantes). The initial pro-inflammatory response also activates a modulating anti-inflammatory reaction consisting of anti-inflammatory cytokines (such as IL-4, IL-10, IL-13). Inflammatory cytokines also impact afferent signals to the nervous system which in turn increase the secretion of anti-inflammatory stress hormones [5] and activate the cholinergic anti-inflammatory pathway [1]. Although these different signals may result in distinct cellular responses depending on the type and severity of the injury, there is a large degree of overlap in the underlying signaling pathways, including MAPK, Jac/STAT, and Ik-B/NF-k β activated during the inflammation.

Animal models of burn injury, cecal ligation and puncture, and endotoxin injection have previously been used to profile circulating mediator concentrations after an insult [6–13]. Gauglitz et al. [6] used male rats receiving a full thickness burn over 60% of the total body surface area (TBSA), where they observed that serum concentrations of several pro- and anti-inflammatory mediators (IL-1 beta, IL-6, IL-10, MCP-1, and CINC) were significantly elevated after burn. Barber et al. [9] studied the relation between burn size (20%, 30%, 40%, and 60% TBSA) and cytokine concentrations (TNA-alpha, IL-1 beta, and IL-6) at one time point (24 h) after burn injury, and observed burn size-dependent increases. Ertel et al. [11] induced sepsis by CLP in male rats and analyzed the TNF, IL-1 and IL-6 cytokines profiles in serum within 20 hour time course after CLP. They observed a persistent elevation of plasma TNF until 10 hours, steadily increasing IL-1 plasma concentrations, and enhanced IL-6 plasma concentrations at all time points compared to the sham group.

It is noteworthy that these studies are limited to one type of insult or a limited number of cytokines. Chemokines and other inflammatory mediators including Leptin and IL18 are gaining more attention in the attempts to characterize the inflammatory responses. For example, elevated concentrations of IL-18, which is a proinflammatory cytokine involved in the regulation of cell-mediated and innate immune responses, have been reported to correlate with organ dysfunction after injury [14]. Peter et al. observed that GCSF, a chemokine mainly produced by monocytes and macrophages, modified the immune system by increasing the white blood cells and decreasing the TNF- α and IFN- γ concentrations in a rat animal model receiving 30 % TBSA burn injury [15]. Leptin is also playing an important

role in regulating energy metabolism. It was observed that Leptin reduced elevated tissue associated myeloperoxidase activity and microscopic damage scores in various tissues including liver, stomach, colon and kidney in rats receiving 30 % TBSA burn [16].

In this study, we used nonlethal rat models of burn injury and cecal ligation and puncture (CLP) to profile the early temporal inflammatory response by measuring 23 different cytokines and chemokines. Moreover, the binding sites of promoter regions of the cytokines and chemokines whose concentrations were significantly altered in the treatment groups were explored to identify putative transcription factors. Comparison of the timing of cytokine/chemokine concentration changes among the groups revealed mediators sensitive to type and extent of injury. Connecting the cytokine/chemokine dynamic patterns to transcription factor activation further provided insights on the regulatory mechanism of inflammation process.

2. Materials and Methods

2.1 Animals

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.

2.2 Experimental Plan

Animals were randomly divided into four groups. Two different insults were investigated: a dorsal cutaneous burn (B) to mimic trauma with no infection, and cecal ligation and puncture (CLP) to mimic infection and sepsis. Control treatments included sham burn and sham CLP. In total four different groups of animals were investigated: sham-burn, burn, sham-CLP and CLP (labeled S, B, SCLP, and CLP, respectively).

2.3 Burn Injury

A full-thickness burn on the dorsal skin corresponding to 20% of the total body surface area (TBSA) was performed as described previously [17]. This model was chosen because it has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant effect on the feeding pattern [18]. Rats were anesthetized by intraperitoneal injection of 80 to 100 mg/kg ketamine + 12 to 10 mg/kg xylazine. All hair removed from the dorsal abdominal area using electric clippers. The animal's back was immersed in water at 100°C for 10 s to produce a full-thickness scald injury over 20% TBSA. Immediately after burns, the animals were resuscitated with 50 mL/kg of saline injected intraperitoneally. Sham burn controls consisted of animals treated identically but immersed in warm water (37°C). Rats were single caged after burn or sham burn and given standard rat chow and water ad libitum until sacrifice. No post-burn analgesics were administered, consistent with other studies with this full thickness burn model since the nerve endings in the skin are destroyed and the skin becomes insensate [19]. Furthermore, after the animals woke up, they ate, drank, moved freely about the cage, responded to external stimuli, and did not show clinical signs of pain or distress.

2.4 Cecum Ligation and Puncture

CLP was used as an infection model because it is thought to closely mimic the physiological changes in human sepsis [20]. Rats were anesthetized and given the analgesic buprenorphine subcutaneously at 0.01 to 0.05 mg/kg. Animals were then placed in supine position and hair was shaved on the abdomen. Bupivacaine (0.125% to 0.25%) was applied

subcutaneously around the incision site for additional perioperative and postoperative analgesia. 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 as to avoid intestinal obstruction. Suture was passed between the body of the cecum and the main artery, so that the latter was not ligated. The cecum was punctured 4 times (not through and through) with a 20 gauge needle and replaced in the peritoneum. The abdominal incision was then sutured in layers using interrupted monofilament sutures. The animal received 10 mL/kg saline intraperitoneally for resuscitation. Sham-CLP controls consisted of animals treated identically without receiving CLP, i.e. they were anesthetized, underwent laparotomy as described above, but no surgical manipulation of the cecum was performed. Rats were single caged after the treatments and given standard rat chow and water ad libitum until sacrifice.

2.5 Cytokine Analysis

Animals were anesthetized at different time points (2, 4, 8, 12, 16, 20, and 24) (n=3 per time point per group) post burn or CLP. Blood samples were immediately collected from the vena cava by heparinized catheters, kept on ice, and then centrifuged at 4500 rpm for 3 min at 4 °C. The serum supernatant was collected and stored at -80 °C until analysis. A MILLIPLEX MAP Rat Cytokine/Chemokine Panel (Millipore) was used for the simultaneous quantification of 23 different cytokines (Eotaxin, G-CSF, GM-CSF, GRO/KC, IFN- γ , IL-10, IL-12 (p70), IL-13, IL-17, IL-18, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IP-10, Leptin, MCP-1, MIP-1 α , RANTES, TNF- α , VEGF) according to the manufacturer's guidelines.

2.6 Data Analysis

We recently developed an algorithm to estimate the area under the curve (AUC) of a variable baseline and compare it with the AUC of the response curve [21]. This method was used to discover significant net responses such as transcriptional regulatory effects in gene expression data. Given the fact that cytokine production may be altered throughout the observation period, the concept of area under the curve can be used to assess the dynamic responses and the deviation from their baseline values [22–23]. This method was selected here since it is proven to be more informative for experiments having high sampling frequencies after the experimental perturbations [21]. In this study, for each cytokine, the overall AUC (area under the cytokine concentration–time curve in treatment group, i.e. B and CLP groups) and baseline AUC (area under the cytokine concentration–time curve in control group, i.e. S group) were calculated numerically [22]. These values were then compared to determine if the overall AUC significantly deviates from the baseline AUC by identifying p-values using a t-distribution and Satterthwaite's approximation for degrees of freedom [23]. Finally, the algorithm proposed by Benjamini and Hochberg [24] was used to determine a data-based p-value threshold controlling the false discovery rate at the level α ($\alpha=0.05$ in this study).

Briefly, AUC was calculated by the trapezoidal rule using the means of replicates, $\bar{X}_{jk}$, at each time point t_j [22] as follows:

$$\begin{aligned}
 AUC_k &= \sum_{j=1}^m w_j \bar{X}_{jk}, \quad k=1 \dots f \\
 \bar{X}_{jk} &= \frac{1}{n_{jk}} \sum_{i=1}^{n_{jk}} X_{ijk} \\
 w_1 &= \frac{1}{2} (t_2 - t_1) \\
 w_j &= \frac{1}{2} (t_{j+1} - t_{j-1}) \quad \text{for } 2 \leq j \leq m-1 \\
 w_m &= \frac{1}{2} (t_m - t_{m-1})
 \end{aligned} \tag{1}$$

where m and f are the total number of time points and number of groups, respectively, and n_{jk} is the total number of replicates or animals at time points t_j and group k . The variance of linear AUC estimator is obtained as:

$$V(AUC_k) = \sum_{j=1}^m w_j^2 \sigma_{jk}^2 n_{jk}^{-1} \quad (2)$$

where σ_{jk} is the standard deviation of the concentration values at time j in group k . A test of null hypothesis of no difference between two AUCs has been presented by Heinzl [23]. The null hypothesis of equality among two AUC's ($k=1, 2$) is given as:

$$H_o: E(AUC_1) - E(AUC_2) = \sum_{j=1}^m w_j \bar{X}_{j1} - \sum_{j=1}^m w_j \bar{X}_{j2} = 0 \quad (3)$$

Assuming t-distribution and Satterthwaite's approximation, t value and degree of freedom, v , are calculated as follows in order to obtain p-value (t_{obs} , v):

$$t_{obs} = \frac{\sum_{j=1}^m w_j \bar{X}_{j1} - \sum_{j=1}^m w_j \bar{X}_{j2}}{\sqrt{\sum_{j=1}^m w_j^2 \sigma_{j1}^2 n_{j1}^{-1} + \sum_{j=1}^m w_j^2 \sigma_{j2}^2 n_{j2}^{-1}}} \quad (4)$$

$$v = \frac{\left(\sum_{j=1}^m w_j^2 \sigma_{j1}^2 n_{j1}^{-1} + \sum_{j=1}^m w_j^2 \sigma_{j2}^2 n_{j2}^{-1} \right)^2}{\sum_{j=1}^m w_j^4 \sigma_{j1}^4 (n_{j1}^2 (n_{j1} - 1))^{-1} + \sum_{j=1}^m w_j^4 \sigma_{j2}^4 (n_{j2}^2 (n_{j2} - 1))^{-1}} \quad (5)$$

Then, the calculated p-values are ordered, $p_{(1)} \leq \dots \leq p_{(z)}$, where z is total number of cytokines. Using Benjamini and Hochberg approach [24], $\hat{l}$ is calculated as:

$$\hat{l} = \max \{1 \leq l \leq z: p_{(l)} \leq \alpha \cdot (l/z)\} \quad (6)$$

If $\hat{l}$ exists, the first $\hat{l}$ null hypotheses are rejected, implying that the cytokines corresponding to $p_{(1)} \leq \dots \leq p_{(\hat{l})}$ are significantly changed.

Heat maps were generated by the “clustergram” function in MATLAB, which was used to cluster the differentially produced cytokines and chemokines (hierarchical clustering). This further enabled to compare and analyze the dynamic patterns of the inflammatory mediators.

2.7 Predicting Putative Transcription Factors

In order to identify potential transcription factors (TFs) that regulate cytokine gene expression, we explored the basic underlying assumption of comparative genomics which states that functional regions evolve in a constrained fashion and therefore at a lower rate than non-functional regions [25–26]. It implies that conserved regions in a set of orthologous sequences survive due to their special functional implications i.e. TF families

associated with binding sites identified on these conserved regions are more likely to function as transcriptional regulators. Promoters of cytokine genes of rats were extracted from the Genomatix database of promoter information with a default length (500bp upstream and 100bp downstream of the transcription start site) unless an experimentally defined length has been reported [27]. Each promoter is characterized by a set of orthologous promoters from the same gene of other vertebrate species, if available (e.g. Homo sapiens, Macaca mulatta, Pan troglodytes, Mus musculus, Equus caballus, Canis lupus familiaris, and Bos Taurus). DiAlign TF [27] with default parameters (core similarity: 0.75, matrix similarity: optimal threshold for each position weight matrix suggested from MatBase [27]) was subsequently applied to identify conserved regions on promoter P and then transcription factor binding sites (TFBSs) that are enriched on corresponding conserved regions from the set of orthologous promoters with a common threshold (70% in this study). In addition, in order to increase the confidence of the predicted binding sites ModelInspector [27] was used to search for a list of cis-regulatory modules (L) from a library of functional, experimentally-verified, modules (MatBase[27]) that match on promoter P. Identified TFBSs on P are then scanned over L and only those that are present on modules in L are selected. Associated TF families with those selected TFBSs are inferred and considered as transcriptional regulators for corresponding cytokines.

3. Results and Discussion

3.1 Animal Weight Changes and Mortality

All animal groups had 100% survival for at least one week following treatment ($n \geq 3$ for each treatment). The time course of whole body weight changes was also monitored. CLP caused a ~10% weight loss, although after 24 hours, the animals started to regain weight at a rate similar to that observed pre-CLP (Figure 1). Sham-CLP did not cause any change in body weight gain rate. These observations are consistent with prior literature using a similar model [17]. Burn injury and sham-burn did not result in any significant change in the rate of body weight gain (data not shown).

CLP in rodents results in a hypermetabolic and catabolic state [17] and has been extensively used as a model of sepsis. While it is generally viewed as more realistic than endotoxin injection models, it is important to note that the response and mortality rate to CLP can vary depending on the specific procedural details (e.g. length of the cecum ligated, size of the needle, and the number of punctures) of the technique used, as well as rat strain [20, 28]. In the procedure used herein, we took care to not ligate the cecal branch of the ileocecal artery, thus preserving viability of the cecum itself, which explains our high survival rate, consistent with observations published by Banta and co workers [17]. We did not want high mortality since our purpose was to compare injury vs. infection induced systemic inflammatory responses in nonlethal and reversible conditions.

3.2 Cytokine Profiles

Cytokine profiles in the Sham group (which did not receive any injury or surgical manipulation) were used as reference to which the profiles obtained for all other groups were compared. Table 1 shows the cytokines which have been filtered out by the AUC method incorporated with Benjamin and Hochberg false discovery rate correction. MCP-1 (Monocyte chemoattractant protein), IL-18 (Interleukin-18), GCSF (granulocyte colony-stimulating factor), GRO/KC (growth related oncogene), IL-10 (Interleukin-10), IL-12P70 (Interleukin-12 or IL-12) and IL-1 α (Interleukin-1 α) were significantly altered in the animals receiving a 20% TBSA burn injury. Concentrations of the chemokines GRO/KC ($P=0.003$), MCP-1 ($P=0.0002$) and GCSF ($P=0.0017$) - which are chemotactic for leukocytes to injured areas- were increased in the burn group. GRO/KC showed a peak around $t=8$ h after the burn

injury whereas MCP-1 steadily increased within 24 hours postburn (Figure 2). Similarly, IL-12 ($P=0.004$), which can be regarded as both a pro- and an anti-inflammatory cytokine, IL-10, an anti inflammatory cytokine ($P=0.004$), and IL-18 ($P=0.0008$), a pro inflammatory cytokine, reached a maximum at $t=4h$. On the other hand, IL-1 α ($P=0.007$), a pro-inflammatory cytokine, seems to be down-regulated after burn.

SCLP, which involves laparotomy without ligation or puncturing of the cecum, caused changes in several markers. It was observed that some of the chemokines, including IP-10 (interferon gamma induced protein) ($P=0.0001$), GRO/KC ($P=0.0004$), and MCP-1 ($P=0.0004$) were significantly different from the Sham group (Table 1). GRO/KC and MCP-1 peaked around $t=8 h$ after SCLP treatment (Figure 3). On the other hand, IP-10 concentrations decreased (Figure 3). Similarly, some pro-inflammatory cytokines, including IL-1 β , IL-17, and IFN- γ ($P = 0.002$, 0.01 and 0.01 , respectively) and Leptin ($P= 0.004$), a hormone regulating energy intake and energy expenditure, were decreased after SCLP treatment.

MCP-1, GMCSF, IP-10, IL-18, IL-12, Leptin, GRO/KC and Eotaxin were significantly altered by CLP treatment compared to the Sham group (Table 1 and Figure 4). IL-18, a pro inflammatory cytokine, was significantly elevated ($P=0.0008$) and demonstrated two peaks at around $t=2 h$ and $t=24 h$. Similar to previous observations, GRO/KC peaked at $8 h$ post-CLP. On the other hand, IL-12 peaked later, at $t= 16 h$, which is also later than the observed IL-12 profile in the burn group (Figure 2). Eotaxin, a chemoattractant protein, also showed significant elevation at $t=16 h$ ($P=0.01$). MCP-1 ($P=0.00002$) significant increased within the $24 h$ post-burn period. Other chemokines, namely GMCSF, IP-10, and Leptin, decreased after the CLP treatment.

Finally we compared the CLP group to the SCLP group to identify infection-specific effects. IL-12, IL-18, Eotaxin, and IL-4 were significantly different (Table 1). IL-4, an anti-inflammatory cytokine, was significantly decreased compared to the SCLP group ($P= 0.01$) (Figure 5). On the other hand, IL-12, IL-18, and Eotaxin were increased in the CLP group compared to the SCLP group (as well as the Sham group).

When baseline values of the control group (S) were used for comparison to the injured animals (B, SCLP and CLP), we observed that GRO/KC and MCP-1 chemokine concentrations were significantly elevated in all treatment groups. Moreover, significant alterations in IP-10, Leptin and Eotaxin profiles in CLP and/or SCLP group (Figures 3 and 4) were also detected. Leptin is a hormone regulating food intake and metabolic functions, and its concentration was decreased in CLP and SCLP groups in this study. Although IL-1 β and TNF- α contribute to the up-regulation of leptin production in animals, results from studies conducted in septic patients are contradictory since both up or down regulation in leptin production could be observed in the patients [29]. MCP-1 and Eotaxin are CC chemokines where the first two cysteines are adjacent to each other. They attract mononuclear cells to sites of chronic inflammation [30]. GRO/KC and IP-10 are chemokines in CXC group (the first two cysteines are separated by an amino acid) which are neutrophil and lymphocytes chemo-attractants and induce granule exocytose, fibroblast differentiation and restrain angiogenesis [30].

IL-18, a pro-inflammatory cytokine, was increased in the early phase of inflammation in burn and CLP groups in this study. It has structural similarities with IL-1 family proteins [31]. It can be expressed by different cell types including macrophages, Kupffer cells, keratinocytes and adrenal cortical cells [32]. Similar to IL-1 and TNF- α pro inflammatory cytokines, IL-18 activates similar signaling molecules [33]; thus, one can speculate that IL-18 might be functionally similar to TNF- α and IL-1 β . In fact, Bohn and co-workers

demonstrated that IL-18 is involved in the regulation of cytokine production during the early phase of bacterial infections and in the clearance of bacteria in mice [34]. IL-12 was another cytokine demonstrating increased concentrations in both the burn and CLP groups in this study. IL-12 plays an important role in the development of T helper (Th) 1 autoimmune responses [32]. It is produced by monocytes, macrophages, dendritic cells, neutrophils and B cells [35]. Administration of IL-12 in chimpanzees by intravenous injection induced increases in plasma concentrations of IL-15, IL-18, and IFN- γ , and a marked anti-inflammatory cytokine response (IL-10, IL-1 receptor antagonist) and secretion of different chemokines [36]. IL-10 is one of the most important anti-inflammatory cytokines which has been studied extensively to characterize the immune responses in different animal models. However, only in the burn group an increase in IL-10 concentration was detected. IL-10 is mainly synthesized by CD4⁺ Th2 cells, monocytes and B cells [37]. IL-10 is regarded as modulator of the pro-inflammatory response by inhibiting production of TNF- α and IL-1 β . IL-10 is also capable of decreasing the IL-18 mRNA expression in monocytes [38].

3.3 Comparison of Injury Models

In this study three different injury models were analyzed. When these are compared, certain mediators exhibit insult-specific behavior whereas others exhibit similar response across all animal groups (Table 2). GCSF, IL-10 and IL-1 α were only identified in the burn group, whereas GM-CSF and Eotaxin were specific to CLP. IL-17, IFN and IL-1 β were only observed in Sham-CLP group which received a sterile surgical treatment. A significant number of overlapping cytokines/chemokines between CLP and other groups were identified, including IL-18, IL-12, IP-10, Leptin, MCP-1 and GRO/KC (Table 2). Among them, MCP-1 and GRO/KC are the only mediators observed in all groups.

We further clustered the temporal profiles of the differentially produced cytokines in all three groups and represented them as heat maps (Figure 6) to elucidate and compare the dynamic patterns. IL-1 α was down regulated in the burn group whereas GM-CSF, Leptin and IP-10 concentrations decreased following the CLP treatment in a similar fashion. In SCLP group, additional cytokines and chemokines were down-regulated including IFN, IL-1 β , IP-10, Leptin and IL-17. In the burn group, IL-12, IL-10, IL-18 and GCSF were up-regulated at the early stage while GRO/KC and MCP-1 at the late stage around t=8–12 h. Similarly, early up-regulation of IL-10 concentration was also reported by Gauglitz et al. [6] whereas they showed MCP-1 concentration was increased at the early stage in the rat model receiving 60 % TBSA burn. Finnerty et al. [7] also elucidated an early up-regulation in GRO/KC concentration in mice following the 35 % TBSA burn. The chemokine/cytokine dynamic patterns in the burn group (Figure 6) were not observed in SCLP or CLP groups in this study. MCP-1 and GRO/KC concentrations were increased at the early stage and moreover MCP-1 concentration showed a persistent elevation in both groups. It is noteworthy that IL-12 profile peaked early around 4 hour postburn, while it peaked much later, at 16 hours following CLP (Figure 6). Similarly, Eotaxin concentration was only increased in the CLP group and also exhibited a peak around t=16 h. However concentrations of both Eotaxin and IL-12 were up-regulated for a short period of time in CLP group.

In this study, we did not observe any significant change in the concentration of TNF- α , IL-1 β and IL-6, in either burn or CLP groups. Similarly, Murphy et al. [39] observed no significant differences between sham and burn (25 % TBSA) mice in the plasma concentrations of TNF- α , IL-6 and IL-10 after the injury. In fact, conflicting observations regarding the cytokine expressions in animal models have been extensively reported in literature. It is obvious that variations in experimental procedures, and size and severity of injuries as well as utilizing different species might result in different consequences in cytokine profiles. Barber and co-workers [9] observed that cytokine concentrations

significantly increased when burn size increased in rats. Walley et al. [10] used mice and three needle sizes (18 GA, 21 GA and 26 GA) for CLP treatments. They observed that as the diameter of the CLP needle decreased, the TNF- α and IL-6 concentrations decreased and IL-10 concentration increased. Klein and co-workers [12] used rats for 30% TBSA burn, and observed that IL-1 beta and TNF α increased after burn. On the other hand, Gauglitz and co-workers [6] utilized rats receiving full thickness burn of 60% TBSA. They observed that serum concentrations of TNF- α were not found to be significantly different although other cytokines or chemokines including IL-10, MCP-1 and GRO (CINC-1) were significantly elevated after burn. We also observed significant elevations in IL-10, MCP-1 and GRO concentrations in rats receiving 20 % TBSA burn injury. Conflicting observations were also reported in the studies conducted in burn or septic patients or human subjects. Interestingly in some studies, a transient increase in TNF- α concentration was observed in burn patients [40]. It has been also shown that IL-1 β was not detected in the plasma of burn patients [41] although its plasma concentration generally increases after burn injury with septic shocks in human or it is positively correlated with burn size [42].

3.4 Putative Transcription Factors

By searching the conserved regions of promoters, we identified putative transcription factors (TFs) of the significantly altered cytokines that might participate in the regulation of their corresponding genes (Table 3). It is well known that many signaling pathways including MAPK, Jac/STAT, and Ik-B/NF-k β cascades where NF-k β , Stat, and C/EBP- β transcription factors play key roles are activated in various tissues in the early phase of burn and septic shocks [12, 43–45]. NF-k β , Stat, and C/EBP- β are well known regulators involved in cytokine production. These were also identified in our analysis (Table 3) and were found to be associated with GRO/KC, MCP-1, Eotaxin and IL-10. It is also remarkable to say that cytokines or chemokines produced by these TFs eventually trigger the signaling pathways activating the similar TFs. IL-18, IL-12 and most of the chemokines activate JAK/STAT and MAPK signaling pathways [46]. IL-10 (which was significantly elevated in the burn group in our study) induces STAT activation which promotes the transcription of Suppressor of Cytokine Signaling 3 (SOCS3), a negative feedback regulator inhibiting many inflammatory cytokines such as TNF- α , IL-6, and IL-1. There are also other putative TFs identified (Table 3) such as ETS, SP1, GATA and VTBP which have been known to regulate the function of immune system and play important role in the inflammation. ETS transcription factors activate immunity-related genes including promoters of IL-12 and IL-18 [47].

Although more complicated experimental analysis is required to identify the functional transcription factors regulating cytokine expression levels, bioinformatics analysis like the one presented in this work provides insights on the signaling system and identifies possible targets for the experimental researches. It is well known that NF-k β regulates the expression of a number of cytokines and chemokines including IL-1, TNF and IL-6 [48]. It is also known that the transcription factor CREB might show an anti-inflammatory behavior by inhibiting NF-k β signaling [49]. We identified that CREB is a putative TF of IL-18 whose concentration was elevated in both burn and CLP groups. On the other hand, IL-1, TNF and IL-6, mainly regulated by NF- k β , were not identified in burn and CLP groups in this study. Another interesting finding is that ETS1 factors might have potential to regulate the MCP-1 and GRO/KC chemokines which had similar temporal profiles. It has also been reported in literature that inhibition of ETS1 up-regulates IL-10 concentration [50]. IL-10 was significantly elevated at the early phase of the inflammatory response in the burn group. However, MCP-1 and GRO/KC concentrations were elevated when IL-10 concentration decreased in the burn group (see Figure 6). On the other hand, in CLP and SCLP groups, we did not observe significant changes in IL-10 concentration, but GRO/KC and MCP-1 were increased at the early stage and more persistent elevated MCP-1 was observed in CLP and

SCLP groups when compared to the burn group (Figure 6). Due to the high interdependency of physiological processes, integrating multiple sources together with the bioinformatics tools in attempt to answer the question to what extent transcription factor activation affects the circulatory inflammatory mediators provide an overview of the global regulation. This is also crucial to elucidate important regulatory points that can be used to modulate the inflammatory state at molecular level.

It is interesting that there is a certain number of putative TFs that might regulate the same cytokine (Table 3). Similarly, a TF might also regulate more than one cytokines. It should be noted that the tissue specificity was not considered in this study to determine the binding sites in the promoters. The sequence of the promoter regions of the cytokine genes was only analyzed to determine the putative TF binding sites. Although a set of TFs has potential to regulate the same cytokine, the question is whether, and how, this affects cytokine expression patterns in different injury models. It should be kept in mind that peripherally circulating cytokines are secreted by various tissues or cells including leukocytes, glial cells and liver, etc. It is well known that inflammatory response of a tissue might be different than that of another tissue, and similarly the host response depends on type of the injury [51]. Temporal and quantitative differences in the activation of TFs in various injury models explain the diversity of cytokines' patterns.

Complex interactions of cytokines or chemokines through the signaling network is a big challenge for studies aiming at determining new therapeutic targets to eliminate deleterious effects of chemokine or cytokine related disease states. Inhibition of certain mediator receptor interactions might be required in order to eliminate the negative effects of disorders caused by inappropriate mediator receptor regulations. However, this might lead to activation or deactivation of undesired upstream signaling pathways. For example, using inhibitors or animal models deficient in certain chemokines-receptors including CXCR2 or CCR1 demonstrated improvement in sepsis formation and sepsis related lethality in animals [52–53]. On the other hand, inhibiting chemokine receptors might also weaken antibacterial resistance of host by up-regulating the IL-10 [54]. Consequently, a comprehensive understanding of underlying mechanisms of the inflammatory response is essential to have a more control over the proposed therapeutic approaches.

The mammalian regulatory landscape is highly complex and redundant conferring highly robust characteristics to the host [55–56]. Although very preliminary, the results of our TF analysis begin to point towards the direction of elucidating the structure and networks of transcription factors as these emerge as major contributors to regulation [57]. The idea of inferring functional interactions by observing the dynamics of regulated signals has been previously explored in the context of liver-specific responses to soluble signals [58], whereas we recently demonstrated how the regulated dynamics can begin to elucidate implicit upstream interactions among transcription factors [59–60]. In this context the information generated through our preliminary analyses enables the initial formulation of putative injury-specific modules of regulatory interactions (Figure 7). Undoubtedly, a lot of work still remains to be accomplished, but we wish to demonstrate that it is indeed plausible to integrate in vivo responses and bioinformatics tools towards the ultimate goal of describing testable hypotheses related to critical regulatory interactions.

4. Concluding Remarks

In this study, we characterized the circulating cytokine profiles in the first 24 hours following 20% TBSA burn injury, SCLP, and CLP. Burn injury alone was used to investigate the effect of a systemic but “sterile” pro-inflammatory insult. CLP was used to investigate the effect of a bacterial infection, also a pro-inflammatory, but different,

stimulus. Since CLP involves laparotomy, a surgical procedure, it was also necessary to include a SCLP group to ascertain the effect of the surgical procedure vs. that of the infection itself. While none of the insults caused mortality, CLP caused a transient decrease in body weight. We found that a certain number of cytokines and chemokines, belonging to the pro- and anti-inflammatory types, were altered by these insults. Furthermore, each insult was associated with a different cytokine signature profile.

Experimental observations can be very noisy. Problems can always arise in multiplex assays because of potential interactions between different antibodies and cytokines, and the presence of varying dynamic mutual-effects between rare and abundant cytokines in the samples [61]. The limited number of replicates in animal experiments can be also an additional complicating factor. In serial sacrifice designs where only one sample is taken from each animal, significant differences in the physiologic concentrations of circulatory proteins between the samples can be observed. Moreover, cyclic fluctuations (or circadian rhythm) in cytokine expressions, which affects the inflammatory response of the body [62], can display 24 h period and even persist in the absence of external timing information [63]. Therefore, due to the dynamic behaviors and fluctuations observed in physiological processes, it was important to utilize an appropriate statistical approach to identify differentially expressed proteins over a time course. Although existing statistical methods such as ANOVA test are more appropriate for static sampling designs, these might not be applicable for time course experiments where detecting differences in the physiological behaviors of two or more groups over time is essential [64]. The AUC is considered as an important indicator for drug availability and assessing the net pharmacological response of a given dose of drug [22–23]. We recently explored this method to analyze gene expression time course data [21]. Similarly, the same concept can be applied for temporal experiments aiming at identifying differentially expressed proteins. This provides a quantitative estimate of overall exposure to cytokines which can be obtained by integrating the concentration curve over time. This also considers the temporal variability in the cytokine concentrations of control groups.

In conclusion, we analyzed the early stage (first 24 hours) serum cytokine and chemokine profiles in moderately injured rats receiving 20 % TBSA burn injury and CLP treatment. We identified cytokines and chemokines which were significantly elevated in the burn and CLP groups. Determining cytokine expression patterns in different animal models receiving different injuries would provide critical insights regarding the complex interactions of those inflammatory mediators at the cellular level enabling a better characterization of the inflammatory response.

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Figure 1.
Body weight change (%) in CLP (solid line) and SCLP (dotted line) groups. $n \geq 10$.

Figure 2.

Cytokine and chemokine profiles in Burn (red dot) and Sham (blue circle) groups. Each circle or dot represents an independent sample, and lines pass through average cytokine profiles at each time point. The white and grey colors represent light and dark cycles respectively.

Figure 3.

Cytokine and chemokine profiles in SCLP (red dot) and Sham (blue circle) groups. Each circle or dot represents an independent sample, and lines pass through average cytokine profiles at each time point. The white and grey colors represent light and dark cycles respectively.

Figure 4.

Cytokine and chemokine profiles in CLP (red dot) and Sham (blue circle) groups. Each circle or dot represents an independent sample, and lines pass through average cytokine profiles at each time point. The white and grey colors represent light and dark cycles respectively.

Figure 5.

Cytokine and chemokine profiles in CLP (red dot) and SCLP (blue circle) groups. Each circle or dot represents an independent sample, and lines pass through average cytokine profiles at each time point. The white and grey colors represent light and dark cycles respectively.

Figure 6.

Heat maps comparing the treatment groups (Burn, CLP and SCLP) with the control group (Sham). Green indicates the lowest level while red indicates the highest and black average level.

Figure 7.

A. The regulatory mechanism between NF- κ B and CREB. In burn and CLP groups, only IL-18 of which CREB is a putative TF was elevated, however no changes in IL-1, IL-6 and TNF concentrations were observed in both treatment groups. **B.** The regulatory mechanism of ETS1. ETS1 was identified as a putative TF of MCP-1 and GRO/KC. In burn group, these two cytokines' concentrations were increased while IL-10 concentration was decreased. Note that straight lines indicate known regulatory mechanisms (based on literature) and dashed lines represent putative mechanisms estimated from this study based on experimental observations and TF analysis.

Differentially produced cytokines and chemokines. Observed P values of cytokines obtained from AUC method which are filtered out by Benjamin & Hochberg criteria are only given.

Table 1

S vs B		S vs SCLP		S vs CLP		SCLP vs CLP		Benjamin & Hochberg	
Cytokines & Chemokines	P value (AUC)	Cytokines & Chemokines	P value (AUC)	Cytokines & Chemokines	P value (AUC)	Cytokines & Chemokines	P value (AUC)	<i>l</i>	$p_{l=a(l/z)}$
MCP-1	0.00023 ($P \leq p_{l=1}$)	IP-10	0.00012 ($P \leq p_{l=1}$)	MCP-1	1.00E-05 ($P \leq p_{l=1}$)	IL-12P70	0.0005 ($P \leq p_{l=1}$)	<i>l</i> =1	0.0022
IL-18	0.0008 ($P \leq p_{l=1}$)	GROK	0.00036 ($P \leq p_{l=1}$)	GMCSF	0.00037 ($P \leq p_{l=1}$)	IL-18	0.001 ($P \leq p_{l=1}$)	<i>l</i> =2	0.0044
GCSF	0.0017 ($P \leq p_{l=3}$)	MCP-1	0.0004 ($P \leq p_{l=3}$)	IP-10	0.0004 ($P \leq p_{l=3}$)	Eotaxin	0.0014 ($P \leq p_{l=3}$)	<i>l</i> =3	0.0065
GROK	0.003 ($P \leq p_{l=4}$)	IL-1beta	0.0024 ($P \leq p_{l=4}$)	IL-18	0.0008 ($P \leq p_{l=4}$)	IL-4	0.009 ($P \leq p_{l=4}$)	<i>l</i> =4	0.009
IL-10	0.004 ($P \leq p_{l=5}$)	Leptin	0.004 ($P \leq p_{l=5}$)	IL-12P70	0.001 ($P \leq p_{l=5}$)			<i>l</i> =5	0.011
IL-12P70	0.004 ($P \leq p_{l=6}$)	IL-17	0.01 ($P \leq p_{l=6}$)	Leptin	0.0022 ($P \leq p_{l=6}$)			<i>l</i> =6	0.013
IL-1alpha	0.007 ($P \leq p_{l=7}$)	IFN	0.011 ($P \leq p_{l=7}$)	GROK	0.012 ($P \leq p_{l=7}$)			<i>l</i> =7	0.015
				Eotaxin	0.012 ($P \leq p_{l=8}$)			<i>l</i> =8	0.017

Table 2

Grouping the cytokines and chemokines based on their concentration changes in three injury models. Some mediators (MCP-1 and GRO/KC) are observed in more than one group whereas some are specific to an injury type.

Cytokines/Chemokines	Sham vs Burn	Sham vs CLP	Sham vs SCLP
GCSF	√		
IL-10	√		
IL-1A	√		
GMCSF		√	
EOTAXIN		√	
IL-17			√
IFN			√
IL-1beta			√
IL-18	√	√	
IL-12	√	√	
IP-10		√	√
LEPTIN		√	√
MCP-1	√	√	√
GRO/KC	√	√	√

Table 3

Putative transcription factors of some cytokines and chemokines observed in burn and CLP groups.

Transcription factors	GRO/KC	MCP-1	Eotaxin	Leptin	IL-18	IL-12	IL-10
ETSF (Human and murine ETS1 factor- E-twenty six family)	✓	✓			✓	✓	
MYOD (Myoblast determining factors)	✓						✓
NFKB (Nuclear factor kappa B/c-rel)	✓		✓				
PBX1 (PBX1 - MEIS1 complexes)	✓						
SORY (SOX/SRY-sex/testis determining and related HMG box factors)	✓						
STAT (Signal Transducer and Activator of Transcription)		✓	✓				✓
PARF (PAR/bZIP family)			✓				
CEBP (CCAAT-enhancer-binding proteins)			✓				✓
SPI1 (GC-Box factors SPI/GC)			✓	✓			
EREF (Estrogen Response Element family)							
VTBP (TATA binding protein factor)			✓		✓		✓
BRNF (Bm POU domain factors)					✓		
CAAT (CCAAT binding factors)					✓		
CREB (cAMP response element-binding)					✓		
FKHD (Fork head domain factors)					✓		
HNF1 (Hepatocyte nuclear factor 1)					✓		
NF1F (Nuclear Factor 1)					✓		
OCT1 (Octamer-binding transcription factor 1)					✓		
GATA (GATA binding factors)				✓		✓	
HAND (Twist subfamily of class B bHLH transcription factors)						✓	
KLFS (Krupple like factor family)				✓			

Note: These are transcription factor families, each of which includes several TFs that have similar binding sites.