Measures of Systemic Innate Immune Function Predict the Risk of Nosocomial Infection in Pediatric Burn Patients

Rajan K. Thakkar, MD,*† Racheal Devine, PhD,† Jill Popelka, BSN, CCRN,† Josey Hensley, BSN, CCRN,† Renata Fabia, MD, PhD,* Jennifer A. Muszynski, MD, MPH,†‡ and Mark W. Hall, MD†‡

Critical injury-induced immune suppression has been associated with adverse outcomes. This acquired form of immunosuppression is poorly understood in pediatric burn patients, who have infectious complication rates as high as 71%. Our primary objectives were to determine if thermal injury results in early innate immune dysfunction and is associated with increased risk for nosocomial infections (NI). We performed a prospective, longitudinal immune function observational study at a single pediatric burn center. Whole blood samples from burn patients within the first week of injury were used to assess innate immune function. Nosocomial infections were defined using CDC criteria. Immune parameters were compared between patients who went on to develop NI and those that did not. We enrolled a total of 34 patients with 12 developing a NI. Within the first 3 days of injury, children whom developed NI had significantly lower whole blood ex vivo LPS-induced TNF α production capacity (434 pg/mL vs 960 pg/mL, $P = .0015$), CD14 $^{+}$ monocyte counts (273 cells/ μ L vs 508 cells/ μ L, $P = .01$), and % HLA-DR expression on CD14 $^{+}$ monocytes (54% vs 92%, $P = .02$) compared with those that did not develop infection. Plasma cytokine levels did not have a significant difference between the NI and no NI groups. Early innate immune suppression can occur following pediatric thermal injury and appears to be a risk factor for the development of nosocomial infections. Plasma cytokines alone may not be a reliable predictor of the development of NI.

INTRODUCTION

Approximately one million pediatric burn injuries occur annually in the United States. Despite advances in burn care, infection-related complications are reported to be as high as 71%, with mortality from burn injury attributed to infections in up to 75% of cases.^{1–7} It is well known that thermal injury leads to a massive inflammatory response,^{1,8–11} though it is increasingly recognized that critical injury is associated with a compensatory *downregulation* of immune function.¹² This form of immune suppression has been shown in a small number of adult studies of thermal injury, though few data exist in burn injured children.^{13–15}

Given the impairment of the skin's barrier function after thermal injury, defense against infectious complications in these patients relies primarily on the cellular elements of the immune system. The "Inflammation and the Host Response

to Injury" collaborative network has investigated immunologic alterations after burn injury. Most of these studies, however, have evaluated adult patients and have been limited to the measurement of plasma cytokine profiles, focusing on systemic inflammation. We and others have demonstrated that systemic inflammation can occur with concurrent suppression of circulating leukocyte function in critical illness and injury, though this is poorly understood in thermal injury. Reduced antigen presenting capacity, as evidenced by reduced expression of human leukocyte antigen (HLA)-DR on circulating monocytes, and reduced capacity of whole blood to produce the pro-inflammatory cytokine tumor necrosis factor (TNF)- α upon ex vivo stimulation with lipopolysaccharide (LPS) have been associated with increased risk for development of nosocomial infection in children with sepsis, cardiopulmonary bypass, and non-thermal trauma.^{12,16–19} Furthermore, limited data in children have suggested a difference in the inflammatory profiles between adult and pediatric burn patients.⁹ Given the major role of the immune response in preventing nosocomial infection, we designed a single-center, prospective, observational study to test the hypothesis that reduced innate immune function will be common and will be associated with the subsequent development of nosocomial infection in pediatric burn patients.

METHODS

This study was conducted at Nationwide Children's Hospital, a freestanding, quaternary care, American Burn Association (ABA)-verified pediatric burn center in Columbus, Ohio. The study was approved by the local Institutional Review

*Department of Pediatric Surgery, Burn Center, Nationwide Children's Hospital, Columbus, Ohio †Center for Clinical and Translation Research, The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, Ohio ‡Division of Critical Care Medicine, Nationwide Children's Hospital, Columbus, Ohio

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Conflict of Interest Statement. The authors have no conflicts of interest to report.

Address correspondence to Rajan K. Thakkar, MD, Department of Pediatric Surgery, Nationwide Children's Hospital, 700 Children's Drive, Columbus, Ohio 43205-2664. Email: rajan.thakkar@nationwidechildrens.org

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Board and informed consent (and assent when appropriate) was obtained from subjects' legal guardians prior to study participation. Inclusion criteria included hospitalization due to acute burn injury, age <18 years, and hospital stay that was expected to last at least 3 days. Exclusion criteria included known immunodeficiency, and current use of systemic immunosuppressive medications. "The sample size for the healthy control subjects was based on the date ranges of admission to create this cohort of patients in such a way as to be temporally matched and age-matched with the thermally injured subjects." We obtained whole blood samples within the first 72 h of the burn injury from enrolled subjects. A subset of patients had longitudinal samples obtained between days 4 and 7. Samples from age-matched healthy controls were also obtained. Healthy subjects were excluded if they met any of the following criteria: recent or current fever, history of chronic inflammatory disease, history of transplantation, current use of antibiotics or systemic immunosuppressive medications, or history of systemic corticosteroid use within the past month.

Immune Function Testing

Ex Vivo LPS-Induced TNF α Production Capacity (TNF α Response). Within 1 h of collection, 50 μ L of heparinized whole blood was added to tubes containing highly standardized LPS stimulation reagent (500 pg/mL LPS from *Salmonella abortus equi*, Enzo Life Sciences, Inc., Farmingdale, NY) and incubated for 4 h at 37°C. The supernatants were then collected and stored at -80°C for batch analysis of TNF α which was quantified using the *Immulinite 1000* automated chemiluminometer (Siemens Healthcare Diagnostics, Deerfield, IL). Stimulation assays were performed in duplicate for each blood sample and values reported represent the average value from each set of duplicates. In patients who had more than one sample from day 0 to day 3, the lowest TNF α response value was used for analysis. LPS stimulation solution was manufactured bimonthly in the Immune Surveillance Laboratory at the Research Institute at Nationwide Children's Hospital and quality controlled to ensure the intrabatch coefficient of variation for TNF α response from healthy donor replicates was <10%.

Flow Cytometry. Whole blood samples for flow cytometry were collected in EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). Absolute CD14⁺ monocyte counts and CD14⁺ monocyte HLA-DR expression were determined by flow cytometry using an LSRII cytometer and analyzed with FlowJo (FLOWJO, LLC Data Analysis Software). Briefly, 100 μ L of whole blood was incubated with human IgG (Invitrogen, Carlsbad, CA) for 10 min at 4°C followed by staining with the appropriate fluorochrome-conjugated specific antibodies or isotype control antibody followed by whole blood lysis. Following centrifugation, cells were washed and fixed with 4% paraformaldehyde (Cytofix, BD Biosciences, San Jose, CA). The following antibodies (BD Biosciences, San Jose, CA) were used: FITC anti-human CD66b (clone G10F5), V450 anti-human CD14 (clone M0P9), BV605 anti-human human leukocyte antigen (HLA)-DR (clone G46-6), and BV605 mouse IgG2a (clone G155-178) isotype control. CD14⁺ monocytes accounts for the vast majority of

circulating monocytes in humans while CD66b is a marker of activation. G46-6 is a monoclonal antibody that specifically binds to HLA-DR. The gating strategy included initial doublet exclusion followed by neutrophil (FITC CD66b) and granulocyte exclusion. Of the remaining cells, CD14⁺ cells were selected, and within that population %HLA-DR⁺ was determined relative to isotype controls. CountBright™ absolute counting beads (Life Technologies, Carlsbad, CA) were used to determine absolute cell counts.

Plasma Cytokine Measurements. Whole blood samples for unstimulated plasma cytokine analyses were collected in heparin tubes (Becton Dickinson). Within 1 h of collection, tubes were centrifuged at 1000 $\times g$ for 5 min and plasma was collected and stored at -80°C for subsequent quantification of interleukin (IL)-2, IL-6, IL-8, IL-10, IL-12, and IL-17 by multiplex assay (Bio-Rad, Hercules, CA) on the Bio-Plex 200 System platform.

Demographics. We collected the following patient information from the electronic medical record (EMR): age, sex, mechanism of burn injury, and total body surface area (TBSA) burn.

Infection Data. Nosocomial infections (NI) were identified through prospective review of the EMR, and were defined as new infections that were identified >48 h from hospital admission according Centers for Disease Control (CDC) criteria.²⁰ Infection testing was obtained at the discretion of the treating clinicians and was not protocolized. Diagnosis of infection was confirmed independently by two surgeons who were blinded to the immune function testing results. Analyses were limited to nosocomial infections diagnosed within 30 days of the initial injury.

Statistical Analysis. The primary outcome variable was the development of nosocomial infection. One-way ANOVA analyses with Dunn's tests were used for continuous variables, and Chi-square or Fisher exact tests were used for categorical variables. Two-way ANOVA was used to compare differences in immune function between serially sampled patients with and without NI over the first week post-injury. Receiver operating characteristic (ROC) curves were created and area under the curve (AUC) analyses were performed to evaluate the relationships between the lowest ex vivo LPS-induced TNF α production capacity, lowest % HLA-DR expression and highest plasma cytokine levels within the first 3 days following thermal injury and the development of a nosocomial infection. The Youden's J statistic was calculated to assess an "optimal" cutpoint for each marker of immune function to determine its relationship to the development of a nosocomial infection. Sensitivities and specificities for these thresholds, along with their associated 95% confidence intervals (CI), are reported. Prism 8 software (GraphPad Inc, San Diego, CA) was used for statistical analyses.

RESULTS

Thirty-four thermally injured subjects and 11 healthy controls were enrolled. Their demographics are shown in Table 1. Of

Tables 1. Demographics

Variable	Burns Without NI	Burns With NI	HC	No NI vs NI	No NI vs HC	NI vs HC
	N = 22	N = 12	N = 11	P	P	P
Age, y	4 [1.9–9.8]	3 [1.5–5]	4 [3.5–10.8]	.99	.99	.56
Male gender (%)	68	60	73	.69	.99	.65
Flame injury (%)	41	42	na	.99	–	–
Scald injury (%)	59	58	na	.99	–	–
Inhalational injury (%)	0	5.8	na	.12	–	–
TBSA burn (%)	12 [10–15]	33 [15–50]	na	.0001	–	–

HC, healthy controls; NI, nosocomial infection; TBSA, total body surface area.

the burn subjects, 12 (35%) developed a nosocomial infection. Patient who developed NI had larger TBSA but were otherwise similar to those without NI. Inhalational injury was rare in the cohort as whole. Infection sites and identified pathogens for subjects with NI are shown in Table 2. Consistent with other studies of nosocomial infection in the intensive care unit, the majority of nosocomial infections (58%) occurred in the lower airway. Bloodstream infections were the second-most common (25% of subjects), including two subjects with bacteremia and one with herpes simplex viremia. The median time to diagnosis of nosocomial infection was 7 days (range: 3–15 days post-injury). There were no deaths in the cohort. All 34 patients had at least one blood sample obtained within 72 h of burn injury, while 17 subjects underwent longitudinal sampling with at least one blood sample obtained between days 4 and 7 after injury.

Immune Function Testing

We observed significantly lower innate immune function in the first 72 h after injury in subjects who went on to develop nosocomial infection (Figure 1). Thermally injured children who recovered without developing NI had higher absolute CD14⁺ monocyte counts in the first 72 h after injury compared to burn subjects who developed NI and healthy controls. Burn subjects who went on to develop NI, by contrast, failed to increase their monocyte counts compared to healthy controls (Figure 1A). HLA-DR expression on CD14⁺ monocytes was also significantly lower in the subjects who developed NI compared to those that did not (54 [41–76] vs 92 [82–95] %, $P = .0206$) and compared to healthy controls (54 [41–76] vs 100 [99–100] %, $P < .0001$) (Figure 1B). Subjects who went on to develop NI also had lower TNF α production capacity compared to those who recovered without NI (374 [124–374] vs 1067 [820–1608] pg/mL, $P = .0004$) and healthy controls (374 [124–374] vs 1122 [835–1584] pg/mL, $P = .0003$) (Figure 1C). The TNF α response was similar between burn subject who recovered without NI and healthy controls.

Among subjects who underwent longitudinal sampling (days 0–3 and 4–7 days sample taken) ($n = 17$), both monocyte HLA-DR expression and the TNF α response were lower at each of these time points in those who developed NI ($n = 9$) vs those who did not ($n = 8$) (Figure 2). These differences were significant at both Day 0 to 3 and Day 4 to 7 after injury.

Subjects who went on to develop NI had higher levels of IL-6, IL-8, and IL-10 in unstimulated plasma within 72 h of

Table 2. Infectious complications

Organism		Source	Post Burn Day Diagnosed
Patient			
1	MRSA	Urine	13
2	<i>Streptococcus pneumoniae</i>	Lung	4
3	<i>S. pneumoniae</i>	Lung	3
4	<i>Haemophilus influenzae</i>	Lung	6
5	MRSA	Blood	9
6	MRSA and Group B beta-hemolytic <i>Streptococcus</i>	Lung	11
7	Radiographic pneumonia	Lung	10
8	<i>Pseudomonas aeruginosa</i>	Blood	15
9	Group A <i>Streptococcus</i>	Lung	3
10	Herpes simplex virus	Blood	8
11	Influenza A	Lung	3
12	Coagulase negative <i>Staphylococcus</i>	Urine	3

Methicillin-resistant *S. aureus* (MRSA).

injury compared to healthy controls, though plasma cytokine levels were not statistically significant between burn subjects who did and did not develop NI (Figure 3).

Areas under the ROC curves to predict the subsequent development of a nosocomial infection were 0.93 and 0.86 for LPS-induced TNF α production capacity and monocyte %HLA-DR expression, respectively. Plasma IL-6 and IL-8 had lower AUCs of 0.79 and 0.78, respectively (Figure 4). The optimal threshold for the LPS-induced TNF α response to predict the development of a nosocomial infection was <624.5 pg/mL (sensitivity, 90%; 95% CI: 62–99%; specificity, 89%; 95% CI: 68–98%). The threshold for monocyte %HLA-DR expression was <79% (sensitivity: 91% [95% CI: 64–99%], specificity: 82% [95% CI: 61–92%]). The threshold for plasma levels of IL-6 and IL-8 were >44.3 pg/mL (sensitivity: 70% [95% CI: 40–89%], specificity: 93% [95% CI: 70–99%]), and >82.8 pg/mL (sensitivity: 60% [95% CI: 31–83%], specificity: 87% [95% CI: 62–97%]), respectively.

DISCUSSION

Infection remains the leading cause of morbidity and mortality after thermal injury.^{1,2} Ours is the first study to characterize the

Figure 1. Innate immune function within the first 72 h following pediatric thermal injury. Circulating absolute CD14⁺ monocyte counts (A), CD14⁺ monocyte HLA-DR expression (B), and ex vivo LPS-induced TNFα production capacity (C) were all significantly lower in children that went on to develop nosocomial infection (NI, $n = 12$) compared to those patients that did not (No NI, $n = 22$). All samples from children with thermal injury were obtained within 72 h after injury. For subjects with multiple samples during that time frame, the lowest value was used in the analyses. Data from age-matched healthy control subjects (HC, $n = 11$) showed higher CD14⁺ monocyte HLA-DR expression and TNFα production capacity compared to subjects in the NI group, though HC had similar absolute CD14⁺ monocyte counts, suggesting a failure to increase CD14⁺ cells after injury in the NI group. Statistical analysis was performed using one-way ANOVA plus Dunn's test. Lines and boxes represent median and interquartile range; whiskers represent the range throughout.

Figure 2. Innate immune function over the first week following pediatric thermal injury. Among subjects sampled on Day 0 to 3 and Day 4 to 7 after thermal injury, ex vivo LPS-induced TNFα production capacity (A) and CD14⁺ monocyte HLA-DR expression (B) were lower over the first week following thermal injury in subjects who went on to develop nosocomial infection (NI, solid squares, $n = 9$) compared subjects that did not develop NI (No NI, solid circles, $n = 8$). Statistical analysis was performed using two-way ANOVA. Symbols represent median values while error bars represent interquartile ranges.

early, functional innate immune response after pediatric burn injury and the first to show an association between reduced immune function and nosocomial infection risk in this population. Our data also suggest that innate immune function testing may have greater prognostic value than measurement of plasma cytokines alone.

Burn injury, like other forms of critical illness and injury, is well known to result in a profound systemic inflammatory response that is characterized by high levels of pro-inflammatory cytokines such as IL-6 and IL-8 in the plasma.^{9-11,15,21} We and others have repeatedly shown, however, that a compensatory anti-inflammatory response often occurs concurrent with this initial pro-inflammatory surge in the setting of critical illness and injury. This counter-regulatory response includes reduction in both antigen presenting capacity and pro-inflammatory cytokine production capacity by circulating innate immune cells such as monocytes. Monocytes play a central role in the innate immune response, with functions that include phagocytosis, intracellular killing, antigen presentation, and cytokine production. Activated monocytes display antigenic peptides on major histocompatibility (MHC)

class II molecules including HLA-DR. These antigen-presenting molecules can then bind to receptors on T cells to participate in lymphocyte activation and perpetuation of the immune response.²² Healthy monocytes should produce TNFα robustly upon stimulation with a toll-like receptor ligand such as LPS. Reductions in monocyte HLA-DR expression and TNFα production capacity are both characteristic of critical illness and injury-induced immune suppression and have been associated with increased risks for the development of nosocomial infection, prolonged organ dysfunction, and death in children with sepsis, cardiopulmonary bypass, and non-thermal trauma.¹⁷⁻¹⁹

In the present study, we show that thermally injured children who fail to increase their absolute monocyte count relative to healthy controls in the first few days following injury are more likely to develop nosocomial infection. A similar increase in peripheral monocyte count was seen in a cohort of adult burn subjects reported by Zhang et al, in which the total number of monocytes was highest on postburn day 1 and then decreased by postburn day 3.²³ The mechanism(s) responsible for failure to increase monocyte counts in our

Figure 3. Plasma cytokine levels within the first 72 h following pediatric thermal injury. Plasma levels of IL-2 (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-12 (E), and IL-17 (F) were similar between thermally injured subjects who went on to develop nosocomial infection (NI, $n = 12$) and those who did not (No NI, $n = 22$) in the first 72 h after injury. Subjects who went on to develop NI, however, had higher plasma levels of IL-6, IL-8, and IL-10 compared to healthy controls (HC, $n = 11$). Statistical analysis was performed using one-way ANOVA plus Dunn's test. Lines and boxes represent median and interquartile range; whiskers represent the range throughout.

subjects who went on to develop NI is unknown “We aim to study this further by evaluating apoptotic pathways using flow cytometry and assessment of plasma biomarkers of apoptosis.”

We also demonstrated reduced expression of monocyte HLA-DR in the first week following burn injury in our subjects who developed NI. This is in agreement with studies of adult trauma and burn patients,^{13,14,24,25} including the observation that, in adult burn patients, the percentage of HLA-DR⁺ monocytes is lower in postburn patients vs controls and is lowest in burn patients who go on to develop

sepsis.^{13,14} Reduced HLA-DR expression tends to recover in adult burn patients who do not develop sepsis, but persists in septic patients.^{13,14} Our results confirm these findings in a pediatric population and is also the first to report a threshold of <79% monocyte HLA-DR expression within the first 3 days of thermal injury to be associated with the development of a subsequent infection.

We have previously shown that reduction in the TNF α response is associated with increased risk for the development of nosocomial infection in children who suffered critical

Figure 4. Receiver operating characteristic curve analysis within the first 72 h of pediatric thermal injury of innate immune function compared to plasma cytokine levels of IL-6 and IL-8. The ROC curves demonstrate early markers of innate immune function (TNFα production capacity and %HLA-DR) are better able to determine the risk of subsequent nosocomial infection as compared to plasma levels of the cytokines IL-6 and IL-8.

traumatic injury.¹⁹ In a cohort of 76 critically injured children, only 10 of whom had burn injury, a TNFα response <520 pg/mL was strongly associated with the subsequent development of nosocomial infection. This represents a significant reduction in the TNFα response relative to healthy controls, which typically had a TNFα response around 1000 pg/mL. Our data demonstrates a similar but slightly higher threshold of <624.5 pg/mL for pediatric thermal injury patients. We speculate that the combination of the loss of skin barrier function and other factors such as less frequent use of empiric antimicrobial agents may make pediatric burn patients more dependent on innate immune cell function for host defense leading to the higher “optimal” cutoff point of <624.5 pm/mL as compared to other pediatric trauma patients.

This study is unique in that we evaluated immune cell function as well as plasma cytokine levels. While cytokines were elevated compared to healthy controls, we found that systemic cytokine levels were less predictive of nosocomial infection risk than were measures of innate immune function. Both the TNFα response and %monocyte HLA-DR expression were significantly different between the NI and no NI groups, and the AUC for both of these measures was higher than those for IL-6 or IL-8 for the prediction of NI. These findings provide additional evidence that immune function parameters may be more informative and potentially more important to measure in future clinical trials in the pediatric burn population, than simple plasma cytokine levels.

Understanding the natural history of immune function in children with burn injury, and the relationship between immune function and nosocomial infection risk, is important because increasing evidence suggests that critical illness and injury-induced immune suppression is reversible through the use of immunostimulatory therapies such as interferon-γ and granulocyte macrophage colony-stimulating factor (GM-CSF).^{26,27} GM-CSF has been used in the oncology population for more than 25 years to increase innate immune cell numbers and function following chemotherapy and bone marrow transplantation. Several small studies have suggested that GM-CSF, in doses lower than those used in oncology, can restore innate immune function in septic adults and children.^{16,28} Several studies have demonstrated the effectiveness of GM-CSF as a topical therapy to promote burn wound healing^{29–31} but studies evaluating its use

systemically in pediatric burn patients are lacking. We are currently conducting two prospective clinical trials of GM-CSF for restoration of immune function in pediatric critical non-thermal injury (NCT01495637) and pediatric sepsis-induced multiple organ dysfunction syndrome (NCT03769844), but these studies both exclude children with thermal injury due to our poor understanding of the immunology of this population.

The present study adds significantly to this understanding and adds impetus for the development of clinical trials targeting thermally injured children. The median time to the diagnosis of the first nosocomial infection in our cohort was 7 days. The presence of a statistically significant reduction in innate immune function in these children as early as the first 72 h after injury suggests that there is a potential window of opportunity for early interventions aimed at restoring immune function. In fact, in our cohort who underwent longitudinal sampling (both days 0–3 and days 4–7 sample taken), both monocyte HLA-DR expression and the TNFα response were lower at each of these time points in those who developed NI versus those who did not. This finding is significant and suggests that future innate immune function testing and perhaps intervention can be conducted at either time interval for those patients at risk of nosocomial infection. If our findings are confirmed in a larger cohort of thermally injured children, the use of early, prospective immune monitoring could allow for more targeted infection surveillance, more informed antimicrobial use, and immunomodulatory approaches to be developed and tested.

There are several limitations to our study. It has a small sample size and is from a single center. Some subjects underwent sampling at a single, early time point either due to the fact that their hospitalization was short-duration or due to lack of availability of study staff. A larger sample size with more frequent, longitudinal assessment in a future confirmatory study will allow for multivariable analyses to adjust for factors such as burn wound size and depth. Despite this, we were able to demonstrate associations between reduced innate immune function and infectious outcomes in our cohort. At the time of this study, the work up for acute infections in our burn population was not protocolized and therefore, it is plausible that some infections could have been missed. We have recently created a clinical guideline to standardize the work up for infections in

pediatric burn patients, which will lend even more rigor to our future work. The number of immune parameters measured in the present study was limited. We cannot therefore comment on elements of innate immune cell function such as migration, phagocytosis, and intracellular killing. We have plans to isolate monocytes from whole blood of pediatric burn patients and attempt to measure phagocytic and intra-cellular killing capabilities. It is possible that these measures would further enhance our ability to predict infection risk. Similarly, it is highly likely that adaptive (lymphocyte-based) immune function is important for host defense in thermally injured children. We are actively investigating this arm of the immune system as well and this will be the subject of future analyses from our group. Lastly, monocyte HLA-DR expression and the TNF α response assays are currently available for research use only in the United States. Though they are being used to drive real-time immune modulation in clinical trials, the generalizability of this approach will depend on high-throughput, rapid turnaround immune function testing becoming available in the clinical laboratory.

This study demonstrates that impairment of innate immune function early after pediatric burn injury, including reduction in monocyte HLA-DR expression, reduction in ex vivo LPS-induced TNF α production capacity, and failure to increase absolute monocyte count, is predictive of the subsequent development of nosocomial infection. Further studies need to be conducted to confirm these findings and to understand the mechanisms underlying innate immune suppression following burn injury. This information will then inform the development and testing of immune monitoring and modulation protocols in this high-risk population with a goal of improving clinical outcomes through normalization of immune function.

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