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The Septic Predictor Index (SPI): a novel platform to identify thermally-injured patients susceptible to sepsis

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Abstract

Background—Over the last decades' sepsis has become the major cause of death in severely burned patients. Despite the importance of burn sepsis, its diagnosis, let alone its prediction is difficult if not impossible. Recently, we have demonstrated burn patients have increased NLRP3 inflammasome activation in white adipose tissue. Here, we aimed to delineate a unique immune profile that can be used to identify septic outcomes in severely burned patients.

Methods—Adult burn patients (n = 37) admitted to our burn centre between June 2013–2015 were enrolled in this study. White adipose tissue from the site of injury and plasma were collected from severely burned patients (>20%TBSA) within 96 hours post thermal injury, indiscriminate of gender or age.

Results—We found that patients exhibiting aberrantly high levels of pro-inflammatory IL-1 β and decreased macrophages at the site of injury are highly susceptible to develop sepsis. Septic patients also had increased anti-inflammatory (IL-10, IL-1RA) cytokines in plasma. The Septic Predictor Index (SPI) was generated as a quotient for the site of injury macrophage proportion and IL-1 β production. All patients that eventually develop sepsis had SPI values >0.5. Septic patients with SPI values >1 all had sepsis onset within 12 days post injury, whereas patients with SPI values between 0.5–1 all had later onset (>12 days).

Conclusion—The SPI can accurately determine sepsis onset in thermally injured patients *a priori* and further enables surgeons to develop clinical studies and focused therapies specifically designed for septic cohorts.

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Conflict of interest declaration

The authors declare no conflicts of interest.

Introduction

Despite their catastrophic origins, patients nowadays usually survive burn injuries. This is in part due to early excision and grafting, implementing critical care protocols and providing nutritional and metabolic support.^{1–4} However, preventable death still occurs and is associated with tremendous impact on families and the health care system. To further improve outcomes it is paramount to understand the major cause of death in burn patients. It was recently shown that the majority of patients who succumb to their burn injury are due to infection or sepsis, which ultimately leads to massive organ failure and death.^{5,6} Additionally, if survived, sepsis delays rehabilitation, patient recovery, substantially increases metabolic demands and results in prolonged hospitalization.⁷ The current approaches to predict sepsis via clinical scores or other biomarkers have yet to be successful and remain elusive.

Outside of the clinical definition of sepsis, quantification of biomarkers from susceptible patients' blood work has been traditionally accepted as a feasible alternative to predict sepsis. These markers are often biological molecules produced in response to microbial infections such as cytokines and chemokines. Cytokines and chemokines are immunological communication molecules that signal the body to activate the body's defense system in order to fight off invading pathogens. Examples of these proteins include tumor necrosis factors (TNF) and various interleukins (IL).⁸ However, these approaches had been ineffective, as the window of time between the detection of these biomarkers and sepsis onset is extremely short.⁹ Thus, no therapeutic intervention can be administered in time to "reverse" sepsis. Novel and reliable scoring that predicts sepsis well in advance of onset would allow patient-focused care in order for prevention to be based on a patient's immune-biochemical trajectory. More importantly, we hypothesize that it will promote novel therapeutic approaches and prospective clinical trials to be undertaken.

Previous work from our group delineated the activation of NLRP3 inflammasome in white adipose tissue of thermally injured patients.¹⁰ As NLRP3 expression is predominately observed in the myeloid population of immune cells¹¹, we surveyed the most abundant tissue-derived myeloid population, macrophages, for NLRP3 inflammasome effector function. Specifically, we assessed white adipose derived macrophage-specific IL-1 β secretion via flow cytometry, as IL-1 β is the effector cytokine produced by NLRP3 inflammasome assembly.¹¹ In this clinical study, we hypothesized that immune appraisal of burn patients at the first surgical intervention can reliably identify patients susceptible to sepsis, which develops subsequently during hospitalization. The advantages of utilizing this approach are that it is more stable and provides a more accurate depiction of immune status in comparison to short-lived blood-based markers.

Methods

Patient cohort and study protocol

This study was approved and performed in accordance with the guidelines and regulations of the Research Ethics Board, Sunnybrook Health Sciences Centre (REB#: 194–2010). Informed consent was obtained from patients or from their Substitute Decision Makers. A

total of 37 thermally injured adult patients (18 years old) with total burn surface area (TBSA) greater than 20% and admitted to our burn center between 2013 and 2015 were enrolled in the study (Supplemental Fig 1), indiscriminate of gender or age. Adipose tissue and blood plasma samples from these patients were collected within 96-hours post thermal injury, and biomarkers were measured from both specimens to generate an acute immune profile post thermal injury. Patient's clinical outcomes and complications were followed and documented until time of hospital discharge (Table 1). Control adipose tissues (normal) were obtained from donors that undergone plastic surgery for tissue reconstruction and used to compare burn patients to normal tissue (n = 12; mean age = 44; males = 58%). Sepsis was defined prospectively by the staff burn surgeons based on the clinical presentation of the patient but also in accordance with the American Burn Association (ABA) guidelines as well as new Critical Care Guidelines (Sepsis-3).^{12, 13}.

Harvesting of stromal vascular fractions from white adipose tissue

Excised adipose specimens from burned patients were immediately transferred to the laboratory, where they were digested with collagenase (Sigma, St. Louis, MO) at 1 mg/mL in serum-free RPMI1640 in a shaking incubator for 2 hours at 37°C. The digest was then strained through sterile gauze to remove particulates, and the cell fraction was collected by centrifugation. The cell pellets were washed multiple times with Hank's Balanced Salt Solution (HBSS), re-suspended in HBSS, and red blood cells were removed by density centrifugation with Lympholyte H (Cedarlane, Burlington, ON), following the manufacturer's protocol. The cell suspension was then passed through a 100 micron strainer (BD Biosciences, Mississauga, ON) and cells were counted.

Sample staining and flow cytometry

Stromal vascular fraction (SVF) derived leukocytes were stained with monoclonal antibodies on ice for 25 minutes, followed by washing and analyzed using BD LSR II Special Order System (BD Biosciences, San Jose, CA, USA). Cells were gated on FSC-A and SSC-A, followed by doublet exclusion (FSC-W×FSC-H, SSC-W×SSC-H). The percentage leukocytes, monocytes/macrophages, T-cells, and IL-1 β producing leukocytes in the SVF were identified using the following fluoro-chrome-conjugated antibodies: anti-CD45 (Brilliant Violet 510™, Biolegend, San Diego, CA); anti-CD14 (PE, eBiosciences, San Diego, CA); anti-CD16 (Anti-Human CD16 Alexa Fluor® 700, eBiosciences), anti-CD3 (PE.Cy7, eBiosciences), anti-IL-1 β (Alexa Fluor® 647, Biolegend), in accordance with the manufacturer's flow cytometry protocol.

All analysis was conducted in an unbiased manner and without any prior knowledge of patient classification (non-sepsis or sepsis). Our primary analysis compared the acute immunologic profile at the site-of-injury between patient cohorts that became septic versus non-septics via flow cytometry. Briefly, leukocytes purified from the SVF were analyzed for cellular heterogeneity and capacity to produce inflammatory cytokines, namely IL-1 β . The gating strategy is outlined in Supplemental Fig 2, where leukocytes were initially gated based on granularity and CD45 (side scatter×CD45), followed by size (forward scatter). These gated cells were then stained for cell surface markers for monocytes and macrophages

(CD14^{hi} CD16^{lo}). Finally, CD14^{hi} cells were assessed for IL-1 β positivity indicating the capacity of these cells to produce this inflammatory cytokine.

Plasma sample preparation and luminex

EDTA-anticoagulated samples were drawn within 96-hours post thermal injury and processed using a standard Percoll-based PBMC isolation from periphery. Plasma samples from non-septic and septic patients were used to compare anti-inflammatory trajectories for Interleukin (IL) 10 and IL-1 receptor antagonist (RA) using a Multiplex platform (Millipore, MA). Experimental kits were all conducted in accordance with manufacturers' protocol. Raw data was processed using Millipore Analyst software. All values are presented as mean $\pm$ SEM and expressed in pg/ml

Statistical analysis

All data was analyzed by one- and two-way ANOVA with a Tukey post-hoc test used when two or more groups were present. Correlations were conducted using Pearson twotailed tests in order to explore the association between septic predictor index (SPI: percentage IL-1 β produced by leukocytes / macrophages in the SVF) and sepsis onset (days post-burn). All graphs were created using Graphpad Prism 6.0 (San Diego, CA) and analyzed statistically using SPSS 20 (IBM Corp., NY, NY), with significance accepted at $p < 0.05$.

Results

Immunologic profile of burned patients assessed by flow cytometry at the injury site

As described in the methods section, the gating strategy for flow cytometric data is outlined in Supplemental Fig 2. The patient cohort that eventually developed sepsis exhibited less macrophage population (CD14^{hi} CD16^{lo} cells) at the site of injury in contrast to non-septic patients (mean = 8.7 ± 1.4 vs. 29.3 ± 5.5 ; $p < 0.05$; Fig 1, A), within 96 hours post thermal injury. However, a greater percentage of these macrophages at the site of injury from patients that eventually become septic were pro-inflammatory IL-1 β +, whereas there were very few IL-1 β + macrophages in the non-septic patients post burn (sepsis: 16.3 ± 3.9 vs. non-sepsis: 2.4 ± 0.74 ; $p < 0.01$; Fig 1, B). This relationship was also observed when septic patients were compared to controls for SVF macrophages and IL-1 β + cells ($p < 0.05$ and $p < 0.001$, respectively).

Systemic immunologic profile of burned patients

We subsequently assessed systemic biomarkers in plasma samples from burn patients via multiplex. Anti-inflammatory cytokines IL-10 and IL-1RA exhibited significant differences between the septic and non-septic patient cohorts (Fig 2, A–B). Specifically, the septic cohort had a significantly higher mean concentration detected in plasma in comparison to the non-septic group for both IL-10 (249 ± 68 vs. 28 ± 5.7 pg/mL; $p = 0.017$) and IL-1RA (183 ± 28 vs. 89 ± 18 pg/mL; $p = 0.014$). These observations were also consistent when comparing septic patients and controls.

Septic Predictor Index

To simplify this technique, we created a ratio of the aforementioned IL-1 β from SVF-derived macrophages and macrophage proportion (proportion of CD14⁺ IL-1 β ⁺ cells / proportion of CD14^{hi} CD16^{lo} cells). This was arbitrarily labeled as the “Septic Predictor Index”. Septicemia patients had significantly greater immune status ratios relative to both normal (mean = 3.3 ± 0.9 vs. 0.09 ± 0.02 ; $p < 0.01$) and non-sepsis burn patients (mean = 3.3 ± 0.9 vs. 0.17 ± 0.05 ; $p < 0.01$; Fig 3, A). All septic patients had ratios that were greater than 0.5 (whereas all non-septic patients had SPI < 0.5). Interestingly, when SPI ratios were plotted as a function of time we saw that patients with a ratio greater than one all had sepsis occur within 12 days post injury, whereas patients with ratios between 0.5–1 had sepsis onset after 12 days (Fig 3, B). Using a Pearson correlation, this association between SPI and sepsis onset was negatively correlated ($r = -0.71$, $p = 0.0013$).

We previously reported that elderly burn patients have delayed immune responsiveness after injury compared to adult counterparts^{14, 15}, thus we wanted to determine if these observations were attributed to an age-effect. Patients were classified as elderly (≥ 65 years of age), as defined by the National Institute of Health and World Health Organization. When comparing SPI values, no significant differences were found between adult and elderly septic patients (Fig 4, A). However, the SPI revealed a similar trend in both adults and elderly that was apparent after stratifying patients into non-sepsis and sepsis groups. Extending this analysis, similar results were obtained when septic and non-septic patients were stratified based on survivorship (Fig 4, B). These findings indicate that the different immune measures at the site of injury observed 96 hours post burn are exclusive to septicemia prediction, and not confounded by neither age nor mortality. Lastly, when comparing the onset of sepsis it was observed that late onset sepsis had significantly lower survival than early onset sepsis (Mantel-Cox = 4.21, $p = 0.040$; Supplemental Fig 3).

Discussion

Presently, this study successfully identified a novel technique that can be used to identify patients susceptible to sepsis by examining excised adipose tissue at the first surgical intervention occurring within 96-hours after injury. Septic patients had reduced macrophage proportion in the SVF, yet a great proportion of these innate immune cells stained positive for intracellular pro-inflammatory IL-1 β . Taken together, these observations were used to create the SPI ratio of determining septicemia in adult burn patients. The importance of this study is that it could enable healthcare providers to implement interventions of all types (e.g. surgical, pharmacological) *a priori* to fight the occurrence of sepsis that occurs later. Despite the relevance being clear, its’ utility requires further exploration and validation.

In this study, we primarily focused on the immune response elicited at the site of injury post burn as the first indicator of sepsis susceptibility. In contrast to conventional plasma biomarkers, assessing the site of injury embodies spatial precision via a highly specific profile localized to the wound area. This differs from biomarkers measured from the bloodstream that have an undecipherable origin. At the site of injury, we observed a reciprocal relationship of macrophage abundance and IL-1 β secretion between the non-septic and septic cohorts. In the septic cohort, there were less macrophages present at the site

of injury, yet a great proportion of these cells were IL-1 β +, relative to non-septic counterparts. This observation was consistent with our previous report of increased caspase-1 activity (a by-product of NLRP3 inflammasome activation that cleaves IL-1 β into its mature form) in the SVF after thermal injury.^{10, 11} Although increases in macrophages at the site of injury have been shown in the past¹⁶, presently we demonstrate that these increases occurring within the first 96 hours are unique to non-sepsis patients. Thus, the imbalanced immune trajectory in sepsis patients may be a compensatory mechanism. Individual macrophages must exert greater function and this high demand in IL-1 β production from a limited number of macrophages can quickly progress to immune exhaustion, which renders a patient incapable of fending off future infections. This hypothesis was also supported by a recent study that showed elderly burn patients have delayed immune activity followed by a hyperactive immune response, which ultimately results in consequential immune paralysis.^{14, 15} Extending this notion of immune inadequacy, animal models of sepsis have demonstrated that “immune-priming” or the administration of a moderate immune challenge using an endotoxin such as LPS prior to subsequent infection results in increased bacterial clearance and overall survival.¹⁷

Although flow cytometry is not the standard assay to determine cytokine secretion (as it does not measure cytokine concentration), it is an acceptable method to deduce origin of cytokine production. Often, intracellular staining of cytokines is done after the application of golgi plug, a protein transport inhibitor to the sample of interest. The treatment of golgi plug to samples typically takes 6 hours, which is the time to ensure that the cytokine is made in these 6 hours and thus become detectable, but not secreted. Interestingly, data presented in this study are patient samples without golgi plug treatment, and exhibited no overt differences when compared to unpublished data where samples were treated with golgi plug followed by staining. Thus, we eliminated the use of golgi plug to this procedure, as it conserves 6 hours to the overall turnaround time to measure the data required for the SPI and further supports the clinical feasibility of this technique.

The immune stimulatory aspect of treating critically ill and burn patients is not novel and has been investigated for quite some time. For example, trials have shown that the administration of granulocyte-colony stimulating factor and granulocyte-macrophage colony-stimulating factor can stimulate inflammation and the immune system.^{18–21} However, despite its acute benefit to leukocyte and neutrophil expansion, these studies failed to demonstrate a therapeutic benefit to trauma-induced sepsis and other complications. Similarly, numerous studies in rodents have demonstrated that treatment with Flt3 ligand stimulates immune response at the wound, increases bacterial clearance and survival.^{22, 23} However, despite its promise, the clinical benefit remains unknown.

The intriguing finding of the smaller macrophage population observed at the site of injury from the septic cohort may be explained by anti-inflammatory cytokines circulating in the bloodstream. The present study showed heightened anti-inflammatory cytokines in plasma of septic patients. It has been shown that IL-10 and IL-1RA not only have antagonizing effects on IL-1 β , but they also dampen the immune response by preventing leukocyte trafficking.^{24–26} Therefore, the elevated IL-10 and IL-1RA levels in septic patients may explain the diminished abundance of macrophages at the site of injury. The trigger for the

aberrant production of anti-inflammatory cytokines, that leads to the prevention of macrophage recruitment to site of injury in septic patients remains unknown. We postulate that a potential cause for this phenomenon may be attributed to the dual role of IL-6: both as a cytokine, and a myokine or energy sensing soluble factor.^{27–29} IL-6 has been shown to be highly elevated post exercising and induces a systemic anti-inflammatory state.^{30, 31} The observed over-activation of inflammation at the site of injury may counteract the systemic state and consequently leads to the immune exhaustion phenotype that we propose in this study.

Although the class of infection (gram-positive and gram-negative) presents differential clinical course in burn patients^{32, 33}, infections from both bacterial origins yielded similar alterations in immune profiles within patient cohorts. It is essential to differentiate that this approach of early appraisal at the site of injury is addressing the immune profile of patients in response to burn, and not determining invading microbes themselves. Although, it is not entirely clear where the source of the sepsis came from, generally in burn patients, sepsis originates from a burn wound infection and develops into pneumonia and subsequent complications.^{34, 35} This was supported in our study by pneumonia occurring in 75% of septic patients, where as in non-sepsis burns it accounted for a 29% incidence (Table 1). However, further elaborating our current profiling in terms of complications is beyond the scope of the current study.

Despite the clear utility and successful identification of a novel predictor of sepsis, the authors would like to note a few limitations of the present study. Though all septic patients showed a consistent phenotype, this study was conducted at a single-center with a relatively small sample size and a larger sample size would enable true sensitivity and specificity in order to highlight its clinical utility. Also, prior to the implementation of the SPI to burn care providers and therapeutic clinical trials, a prospective validation study will ultimately be required. The current study investigated adipose tissue from the site and injury and although it would have been interesting to compare samples from other remote regions, this was not feasible. However, we recently showed in a rodent study of burn-sepsis that even epididymal adipose tissue has the same immune and inflammatory response as the site of injury (unpublished data). Presently, the SPI was able to identify sepsis and non-sepsis groups regardless of bacteremia, however future studies should investigate whether the SPI is able to distinguish systemic inflammatory response syndrome (SIRS) from sepsis to broaden its therapeutic scope.

In conclusion, the present study highlights a rapid and efficient approach to individually appraise the immune system in order to identify patients susceptible to future sepsis onset. There are various approaches that have shown to alleviate the hypermetabolic response in burn patients^{37–40}, however there is a lack of targeted-therapy that targets an individual cell population to alter its trajectory in order to bypass detrimental outcomes. Future studies should extend our findings and focus on fine-tuning the immune-metabolic dynamic such that patients could simultaneously maintain both a functional metabolic and immune system post thermal injury in order to elicit appropriate protection against microbial infections and sepsis all together.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Patients that develop sepsis onset demonstrate differential acute immunological profile to non-septic cohorts at site-of-injury

Patients shown in the figure had SVF-derived leukocytes harvested from excised adipose tissue within 96 hours post burn. These leukocytes were analyzed from immune cell and cytokine frequencies via flow cytometry. Symbols represent data from individual patients, and small horizontal bars represent mean values. Statistics were analyzed via 2-way ANOVA. (A) Macrophages proportion (CD14^{hi} CD16^{lo} cells) at the site of injury; (B) proportion of IL-1 β from CD14^{hi} cells at the site of injury. Control adipose tissues (normal) were obtained from donors that undergone plastic surgery for tissue reconstruction. Data expressed as mean $\pm$ SEM. *, ** & *** = $p < 0.05$, $p < 0.01$ & $p < 0.001$, respectively.

Figure 2. Patients that develop sepsis onset exhibit differential acute systemic immunological profile to non-septic cohorts

Plasma sample from burn patients were tested for acutely released soluble factors post thermal injury via ELISA-based assay. Symbols represent data from individual patients, and small horizontal bars represent mean values. Statistics were analyzed via 2-way ANOVA.

(A) Plasma IL-10 concentration detected in burned patients; (B) plasma IL-1RA concentration detected in burned patients. Control plasma samples (normal) were obtained from healthy donors. Data expressed as mean $\pm$ SEM. *, ** & *** = $p < 0.05$, $p < 0.01$ & $p < 0.001$, respectively.

Figure 3. Immune profiling burned patients to predict sepsis onset

(A) Frequency of IL-1 β produced by the macrophage and macrophage proportion at the site of injury is expressed as a quotient to generate the septic predictor index (SPI = proportion of CD14⁺ IL-1 β ⁺ cells / proportion of CD14^{hi} CD16^{lo} cells). Symbols represent data from individual patients, and small horizontal bars represent mean values. Statistics were analyzed via two-way ANOVA. (B) Pearson correlation of the SPI and the individual onset of sepsis in exclusively burn-sepsis patients. Early and late onset sepsis was divided based on the 12th day post burn (dashed vertical line). Early onset sepsis had SPI values greater than 1 (dotted horizontal line), whereas late onset sepsis were between 0.5–1. Thus, the magnitude of the SPI predicted acute or delayed sepsis onset. Data expressed as mean $\pm$ SEM. *, ** & *** = $p < 0.05$, $p < 0.01$ & $p < 0.001$, respectively.

Figure 4. Analysis of the immunologic profiles of burned patients stratified by age and mortality Frequency of IL-1 β produced by the macrophage and macrophage proportion at the site of injury is expressed as a quotient to generate the septic predictor index (SPI = proportion of CD14+ IL-1 β + cells / proportion of CD14^{hi} CD16^{lo} cells). The immune status ratio was stratified based on (A) age and (B) mortality revealing that albeit different, septic burn patients behaved similarly regardless of age or survivorship. Data expressed as mean $\pm$ SEM. *, ** & *** = $p < 0.05$, $p < 0.01$ & $p < 0.001$, respectively.

Table 1

Demographics, burn injury characteristics, and outcomes.

	Non-Sepsis	Sepsis	P
No. of patients	17	20	
Age, mean $\pm$ SD	50 $\pm$ 17	45 $\pm$ 19	0.79
Male, No. (%)	13 (77%)	14 (70%)	0.23
TBSA, mean $\pm$ SD	43 $\pm$ 19	41 $\pm$ 11	0.90
Inhalation injury, No. (%)	9 (53%)	16 (67%)	0.29
Etiology, No. (%)			
Flame	13 (77%)	19 (95%)	0.052
Scald	4 (24%)	-	-
Other (chemical, contact, electrical)	-	1 (5%)	-
Complications, No. (%)			
Pneumonia	5 (29%)	15 (75%)	0.006
Heart failure	-	1 (5%)	0.57
Liver failure	-	2 (10%)	0.32
Respiratory failure	1 (6%)	3 (15%)	0.17
Renal failure	1 (6%)	8 (40%)	0.005
MODS	2 (12%)	3 (15%)	0.17
LOS ^a , median (IQR)	21 (18–52)	33 (23–59)	0.03
LOS/TBSA ^a , median (IQR)	0.79 (0.5–1.1)	0.94 (0.7–1.5)	0.46
Mortality, No. (%)	4 (24%)	4 (20%)	0.48

Numbers may not add to 100 due to rounding

^aAnalysis restricted to patients alive until discharge

TBSA, total body surface area; LOS, length of stay.