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The dynamics of the early inflammatory response in double-hit burn and sepsis animal models

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ABSTRACT

Severe burn trauma is generally associated with bacterial infections, which causes a more persistent inflammatory response with an ongoing hypermetabolic and catabolic state. This complex biological response, mediated by chemokines and cytokines, can be more severe when excessive interactions between the mediators take place. In this study, the early inflammatory response following the cecum ligation and puncture (CLP) or its corresponding control treatment (sham-CLP or SCLP) in burn (B) male rats was analyzed by measuring 23 different cytokines and chemokines. Cytokines and chemokines, including MCP-1, IP-10, leptin, TNF-α, MIP-1α, IL-18, GMCSF, RANTES and GCSF were significantly altered in both B+CLP and B+SCLP groups. IL-10 and IL-6 were significantly up-regulated in the B+CLP group when compared to the B+SCLP group. Down regulation of leptin and IP-10 concentrations were found to be related to surgery and/or infection. IL-18 and MCP-1 were elevated in all groups including previously published single injury models receiving similar treatments. In this study, insult-specific mediators with their characteristic temporal patterns were elucidated in double hit models.

1. Introduction

Burn injury initiates a biological cascade of events where different inflammatory mediators take place. The local mediators and endotoxins secreted by damaged tissues induce expression of pro-inflammatory cytokines (IL-1, TNF-α, IL-18, IL-6 and IL-12), chemokines (Eotaxin, G-CSF, GM-CSF, GRO/KC, MCP-1, MIP-1α and RANTES), and anti-inflammatory mediators (IL-4, IL-10, IL-13, stress hormones and cholinergic anti-inflammatory). Inflammation is known as a self limiting process but persistent response can lead to excessive tissue injury. Although burn wound is an important portal of entry for microbes [1], pathogen originating from gastrointestinal tract is one of the most serious complications, which is not fully understood. Experimental observations demonstrate that there is a loss of physical barrier function in gastrointestinal tract after burn injury, which might be because of physical disruption of the mucosal barrier, intestinal over-growth of bacteria and suppression of the immune defense [2]. Increasing endotoxin concentrations secreted by damaged tissues and inflammatory cytokines in the circulation can behave as immunosuppressive agents which decrease T-cells population [3,4], and affect the mesenteric lymph [5,6]. Decreases in intestinal blood flow due to the vasoconstriction [2] and increase in nitric oxide and free radicals production stimulated by cytokines and endotoxin agents [7–9] further damage the endothelial cells in gut/digestive system and minimize the mucosal barrier, which increases the intestinal permeability.

Animal models of burn injury, cecal ligation and puncture, and endotoxin injection have previously been used to profile circulating mediator concentrations after the insult. Murphy et al. [10] observed no significant differences between control (sham) and burn mice receiving 25% total body surface area (TBSA) burn in the plasma concentrations of TNF-α, IL-6 and IL-10 after the injury. Barber and co-workers [11] showed that cytokine concentrations significantly increased when burn size increased in rats. Walley et al. [12] used mice and three different needles (18 GA, 21 GA and 26 GA) for CLP treatments. They elucidated 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 [13] 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 [14] utilized rats receiving full thickness burn of 60% TBSA. They showed that serum concentrations of TNF-α were not found to be significantly different, on the other hand other cytokines were significantly elevated after the burn. Although these studies have elucidated important information regarding the circulatory cytokine concentration changes, they are limited to one type of insult or a limited number of cytokines. There are other mediators that should be further characterized during the inflammation. It

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was 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, a protein important in regulating the energy metabolism, was found to reduce 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].

Variations in experimental procedures, and size and severity of injuries [11–14] as well as utilizing different species [10] might result in different inflammatory response. Interactions of cytokines and chemokines with their corresponding receptors cause very comprehensive signaling patterns. Therefore, quantitative and temporal differences in the secretion of mediators during the inflammation result in different host response. Consequently, it is very challenging to determine new therapeutic targets to eliminate deleterious effects of inflammation by interfering with the signaling pathways. For example, it has been shown that utilizing some inhibitors or animal models deficient in certain chemokines receptors might protect the host and improve the mortality [17–20], however this might also weaken the antibacterial resistance of host [21,22]. Published studies in the literature have already revealed that many signaling pathways such as MAPK, JAK/STAT, and IkB/NF-kappaB cascades where transcription factors including NF-kappaB, Stat, and C/EBP-β play critical role are activated in various tissues after burn and sepsis [13,23–25]. Searching the conserved regions in the cytokine promoters of different species also revealed that there are other putative transcription factors which might regulate the corresponding cytokines [26]. The large degree of overlap between transcription factors which are activated by cytokines results in complex and unpredictable responses. Therefore a comprehensive understanding of these complex responses is required to have a more control over the proposed therapeutic approaches.

We have previously analyzed single injury rat models receiving a "sterile" cutaneous dorsal burn on 20% of the total body surface area (TBSA) or a cecum ligation and puncture treatment (CLP) to induce infection [26]. It was shown that MCP-1, GROK/KC, IL-12, IL-18 and IL-10 were significantly altered in both burn and CLP groups. It was also elucidated that leptin and IP-1 concentrations were decreased in both CLP and sham-CLP groups. In the current study, by monitoring the response of 23 different cytokines and chemokines, we further analyzed a double-hit animal model receiving a 20% TBSA burn injury followed 2 days later by CLP treatment, which has been proposed by Banta and coworkers to mimic the patho-physiological changes observed in burn-septic patients [27]. Determining various cytokine or chemokine concentrations in serum and their characteristic patterns during the shock stage of inflammation in double-hit animal models would provide a better understanding of underlying mechanism of inflammatory response. These studies are important since they provide insights to decide whether a strategy is worth evaluating in the clinical arena, and if so, under what circumstances the use of that strategy may be limited in a particular group of patients.

2. Materials and methods

2.1. Animal model

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

A detailed experimental design is illustrated in Fig. 1. Experimental procedures for double-hit animal models have been described previously in detail [27]. Rats first received a full-thickness burn on an area of the dorsal skin corresponding to 20% of the total body surface area (TBSA). This model has nearly 100% long-term survival, no evidence of systemic hypoperfusion, and no significant effect on feeding pattern [28–30]. Rats were anesthetized by intraperitoneal injection of 80–100 mg/kg ketamine + 12–10 mg/kg xylazine, and all hair removed from the dorsal abdominal area using electric clippers. The animal's back was immersed in water at 100 °C for 10 s to produce a full-thickness scald injury covering 20% TBSA. Immediately after burns, the animals were resuscitated with 50 mL/kg of saline injected intraperitoneally. Negative controls (sham group) consisted of animals treated identically but immersed in warm water (37 °C). Rats were single caged after burn and given standard rat chow and water ad libitum until sacrifice. No post-burn analgesics were administered, consistent with other studies since the nerve endings in the skin are destroyed and the skin becomes insensate in full thickness burn model [31–33]. Furthermore, after animals woke up, they ate, drank and moved freely about the cage, responded to external stimuli, and did not show clinical signs of pain or distress.

Infection was induced by cecal ligation and puncture (CLP) 2 days after the burn injury (Fig. 1). CLP is an animal model that mimics the physiological changes in human sepsis [34] and clinically relevant since it induces inflammatory response. Rats were first anesthetized, and then the analgesic buprenorphrine was given subcutaneously at 0.01–0.05 mg/kg. Animals were then placed in supine position and hair was shaved on the abdomen. Bupivicaine (0.125–0.25%) were applied 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 that intestinal obstruction was not produced. We took care to not ligate the cecal branch of the ileocecal artery, thus preserving viability of the cecum itself, in order to increase the survival rate. The cecum was punctured four 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. Negative controls (sham CLP or SCLP) consist of animals treated identically without receiving cecal ligation and puncture, i.e. they were anesthetized, undergo laparotomy as described above, but no surgical manipulation of the cecum was performed. Rats were

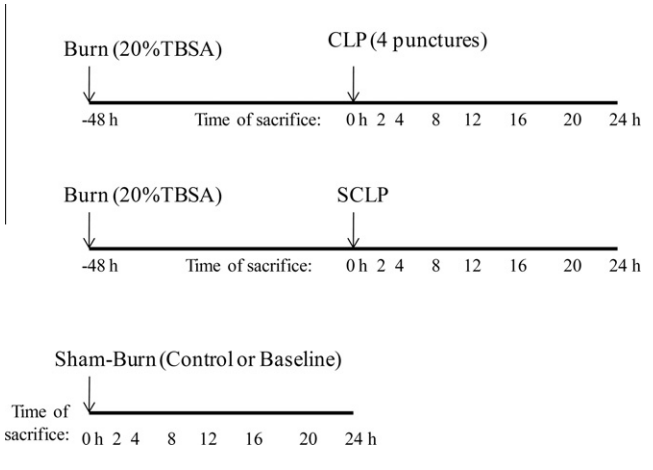

Fig. 1. Experimental plan.

single caged after the treatments and given standard rat chow and water ad libitum until sacrifice.

2.2. Cytokine analysis

Animals were anesthetized and sacrificed at different time points (2, 4, 8, 12, 16, 20 and 24) ($n = 3$ per time point per group) (Fig. 1). Blood samples collected from *vena cava* by heparinized catheters were stored on ice until serum preparation. Serum was separated by centrifugation at 4500 rpm for 3 min at 4 °C and stored at −80 °C until analyzed. 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-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IP-10, leptin, MCP-1, MIP-1α, RANTES, TNF-α, VEGF) using the manufacturer's manual.

2.3. Data analysis

Many existing methods, such as ANOVA, do not take the time scale into account, i.e. the inherent ordering and spacing provided by the time points are ignored, since each time point is treated as different condition with perfectly balanced repeated measures (the same number of observations). On the other hand, in general, there are limitations in more complicated biological studies where animals are used, such as missing observations in some points, limited number of repeats, or unbalanced repeats among time points. Therefore, in order to detect differences in the physiological behaviors of two or more groups, the concept of area under the curve (AUC) and the method (EDGE) proposed by Storey and Tibshirani [35] were applied to analyze the time course experimental data. The method EDGE explores the goodness of a time-dependent curve fit (this can be a polynomial basis or cubic spline basis) to a series of data points. Therefore, this method is very powerful when temporal differences between the groups are investigated. On the other hand, we have already explored the AUC method to assess the dynamic responses of cytokines and chemokines [26] and to analyze the time course microarray data [36]. This method was used to compare the areas under the cytokine concentration–time curves belonging to different groups, thus it is a very useful tool when the overall response of a cytokine to a particular condition is investigated [26].

In this study, three different groups (two "double hit" models which are Burn+CLP and Burn+SCLP, and a control group or sham group receiving no injury or any surgical treatment) were compared (Fig. 1). In AUC method, for each cytokine, the overall AUC (area under the cytokine concentration–time curve in treatment groups, i.e. B+SCLP and B+CLP groups) and baseline AUC (area under the cytokine concentration–time curve in control group) were identified numerically with their standard deviations [26]. Then these values were compared if the overall AUC significantly deviates from the baseline AUC by identifying P-value using t-distribution (two-sided) and Sattertwaite's approximation for degree of freedom [26]. A brief explanation was given in the "Supplementary materials". Storey's method, on the other hand, has been already implemented under the EDGE software package [37]. The statistical test used is analog of F statistics that compares the goodness of fit of the model under the null hypothesis to that under the alternative hypothesis. The null hypothesis model is obtained by fitting a curve to the two or more groups combined, and the alternative hypothesis model is obtained by fitting a separate curve to each group. For the consistency of analysis, the algorithm proposed by Benjamini and Hochberg [38] was used to determine a data-based p-value threshold controlling the false discovery rate at the level of 0.05 for both AUC and EDGE methods.

In order to compare and analyze the dynamic patterns of the inflammatory mediators further, heat maps were generated by the "clustergram" function in MATLAB which was used to cluster the differentially produced cytokines and chemokines (hierarchical clustering).

3. Results

3.1. Animal weight changes and mortality

We have previously showed that "single hit" models receiving 20% TBSA burn injury or CLP with four punctures survived within 1 week [26]. In this study, "double hit" animal models receiving 20% TBSA burn injury followed 2 days later CLP with four punctures did not show any significant mortality. Similar to previous findings [26,27], CLP approximately caused 10% weight lost on each individual burn-animal. 20% TBSA burn and sham treatments did not resulted in any significant weight lost (data not shown). CLP induces hypermetabolic and catabolic state and it is more realistic than endotoxin injection. However, the mortality rate following the CLP depends on the strain and the experimental procedures used such as length of the cecum ligated, size of the needle, and the number of punctures. It is noteworthy to mention that in this study ligating the cecal branch of the ileocecal artery was avoided to preserve viability of the cecum itself, which resulted in high survival rate, consistent with previous observations [26,27].

3.2. Cytokine profiles in B+SCLP group

Cytokine profiles in control group which did not receive any injury such as burn or surgical application was compared with the profiles obtained from other groups (B+SCLP and B+CLP). SCLP treatment, an abdominal surgery without puncturing the cecum, also results in inflammatory response [26]. Fig. 2 shows the P-values obtained from AUC and EDGE methods when control group was compared with B+SCLP group. P-value threshold determined by Benjamini and Hochberg algorithm [38] to control the false discovery rate in the cytokine data was found to be less than 0.02 in both methods. Both AUC and EDGE methods identified that MCP-1, GMCSF, TNF-α, MIP-1α and GRO/KC were found to be significantly altered after the SCLP treatment in burn animals. In addition to these, the AUC method also determined that overall responses of IP-10, leptin, IL-12P70 (or IL-12), IL-18 and GCSF were significantly changed in B+SCLP group (Fig. 2), although comparison of time-dependent curve fits of the cytokines/chemokines by EDGE identified a significant difference in the profiles of RANTES. MCP-1, GMCSF, IP-10, GCSF, RANTES and GRO/KC are chemotactic cytokines. In general, their functions are to recruit the white blood cells to the site of damaged tissue and activate the immune cells. TNF-α and IL-18 are pro-inflammatory cytokines. IL-12 is, on the other hand, both pro- and anti-inflammatory cytokine. leptin is a hormone controlling the energy metabolism of the body.

To better elucidate the dynamic patterns of the inflammatory mediators and compare them, we further clustered the temporal profiles of the differentially produced mediators and represented them as heat maps (Fig. 3). A detailed representation of original data is provided in "Supplementary materials". IL-18 and TNF-α showed a similar dynamic pattern in B+SCLP group. Concentrations of these two pro-inflammatory cytokines were up-regulated at the early stage of inflammation, and they were elevated until 12 h post-treatment. Concentration profiles of IP-10 and leptin were also found to be similar. They were persistently suppressed following the SCLP treatment in burn group. GMCSF is a chemokine whose concentration was also decreased after the treatment. Concentration of IL-12 started to increase around 4 h following the

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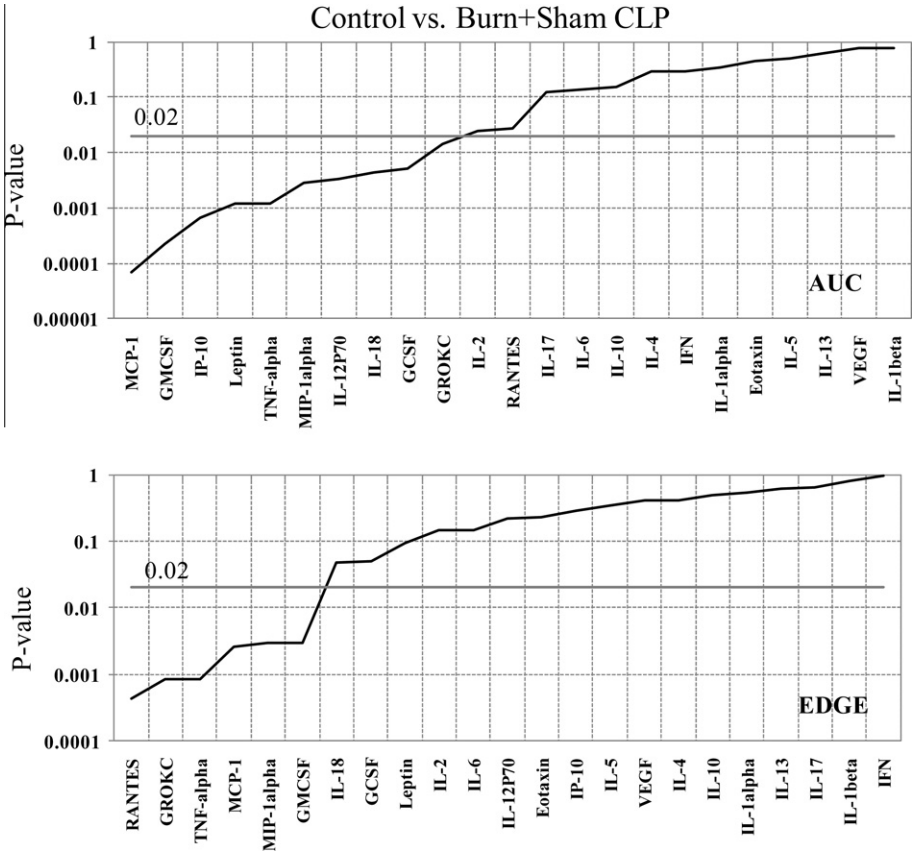

Fig. 2. *P*-values identified by the AUC and EDGE methods when control group was compared to B+SCLP group.

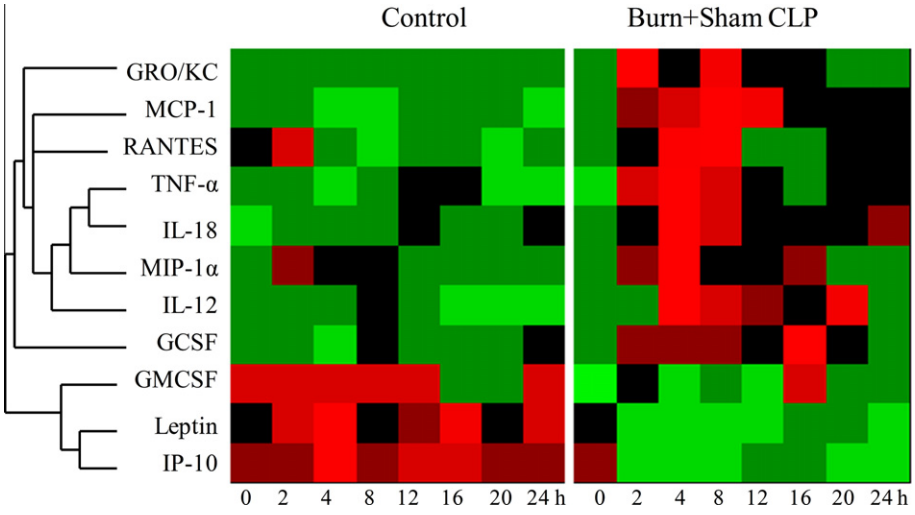

Fig. 3. Comparison of cytokine and chemokine profiles in control and B+SCLP groups. Green indicates the lowest level while red indicates the highest and black average level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SCLP treatment. The chemokines which are GRO/KC, MCP-1, MIP-1α and GCSF were elevated at the early stage. Concentrations of most of the cytokines and chemokines including GRO/KC, MIP-1α, IL-12 and GCSF went back to their baseline values around 24 h post-treatment.

3.3. Cytokine profiles in B+CLP group

The B+CLP group was compared to control group. The *P*-values identified by the AUC and EDGE methods were given in Fig. 4. Benjamini and Hochberg correction method with a false discovery rate of 0.05 also identified that the *P*-values less than 0.02 were significant in both methods when the cytokine data of B+CLP group was compared to that of control group. The AUC and EDGE methods identified that GCSF, MIP-1α, MCP-1, RANTES, IL-6 and IL-10 were found to be significantly changed in B+CLP group. Moreover, the AUC method further determined that overall responses of IP-10, leptin, IL-18, and TNF-α were significantly altered (Fig. 2). On the other hand, EDGE identified that temporal profiles of GMCSF and VEGF in B+CLP group were significantly different than that of

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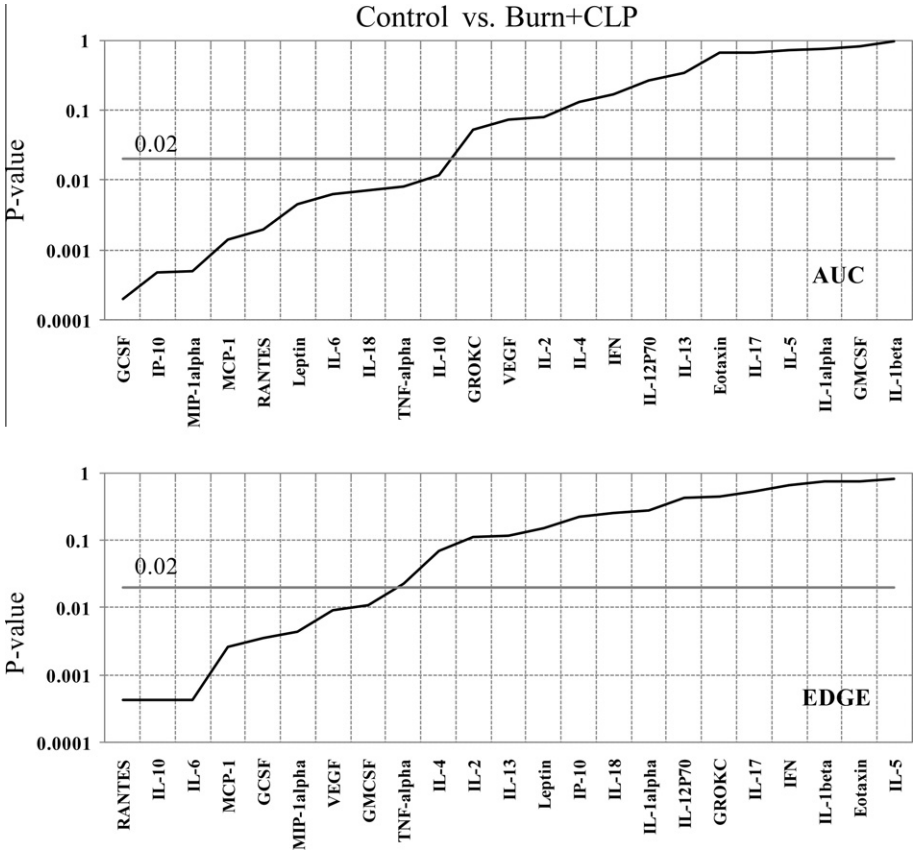

Fig. 4. *P* values identified by the AUC and EDGE methods when control group was compared to B+CLP group.

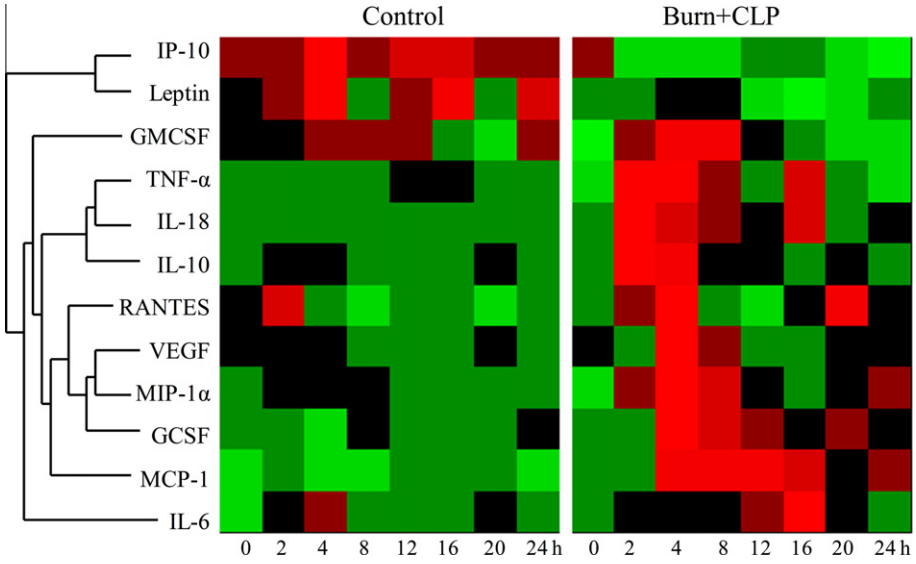

Fig. 5. Comparison of cytokine and chemokine profiles in control and B+CLP groups. Green indicates the lowest level while red indicates the highest and black average level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control group, which was not observed by AUC method. VEGF is a chemotactic protein for macrophages and granulocytes. IL-6 whose concentration was significantly altered in B+CLP group is a well known inflammatory cytokine which has both pro- and anti-inflammatory behaviors. IL-10 is also observed in this group, and this cytokine is a well studied anti-inflammatory cytokine.

Fig. 5 shows the dynamic patterns of the cytokines and chemokines which have been clustered. Similar to the observation in B+SCLP group, it was identified that IL-18 and TNF-α had the same concentration profiles in B+CLP group. These two cytokines were elevated at the early stage of the inflammation (around 2 h post-treatment). They further exhibited a second peak around 16 h. However, their concentrations went back to their baseline values around 20 h post-treatment. IL-18 is functionally very similar to IL-1 cytokine. These pro inflammatory cytokines, involving TNF-α as well, activate the same signaling cascades including MAPK and

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NF-kappaB signaling pathways. Moreover, cytokine clustering analysis elucidated that IP-10 and leptin had similar dynamic pattern in B+CLP group too. They were also persistently suppressed following the CLP treatment in burn group. The anti inflammatory cytokine, IL-10, was up-regulated at the early stage of 24 h post-injury period. However, the elevation in its concentration was not found to be persistent. IL-6 which is a late inflammatory cytokine showed a peak around 16 h. Its concentration level went back to the baseline value at the end of 24 h post-injury period. RANTES, MIP-1α, GCSF, VEGF and MCP-1 are the chemokines whose concentrations have been increased around 4 h post-injury. Their concentrations were, in general, found to be elevated for 24 h post-injury period when compared to their baseline values, and moreover GCSF and MCP-1 were increased more persistently.

When B+SCLP group was compared to B+CLP group (Fig. 3 vs. Fig. 5) to identify the infection related cytokines/chemokines in burn animal, it was elucidated that IL-10 and IL-6 concentrations were only observed in B+CLP group. Although GRO/KC and IL-12 were significantly altered in B+SCLP group, there is a certain number of cytokines/chemokines including MCP-1, TNF-α, IL-18, MIP-1α, GCSF, leptin, GMCSF, RANTES and IP-10 significantly changed in both groups. The concentrations of IP-10 and leptin were decreased in both B+SCLP and B+CLP groups. In general, MCP-1, MIP-1α, TNF-α and IL-18 started to be elevated at the early stage of inflammatory response in both groups (Figs. 3 and 5), however, dynamic patterns of TNF-α and IL-18 clearly showed that they exhibited a second peak in B+CLP group, which was not observed in B+SCLP group. In both groups, MCP-1 concentration was increased persistently. GRO/KC was significantly increased in B+SCLP group, however it was not selected in B+CLP group although its *P*-value was found to be less than 0.05 by AUC method (Fig. 5). IL-10 and IL-6 were identified as CLP-related cytokines in burn group which were elevated at the early stage and late stage, respectively, for a short period of time. On the other hand, IL-12 started to increase around 4 h post injury in B+SCLP group, which was not observed in B+CLP group.

4. Discussion

In this study, short term cytokine profiles in "double hit" models were elucidated. Animals received 20% TBSA burn injury followed 2 days later by CLP or SCLP treatment. CLP model was used as an infection model because it is thought to closely mimic the physiological changes in human sepsis following the severe burn [34]. Consistent with the previous studies [26,27] no significant mortality was observed although CLP resulted in approximately 10% weight loss on each animal (data not shown).

It is important to utilize an appropriate statistical approach to identify differentially expressed proteins over a time course because of the dynamic behaviors and fluctuations observed in physiological processes. Experimental observations can be noisy, which is typically observed in multiplex assays due to the dynamic interactions of different proteins in the assays. Moreover, intrinsic regulatory mechanisms such as circadian rhythm observed in the cytokines' expressions over time, which affects the inflammatory response of the body, result in more complex temporal profiles. Therefore, we applied different methods, namely AUC and EDGE methods, to detect differences in the physiological behaviors of two or more groups over time. AUC is considered as an important indicator for drug availability and assessing the net pharmacological response of a given dose of drug [39,40]. We have already used this method to assess the dynamic responses of cytokines and chemokines [26] and to analyze gene expression time course data [36]. Similarly, the same concept can be applied in this study to provide a quantitative estimate of overall exposure to cytokines which can be obtained by integrating the concentration curve over time. The method of EDGE, on the other hand, compares the goodness of fitting a curve to the combined data of different groups with that of fitting a separate curve to each group. In spite of the differences in the underlying mechanisms, these two methods successfully identified a number of the same cytokines significantly changed in "double hit" models compared to the baselines described by control group (Figs. 2 and 4). Moreover, utilizing different statistical approaches is much more powerful. For example, AUC could identify that overall responses of IP-10, leptin, IL-18, and TNF-α were significantly altered in B+CLP group when compared to control group, which was not captured by EDGE (Figs. 2 and 4). On the other hand, EDGE elucidated temporal differences in the profiles of RANTES in B+SCLP group, and VEGF and GMCSF in B+CLP group (Figs. 2 and 4), which was not observed by AUC method.

Although, in both B+CLP and B+SCLP groups the same cytokines and chemokines including MCP-1, TNF-α, IL-18, MIP-1α, GCSF, leptin, RANTES, GMCSF and IP-10 were significantly altered, IL-6 and IL-10 were only changed in B+CLP group (Figs. 3 and 5). Moreover, dynamic profiles of TNF-α and IL-18 in B+CLP were found to be different than those of B+SCLP (Fig. 5). TNF-α and IL-18 were always found to be in the same cluster. They were up-regulated at the early stage ($t = 2$ h) and they showed a second peak at around 16 h in B+CLP (Fig. 5). However, the elevation in their concentrations was slightly more persistent and they did not show a second peak in B+SCLP group. TNF-α and IL-18 are pro- inflammatory cytokines and they are functionally similar. They activate the same signaling molecules including NF-kappaB and p38 MAPK which induce expression of various cytokines and chemokines [41,42] and augment the host defense mechanisms against infection [43,44]. Similarly, IL-10 was also increased at the early stage, but it did not exhibit a second peak in B+CLP group. IL-10 induces STAT3 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, IL-1 and IL-18. Early expression of IL-10 in B+CLP might be a protective reaction to regulate the excessive response of pro-inflammatory mediators. Therefore, this might have decreased the TNF-α and IL-18 concentration in B+CLP group. However no IL-10 was identified around 16 h post injury when TNF-α and IL-18 exhibited a second peak for a very short period of time in B+CLP group. On the other hand, IL-10 concentration was not increased or altered in B+SCLP group that might explain the profiles of TNF-α and IL-18 concentrations which exhibited a slightly persistent increase in B+SCLP group.

There are a number of chemokines including MCP-1, RANTES, MIP-1α, VEGF and GCSF which were up-regulated at the early stage of inflammatory response in B+CLP group (Fig. 5). MCP-1 showed a more persistent elevation. Chemokines, chemo-tactic cytokines having similar protein structures, are mainly involved in recruiting a large variety of white blood cells to injured area. They might also serve as pro and anti inflammatory mediators. They in general interact with G-protein coupled receptors to induce cell migrations and activation [45,46]. Chemokine receptor activation results in cellular cascades, production of inositol triphosphate including the release of intracellular calcium and activation of protein kinase C and binding proteins of Ras and Rho families involved in cell motility [45–48]. Soriano and co-workers found that chemokines, after binding transmembrane G protein coupled receptor, activate JAK/STAT signaling pathway to trigger chemotactic responses [49].

The concentration of IL-6 increased at the late stage of post-injury period ($t = 16$ h) in B+CLP group (Fig. 5). On the other hand IL-12 was increased in B+SCLP group (Fig. 3). IL-6 and IL-12 activate similar signaling cascades including JAK/STAT, and MAPK signaling pathways [50–52]. Both of them exhibit pro- and anti-inflammatory properties since they induce the Suppressor of Cytokine Signaling proteins (SOCS) through JAK/STAT signaling pathway. It is noteworthy that when pro-inflammatory cytokines including IL-18 and TNF-α showed a second peak, IL-6 was up-regulated in B+CLP group

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(Fig. 5). Moreover, the persistent elevation of MCP-1 was started to be down regulated around 16 h. Therefore, IL-6 might be an important inflammatory modulator by activating the inflammatory cascades at moderate level and repressing the excessive expression of pro-inflammatory mediators. Moreover, IL-6 might be seen in more severe injuries when compared to IL-12, since it was not observed in B+SCLP group. However IL-12 having similar functions was only observed in B+SCLP group which is a moderately severe injury model. It has been demonstrated that IL-12 treatment increased the survival rate of mice models receiving burn and CLP treatments [53]. Further studies to compare the ameliorative effects of IL-6 and IL12 to control the inflammatory response in burn and septic animals are warranted.

In the current study, we compared the cytokine and chemokine profiles following the CLP or SCLP treatment in burn animals (double-hit models) with the baseline profiles corresponding to the control group. In our previous study [26], we also compared short term cytokine and chemokines profiles in "single hit" models receiving only 20% TBSA burn injury or CLP with four punctures.

The concentrations of MCP-1 and IL-18 were significantly increased in all single and double injury models. GRO/KC was also significantly increased in single injury models as well as B+SCLP group. Leptin and IP-10 were decreased following the SCLP and CLP treatments, which implies that the alterations in these mediators might be the response of body to surgery and infection. In double hit model of B+CLP, an increased number of mediators have been identified, including well studied cytokines such as TNF-α, IL-6, and IL-10.

CLP which results in hypermetabolic and catabolic states in rodents [27], very similar to septic patients, has been extensively used to understand the effects of septic complications in the living system. SCLP, the control of CLP, is a sterile surgical treatment where cecum is not ligated and punctured. In our previous study [26] we showed that single hit model receiving SCLP alone depicts an inflammatory response. It was observed SCLP resulted in decreased IP-10 and leptin, and increased GRO/KC and MCP-1 profiles [26]. Similar observations were also obtained in double hit model B+SCLP in this study. Moreover, other cytokines and chemokines including IL-18, TNF-α, IL-12 and RANTES were also elevated, which implies that a second injury results in different cytokine profiles. Abdominal surgery in human causes systemic inflammation, leukocyte reprogramming and alters expression of toll-like receptors which play a key role during the inflammation [54]. A range of cytokines, overproduction of which might have detrimental effects on organ function and contribute to the enhanced risk of septic complications, is sequentially released into the peritoneal fluid following abdominal surgery [55]. Therefore, it is very critical to understand the mechanism of inflammation after surgical treatments to eliminate its undesirable consequences since inflammatory response caused by any surgery might be different in the patients who have received any other injury beforehand.

Proposing a therapeutic strategy for burn and septic patients is quite challenging due to the complex interactions of cytokines or chemokines through a very redundant and interconnected network. Therefore, a comprehensive understanding of the behaviors of inflammatory mediators following various injuries is essential. Unfortunately, the results from experimental researches and clinical trials of immunomodulatory therapies are quite controversial. As suggested by Vincent et al., the timing and dose of these interventions are critical, and single therapy might not be ineffective [56]. Moreover, variations in experimental procedures, and size and severity of injuries and utilizing different species might also result in different outcomes. Holzheimer et al. investigated the relationship between the circadian rhythm and cytokine production [57]. They showed that cytokines' production was altered by the time of injury. These quantitative and temporal differences in the secretion of endotaxins by the damaged tissues as well as the physiological dynamics of host body result in more complex and unpredictable responses to the injuries. Therefore, it is essential to further characterize the dynamic profiles of inflammatory mediators in various animal models. Herein, we used non-lethal animal models which eventually recover from the injuries. Therefore, it can be speculated that the inflammatory response of host body to the injuries should be protective and under control. We have observed that IL-18 and MCP-1 have been significantly up-regulated in our animal groups, which might be essential to induce cell mediated immunity. It has been shown that administration of IL-18 significantly improved the survival rates of animals [58]. MCP-1 is also known as an important mediator for the development of burn associated type 2 T-cell response [59]. Another interesting observation in this study is that IP-10 and leptin concentrations were down-regulated in both B+CLP and B+SCLP groups (Figs. 3 and 5). Leptin is playing an important role in regulating energy metabolism and reducing the food intake. Although it has been shown that leptin reduced elevated tissue associated myeloperoxidase activity in burn animals [16], the reason of down-regulation of leptin in this study may be to reverse the CLP or surgery induced anorexia, which might be a protective response. Similarly, down-regulation of IP-10, a chemo-tactic cytokine, can be also a protective response in order to balance the excessive up-regulation of other chemokines. Anti-Leptin and anti-IP-10 treatment strategies should be further investigated to elucidate their therapeutic effects. In our study, we observed that IL-6, IL-10 and TNF-α were up-regulated in B+CLP group. It has been previously shown that IL-6 secretion correlates with the severity of injury [60,61]. However, interfering with these cytokines has been relatively disappointing with regard to identifying treatments to improve the survival [56,62,63]. These cytokines play key roles therefore these treatments may inhibit the host defense functions or result in inadequate inflammatory response. However, administration of a moderate mediator exhibiting both pro and anti inflammatory behaviors (such as IL-12) might balance the system and improve the host immune functions. In our study, IL-12 was observed in B+SCLP model, relatively less severe injury model. This cytokine can activate both pro- and anti-inflammatory pathways and treatment of burn animals with IL-12 have significantly improved the survival rates in previous studies [64,65].

In summary, a systematic analysis of dynamic patterns of systemic inflammatory mediators was shown by utilizing rat models receiving 20% total body surface area (TBSA) scald burn injury followed by cecal ligation and puncture (CLP) treatment. It was observed that MCP-1 and IL-18 in all animal groups including previously published single injury models and GRO/KC in most cases were up-regulated following the insult(s). Alterations in leptin and IP-10 concentrations are mostly related to surgical or CLP response. On the other hand, IL-6 was only observed in B+CLP group whereas IL-12 was detected in B+SCLP group. It is essential to further analyze the effects of these cytokines/chemokines' treatments on the injury models to gain a more comprehensive understanding of these complex physiological changes and to propose therapeutic approaches to combat the deleterious consequences of burn and septic shocks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2011.07.001.

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