The Role of NLPR3 Inflammasome in Response to Thermal Injury and Sepsis

by

Mile Stanojčić

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

The Institute of Medical Science
University of Toronto

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Abstract

Although the induction of acute-phase inflammation after burn injury is essential, if prolonged it can induce increased risk of infection and sepsis. The NLRP3 inflammasome has established itself as an integral mediator of the acute phase response, however there is a current lack of understanding how it is involved in the inflammatory response after burn injury.

In this study we investigated the role of the NLRP3 inflammasome in burn and septic patients and further aimed to determine if its components can be used to predict sepsis. In Chapter 2 we established the hyperinflammatory response in adult burn and septic burn patients. We also showed that there is increased NLRP3 inflammasome activation and expression in white adipose tissue (WAT) of burn patients and that its pro-inflammatory by-products increase with increasing injury severity.

In Chapter 3, we studied the effect that NLRP3 knockout would have on survival and the immune response in rodents. Interestingly, knocking out NLRP3 inflammasome resulted in increased bacterial clearance and survival in rodents. However, it also induced greater acute responsiveness at the site of injury and WAT characterized by greater macrophage and neutrophil infiltration, ER stress, apoptosis/pyroptosis, and increased systemic inflammation.
Differential effects were found in other organs with an overall faster return to baseline in inflammatory mediators. In septic burn patients, we found peaks in WAT NLRP3 gene expression occurring later than non-septic’s.

Extending these findings in Chapter 4, we used the major source of NLRP3 inflammasome (macrophages) and its by-product (IL-1β) to generate the Septic Predictor Index to identify burn patients susceptible to sepsis. Patients with Index ratios greater than 0.5 had reduced macrophages proportions in WAT that were producing excess amounts of pro-inflammatory IL-1β, and subsequently developed sepsis. Collectively, these results suggest that the NLRP3 inflammasome is a critical acute-phase mediator in septic burn patients and despite its utility to identify patient susceptible to sepsis, its mechanism is complex yet protective of sepsis development.
Acknowledgments

I would like to thank Drs. Nathens and Emmenegger from my committee for their continued guidance with my thesis and professional development, and Dr. Simor whose generosity and accommodation within microbiology made the two-hit model possible. Most importantly, I would like to thank my supervisor and mentor Dr. Marc Jeschke. You gave me an opportunity to be part of your vision of burn research in Canada and I am grateful for everything we have accomplished. I learned just as much professionally as I did personally, both of which were invaluable.

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No matter what transpired, I will always remember: W.I.T.
Contributions

Alexa Parousis assisted with the western blot experiment found in Chapter 2 (Figure 2-6).

Dr. Peter Chen assisted with the flow cytometry analysis in Chapter 4 (Figure 4-2).
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>American Burn Association</td>
</tr>
<tr>
<td>AIM2</td>
<td>interferon-inducible protein AIM2 or absent in melanoma 2</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATF-6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
</tr>
<tr>
<td>CARTD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CASP</td>
<td>colon ascendens stent peritonitis</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>CLP</td>
<td>cecal ligation puncture</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular patterns</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>human leukocyte antigen D related</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1 beta</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
</tbody>
</table>
IP-10 interferon gamma-induced protein 10
IRE-1 inositol-reaching enzyme 1
JNK c-JUN N-terminal kinases
LD50 lethal dose (50% mortality)
LOS length of stay
LPS lipopolysaccharide
mcL microliter
MCP-1 monocyte chemotactic protein 1
mg/dL milligram per deciliter
MIP-1α macrophage inflammatory protein 1 alpha
MIP-2 macrophage inflammatory protein 2
mmHg millimeter of mercury
MOF multiple organ failure
MSU monosodium urate
NACHT nucleotide-binding and oligomerization domain
NF-κB nuclear factor-κB
NLR nod-like receptor
NLRP3 nucleotide-binding domain, leucine-rich-containing family, pyrin-domain-containing-3
NLRP3−/− nucleotide-binding domain, leucine-rich-containing family, pyrin-domain-containing-3 deficient mice
PA Pseudomonas aeruginosa
PaCO₂ partial pressure of carbon dioxide
PAMP pathogen-associated molecular patterns
p-eIF2α phospho-eukaryotic translation initiation factor 2 alpha
PERK protein kinase RNA-dependent-like ER kinase
PBMC peripheral blood mononuclear cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>qSOFA</td>
<td>quick sequential [Sepsis-related] organ failure assessment</td>
</tr>
<tr>
<td>rhGH</td>
<td>recombinant human growth hormone</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SPI</td>
<td>Septic Predictor Index</td>
</tr>
<tr>
<td>SVF</td>
<td>stromal vascular fraction</td>
</tr>
<tr>
<td>TBSA</td>
<td>total body surface area</td>
</tr>
<tr>
<td>TG</td>
<td>triglycerides</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WT</td>
<td>C57BL/6 wildtype mice</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

The contents of this chapter have been published in Surgical Clinics of North America, Current Opinion in Critical Care, Annals of Surgery:


1.1 Thermal Injuries

1.1.1 Introduction to Thermal Injuries

Burn injury is a devastating form of trauma and occurs when cells of the skin or other tissues are destroyed by flames, hot liquids (scald burn) or hot solids (contact burn) (Peck, 2011). It is estimated globally that 11 million cases of burns require medical attention and account for approximately 300,000 deaths (Peck, 2011). In the United States, non-fatal burn injuries requiring medical attention are attributed to nearly 500,000 cases annually (DeKoning et al., 2009). International studies from the United Kingdom suggest that admission proportions are lower with 20.6 per 100,000 population between 2005-2009 (Dunn, 2010). In terms of hospitalizations, burns account for over 40,000 annual admissions in the United States alone (Li et al., 2015). Thus, burns are a growing global concern and despite their prevalence they are easily neglected and are a forgotten trauma.

Children and elderly are particularly vulnerable to burn injuries. In 2006, burns and fires were reported as the third leading cause of unintentional injury and death in the United States for children 1 to 9 years old (National Center for Injury Prevention and Control, 2009). Factors contributing to the likelihood of a child getting burned include knowledge of the risk of burns, mother’s education/literacy, installation of smoke detectors, access to fire extinguishing chemicals and access to health care services (Peden et al., 2008). Older adults are more prone to injury and as a result are at a higher risk of burn injuries. It was previously reported that burns in elderly was the fourth leading cause of death (National Center for Injury Prevention and Control, 2009) and they are the second population at greatest risk for death due to residential fires after
very young children (Marshall et al., 1998). In addition, older adults do not tolerate burns as well as children and adults. Alden and colleagues previously reported that even small scald burns (7% total body surface area) resulted in 22% mortality (Alden et al., 2007). In fact, the LD$_{50}$ (percentage of total body surface area that is required to be burned for 50% mortality) in children is above 90% whereas in patients >70 years old it is less than 40%, and in patients over 80 it is under 20% (Peck, 2011). Despite a declining prevalence of burn injuries globally and key advances in therapeutic strategies to improve patient outcome, they still cause significant morbidity and mortality in patients (Peck, 2011; Li et al., 2015).

Although the treatment of severely burned patients has been continuously improving, death from such injuries is still a primary concern with more than 4,500 deaths per one million burn injuries yearly in the United States (William et al., 2011). Primary factors known to determine mortality in burn patients include age, burn size, inhalation injury and sepsis (Kraft et al., 2012; William et al., 2009). In the past, patients were most likely to succumb to their injuries due to age, severity of burn and inhalation injury, all factors that are uncontrollable (Wolf et al., 1997). However, presently the paradigm has shifted as resuscitation improved and now sepsis is the leading causes of death in severely burned patients (Williams et al., 2009). This in part is attributed to the loss of the skin barrier after injury that results in a heightened risk of wound infections. Sepsis continues to challenge burn-trauma patients in part due to difficulty detecting, defining and treating it.

Early recognition and treatment of sepsis are imperative to prevent mortality. However, in most cases it is not that simple. Taken together, sepsis is a complicated aspect of critical care of burns and the following section will provide a detailed understanding of the classification and pathophysiology.
1.2 Sepsis

1.2.1 Introduction to Sepsis

In general, sepsis and septic shock continue to burden the healthcare system and are among the greatest medical concerns with a mortality rate ranging from 30 to 50% in North America (Angus et al., 2001). There are over 18 million septic patients each year, and 30% of those are attributed to burn infections (Perman et al., 2012). It is the most common cause of death in intensive care units and has been shown to result in an annual health care cost of $14-16 billion (Angus et al., 2001; HCUP, 2008). Between 1979 and 2000, sepsis accounted for an incidence rate of 500,000 cases in the United States. More recently, that figure has increased onwards of 750,000 cases annually (Funk et al., 2009). Sepsis can be defined as systemic inflammatory response syndrome (SIRS) occurring in the presence of an infectious source (LaRosa and Opal, 2008). The most frequently observed gram-positive and gram-negative bacteria in septic patients include Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella, and Pseudomonas aeruginosa (Opal et al., 2003; Ramanchadran, 2013; Ranvieri et al., 2012). It has long been understood that gram-negative bacteria are the most common sources of bacteremia in sepsis, however, Martin and colleagues showed that in the last 25 years there has been a shift in gram-positive dominance with an average annual increase of 26% in cases within the study period (Martin et al., 2003).
1.2.2 Sepsis Classification

In 1992, as part of an international effort to standardize the classification process, the American College of Chest Physicians and Critical Care Consensus Conference proposed diagnostic guidelines for systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock (ACCP/SCCMCC, 1992). Generally speaking, the SIRS response is triggered by non-traumatic causes; sepsis can be considered the activation of SIRS inflicted by microbial infections (e.g. bacterial, fungal or viral); severe sepsis has the inclusion of multiple organ dysfunction and lastly, the manifestation of hypotension with a lack of responsiveness to fluid resuscitation is septic shock. The specific criteria for each condition are listed below. In 2003, another consensus panel revisited these criteria in an attempt to clarify categorical ambiguity. It was determined that some of the symptoms of SIRS, such as tachycardia, manifest in other septic and non-septic conditions and are poor at differentiating it from other conditions. Thus, the terms “sepsis” and “severe sepsis” can be used interchangeably when there are organ complications present as a result of infection (Levy et al., 2003). More recently, the “Surviving Sepsis Campaign” produced updated guidelines in an effort to improve the management of sepsis (Rhodes et al., 2017). Suggestions were noted for all categories of sepsis with an emphasis placed on early quantitative resuscitation of the septic patient within the first 6-hours of positive blood cultures. In light of the multiple paradigm shifts in defining clinical sepsis described above, below is a summary of the innate immunological alterations that manifest during sepsis.

Systemic Inflammatory Response Syndrome (SIRS)

A patient must demonstrate at least 2 of the following criteria:

- Temperature less than 36°C or greater than 38.3°C
• Heart rate greater than 90 beats/min
• Respiratory rate greater than 20 breaths/min or PaCO\textsubscript{2} less than 32 mmHg
• White blood cell counts less than 4000 cells/\mu L, greater than 12000 cells/\mu L or more than 10% bands

\textit{Sepsis}: SIRS with the inclusion of an infection.

\textit{Severe Sepsis}: Sepsis with the association of hypotension, hypoperfusion, or multiple organ dysfunction.

\textit{Septic Shock}: Sepsis with hypotension below 90 mmHg or a deviation from baseline of 40 mmHg or greater, despite fluid resuscitation.

In burns, sepsis is difficult to detect and conclusively diagnose by conventional standards and this classification will be discussed in subsequent Chapter 1.3.

\section{1.3 Sepsis and Burns}

\subsection{1.3.1 Infections and Burns}

It is estimated that between 50 to 75\% of burn-related deaths are a direct result of infections and related complications (Atiyeh et al., 2005). With the excision of the skin’s natural protective barrier, burn wounds are vulnerable portals of microbe infiltration. Unfortunately, any form of infiltration can lead to serious infection due to the ability of a burn injury to suppress the immune
system. These infections can leak into the bloodstream and spread throughout the circulatory system, causing bacteremia as well as septic shock (Cohen et al., 2004). Burn wound infections are a primary concern during the course of treatment due to their potential to create serious complications that will increase the risk of mortality (Church et al., 2006). Bacteria like *Pseudomonas aeruginosa* (PA), which may come from endogenous flora or the environment, are capable of forming biofilms at the wound site that enables the formation of persister-type cells (Stoodley et al., 2002). *Staphylococcus aureus* is another common bacteria of concern in post-burn patients due to its ability to generate many virulence factors that allow it to thrive (Haraga et al., 2002). Bacteria can invade the tissue and make it more difficult to heal or treat the actual burn. Furthermore, the burn wound may become infected after surgery if a donor graft or the excised host tissue is not epithelized properly. Even so, the greatest threat with burn injuries is septic shock or organ dysfunction, which accounts for 54% of deaths post-burn (Fitzwater et al., 2003; Mason et al., 1986). This sepsis is usually caused by invasion of the wound site that may result in infection, which spreads to other areas of the body. Sepsis is accompanied by a multitude of symptoms including hyperglycemia, hypotension and either hypothermia or hyperthermia (Church et al., 2006). New treatment methods allow earlier detection of possible infections, which means they can be addressed before serious complications ensue.

While burns induce immunosuppression, the presence of bacteria and infections results in a protective immune response to fend off pathogens, however in burn patients this response has been shown to be prolonged and exacerbated. The following section will highlight the inflammatory response manifested during the post-burn response.
1.3.2 Sepsis and Burns

In 2007, the American Burn Association Consensus Conference was held and updated the definition of sepsis and infections in burns (Greenhalgh et al., 2007). Sepsis can be considered a change in the burn patient that “triggers” the concern for infection. These triggers must include one of the following:

I. Temperature $>39^\circ\text{C}$ or $<36.5^\circ\text{C}$

II. Progressive Tachycardia
   • Adults $>110$ beats per minute

III. Progressive Tachypnea
   • Adults $>25$ beats per minute not ventilated ($>12$ L/min ventilated)

IV. Thrombocytopenia
   • Will not apply until 3 days after initial resuscitation
   • Adults $<100,000$/mcL

V. Hyperglycemia
   • Occurs in the absence of pre-existing diabetes mellitus
   • Untreated plasma glucose $>200$ mg/dL
   • Insulin resistance ($>7$ units of insulin/hr IV, $>25\%$ increase in insulin over 24 hrs)

VI. Inability to continue enteral feeding $>24$ hours
   • Abdominal distension
   • Enteral feeding intolerance (two times feeding rate in adults)
   • Uncontrolled diarrhea ($>2500$ mL/day in adults)

In addition, it is required that an infection is documented, this includes:

A. Culture positive infection, or
B. Pathologic tissue source identified, or
C. Clinical response to antimicrobials

More recently, the Third International Consensus Definitions of Sepsis and Septic Shock (Sepsis 3) was held to update the terms and definition of sepsis (Singer et al., 2016). An operational diagram of the clinical criteria to identify patients with sepsis can be found below (Figure 1-1).
Figure 1-1. Operational diagram of clinical criteria to identify patients with sepsis and septic shock. (adapted from Singer et al. 2016).
1.4 Immune Pathophysiology of Sepsis

The traditional and most widely accepted immunological characteristic during the septic response is hyper-inflammation initiated from multiple factors. More recently, this paradigm has shifted with a competing theory regarding a compensatory response. Both of these theories along with the pathophysiology of sepsis will be discussed in this section.

Upon pathogen exposure, the host immune response initiates a surge of pro-inflammatory cytokines that collectively encompass the “cytokine storm”. Endotoxins found on the bacterial cell wall serve as danger-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) that are detected by recognition receptors (PRRs) of the innate immune system (Calfee et al., 2010). For example, the LPS component of gram-negative PA is recognized by Toll-like receptor 4 (TLR4) on the cell surface of antigen presenting cells (monocytes/macrophages), which in turn stimulates the production of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), Interleukin (IL)-1β, and IL-6. The severity of this response has been attributed to numerous factors including comorbidities, pathogen load and virulence (Hotchkiss et al., 2003). This elicits a cascade of systemic and tissue-specific events including homing of chemokines to the injury site, phagocytosis and vascular damage (Boomer et al., 2013; Casey, 2000; Giamarellos-Bourboulis and Raftogiannis, 2012). This response is not limited to the site of infection; elevated levels of IL-6 stimulate the hepatic production of C-reactive protein, which functions as a acute phase reactant and a biomarker of interest for predicting sepsis (Levy et al., 2003; Christ-Crain et al., 2005; Gabay and Kushner, 1999). This sequence of events contains numerous alterations on immune cell behavior and function, which is outlined in Table 1-1.
Table 1-1. Overview of SIRS and Sepsis Effects on Innate and Adaptive Immune Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Apoptosis</th>
<th>Cytokine Production</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Macrophage</td>
<td></td>
<td>↑↓ Pro- (early/late)</td>
<td>Hotchkiss et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Anti-</td>
<td>Cavaillon and Annane, 2006</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>King et al., 2014</td>
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<td></td>
<td></td>
<td></td>
<td>Hotchkiss et al., 2002</td>
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<tr>
<td>Dendritic cell</td>
<td>↓</td>
<td>↓</td>
<td>Hotchkiss et al., 2002</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>↓</td>
<td>↑↓ Pro- (early/late)</td>
<td>Drifte et al., 2013</td>
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<td></td>
<td>(delayed</td>
<td>↑ Anti-</td>
<td>Taneja et al., 2004</td>
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<td></td>
<td>apoptosis)</td>
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<td></td>
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<tr>
<td>Natural Killer cell</td>
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<td>↓</td>
<td>Reviewed in Stearns-Kurosawa et al., 2011</td>
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<td>T-cell</td>
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<td></td>
<td>Venet et al., 2004</td>
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<tr>
<td>B-cell</td>
<td>↑</td>
<td>↑ antibody production</td>
<td>Hotchkiss et al., 2001</td>
</tr>
</tbody>
</table>
Another paradigm that is heavily criticized is the delayed and potentially prolonged anti-inflammatory response that succeeds initial inflammation after injury. Despite countless literature supporting SIRS, it does not fully explain all pathological alterations that occur in trauma patients or the lack of clinical benefit of reducing hyperinflammation and/or inflammatory mediator release (Adib-Conquoy and Cavaillon, 2009). Thus, this raised the possibility of a biphasic model of sepsis. This systemic deactivation of the inflammatory response was later described as the compensatory anti-inflammatory response syndrome (CARS) (Bone et al., 1997). Exploring the possibility of a biphasic response to sepsis supports the increased susceptibility to secondary complications and variable immune dysfunction that occurs during the course of infection (Boomer et al., 2013; Cavaillon and Annane, 2006). Specifically, CARS has been described to include the following components (Bone et al., 1997; Ward et al., 2008):

- Reduced pro-inflammatory cytokine response by activated monocytes
- Lymphocyte apoptosis and dysfunction
- Reduction in monocyte HLA-antigen presenting receptors
- Increased expression of anti-inflammatory IL-10

The cytokine components of CARS include release of IL-10, IL-1 receptor antagonist (IL-1RA) and transforming growth factor-β (TGF-β) (Fisher et al., 1992; Marchant et al., 1994; Marie et al., 1996), collectively representing a normal homeostatic response to limit inflammation (Munford and Pugin, 2001). Other studies have used the CARS model to show that septic patients with low proportions of HLA-DR (a measure of immune challenge and hallmark of sepsis) produced low amounts of pro-inflammatory cytokines in response to bacterial challenge (Astiz et al., 1996). IL-10 levels have also been shown to correlate with multiple organ
dysfunction and mortality (Doughty et al., 1998). Lastly, when considering both models of the septic response, a high IL-10 to TNF-α ratio was a predictor of mortality in patients admitted with fever (van Dissel et al., 1998). Contrasting evidence for the capacity of anti-inflammatory soluble mediators to have therapeutic value has generally been limited to animal models, which will be discussed in further detail below. A prospective single-center clinical study conducted by Gomez and colleagues did not show any support for a two-phase model underlying the pathophysiology of sepsis and as expected, were only able to conclusively show immune variables behaved in a “mixed and time-dependent manner” (Gomez et al., 2013). Thus, neglecting all of the aforementioned studies supporting both sides of the spectrum, the compensatory response can be simplified to an adaptive reaction of the immune system to dampen inflammation (refer to Table 1-2 for summary of immune mediators). However, the distinct elevation in anti-inflammatory cytokines at early stages of trauma raises questions regarding the “adaptive” nature of this response.

As previously mentioned, due to the loss of the skin barrier burn patients are at increased risk for developing infections and this ultimately increases susceptibility to sepsis and associated complications. The following section will highlight infections in burn patients.
Table 1-2. Overview of CARS Effect on Innate and Adaptive Immune Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Apoptosis</th>
<th>Cytokine Production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td></td>
<td>↑ Anti-</td>
<td>Opal et al., 2000</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>↑</td>
<td>↑ Anti-</td>
<td>Ward et al., 2008</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>↓</td>
<td>↓ Pro-</td>
<td>Reviewed in Hotchkiss and Karl, 2003</td>
</tr>
<tr>
<td>Natural Killer cells</td>
<td>↑</td>
<td>↓</td>
<td>Reviewed in Hotchkiss and Karl, 2003</td>
</tr>
<tr>
<td>T-cells</td>
<td>↑</td>
<td>↓</td>
<td>Reviewed in Adib-Conquy and Cavaillon, 2009</td>
</tr>
<tr>
<td>B-cells</td>
<td>↑</td>
<td>↓</td>
<td>Reviewed in Adib-Conquy and Cavaillon, 2009</td>
</tr>
</tbody>
</table>
1.5 Inflammation and Burns

1.5.1 Inflammatory Response in Burns

The inflammatory response that is initiated immediately post-burn can last for nearly a year (Jeschke et al., 2011). The trauma of a severe burn injury induces distinct systemic inflammatory and immunological responses primarily mediated by cytokines, as shown in pediatric and adult burn patients (Gauglitz et al., 2008a; Jeschke et al., 2011; Nyhlen et al., 2004). Cytokines are a group of proteins with autocrine and endocrine activities that facilitate communication between different cell types, including those that mediate immune function, angiogenesis, cell proliferation and apoptosis. They also regulate homeostasis and cellular repair through effects on cell growth and differentiation via receptor activation. The expression profile of a variety of cytokines and chemokines’ are distinct in post-burn patients (Jeschke et al., 2014a).

Various cytokines, such as IL-1, IL-6 and TNF, have been utilized as markers related to the severity of burn injury (de Bandt et al., 1994; Endo et al., 1992; Ueyama et al., 1992). Some of the early markers associated with the initial inflammatory (acute phase) response include IL-6, IL-8, IL-10, TNF-α and IL-1β (Jeschke et al., 2014a). There are varying levels of these cytokines in patients that can be used to distinguish survivors and non-survivors. Specific markers of non-survivors include the elevated expression of IL-6 and IL-8, as well as granulocyte-colony stimulating factor (G-CSF) and monocyte chemotactic protein 1 (MCP-1) (Jeschke et al., 2014a). Previous work on a large prospective study of 242 burn patients showed that the entire inflammatory response was profoundly altered up to 2-3 years post-burn (Jeschke et al., 2008a).
Pro-inflammatory cytokine synthesis or “cytokine storm” is a systemic response to manage deleterious effects of thermal injury in an attempt to restore homeostasis. However, when this response is prolonged post-trauma it can result in stress-induced hyperglycemia, insulin resistance, hypermetabolism and catabolism, which are precursors to organ dysfunction and lead to increased risks of infection, sepsis and death (Jeschke et al., 2014a; Jeschke and Herndon, 2014b; Van den Berghe et al., 1997). The prevention of excessive inflammation is a key component of post-burn treatment because of its dynamic relationship with the hypermetabolism and impaired glucose metabolism that are also occurring.

In addition to local inflammation, severe dermal burns are known to induce SIRS in the acute phase after injury, which also correlates with a higher risk of organ failure (Ipaktchi et al., 2006). The innate immune system, particularly the mononuclear phagocytotic system, plays a major role in the response to burn injury (Xiu and Jeschke, 2013). These mononuclear phagocytic cells include monocytes and macrophages. They are professional phagocytes and the first cellular responders in severe burn injury. They are not only able to internalize and digest bacteria and other dead cells and scavenge toxic compounds produced by metabolism, they also contribute to remote organ damage and failure (Hume et al., 2002; Souto et al., 2011).

Severe thermal injury is associated with a dysfunctional phenotype and function of monocytes and dendritic cells (DCs), which results in significant immunosuppression in severely burned patients (Xiu and Jeschke, 2013). Immunosuppression from burn injury is thought to contribute to the development of sepsis. Hypo-responsive monocytes (down-regulated HLA-DR⁺ monocytes) and DC depletion are the hallmark of the immunosuppression in severe burn injury (Xiu and Jeschke, 2013). The expression of HLA-DR on DCs and circulating monocytes rapidly decrease starting as early as 2-3 days post-injury. Clinical studies have also demonstrated a
significant reduction of circulating DCs (including myeloid and plasmacytoid DCs) in the peripheral blood of severely burned patients (Williams et al., 2012; D’Arpa et al., 2009).

Post-burn immune suppression is a major determinant that contributes to patient morbidity and mortality. One of the main contributors to adverse outcomes is the complex yet overwhelming inflammatory and metabolic changes that occur in response to injury (Jeschke et al., 2011). Consequences of this extreme and persistent inflammation include increased body temperature, proteolysis, lipolysis, hyperdynamic circulation, and profound depression of the immune system increasing risk of infection (Jeschke et al., 2011). Occuring in parallel to the hyperinflammatory response are severe and prolonged increases in glucocorticoids and catecholamines production that are now thought to underlie the hypermetabolic state seen in burns (Gore et al., 2006). In other words, the hypermetabolic response is driven by an increase in hormone production creating an overactive state that speeds up metabolic rate and promotes muscle loss as well as insulin resistance due to impaired glucose regulation (Williams et al., 2011). This profound and persistent hypermetabolism is an ongoing challenge in burn care treatment (Jeschke et al., 2004), which will be discussed further in the subsequent section.
1.5.2 Hypermetabolic Response in Burns

Arguably, the profound hyperinflammatory and hypermetabolic response that occurs after burn injuries is the main characteristic that distinguishes itself from other forms of trauma. In fact, greater burn injury severity has been shown to result in an increased pro-inflammatory response that contributes to muscle catabolism (Jeschke et al., 2007b). Thus, in order to understand the complexity of the post-burn response both immune and metabolic alterations need to be considered. In the early 1940s nutritional scientist Cuthbertson introduced the “ebb” and “flow” phases after injury to describe metabolic changes occurring after major trauma (Cuthbertson et al., 1942, Figure 1-2). First, the “ebb” or shock phase is characterized by reduced total metabolism and diminished peripheral blood volume in circulation. The “flow” or traumatic inflammation phase begins 3-5 days after injury with increases in metabolic activity and various aspects of circulation (hyperaemia, exudation, leucocytic migration, etc.) to collectively stimulate a repair process. Critical illness or injury results in an extensive and persistent hypermetabolic response, which drives catabolism far longer than the duration of the initial insult (Mongardon and Singer, 2010). The hallmarks of this response are supraphysiologic metabolic rates including profoundly accelerated proteolysis, lipolysis, glycolysis, liver dysfunction, insulin resistance, and loss of total and lean body mass; fatal physiologic exhaustion occurs if this hypermetabolic response is left untreated (Goldstein and Kopin, 2007; Jeschke et al., 2008a; Williams et al., 2009b). Mediators of these complex responses are cytokines, acute phase and constitutive proteins, as well as hormones. All these mediators are altered upon onset of any acute critical illness and remain abnormal for a much more prolonged period of time than previously thought (Jeschke et al., 2008a; Jeschke et al., 2011).
Figure 1-2. Posttraumatic metabolic alterations: Ebb and Flow phases (Cuthbertson, 1942)
When circulating levels of gluconeogenic hormones, glucagon, cortisol, and catecholamines are elevated in response to critical injury, inefficient liver glucose production is stimulated alongside substantially increased lipolysis, leading to futile substrate cycling (Jeschke et al., 2008a; Williams et al., 2009b). Recent studies in critically ill and severely burned patients demonstrate significant derangements in energy-producing and mitochondrial pathways inclusive of increased gluconeogenesis, glycogenolysis, lipolysis, and elevated glucogenic precursor circulation. Impaired insulin sensitivity and hyperglycemia result in post-receptor insulin resistance (Gauglitz et al., 2009a; Jeschke et al., 2004). Lactate, the anaerobic glycolysis end-product, is recycled to the liver to stimulate the production of more glucose via gluconeogenic pathways (Herndon et al., 2004). Interestingly, significant elevations in serum insulin, serum glucose and insulin resistance have shown to persist for at least three years post-burn (Jeschke et al., 2008a; Gauglitz et al., 2009a). A summary of the post-burn hypermetabolic alterations occurring in different organs can be found in Figure 1-3.
Figure 1-3. Pathophysiological alterations in multiple organs after burn trauma.

The data summarized in the figure is extrapolated from previously published data (Aili et al., 2001; Heszele et al., 2004; Jeschke et al., 2000; Spies et al., 2002; Hart et al., 2001; Jeschke et al., 2007a; Herndon et al., 2001; Hart et al., 2002; Jeschke et al., 2008b; Bailey and Turner, 1996; Deane et al., 2009; Williams et al., 2009c; Patsouris et al., 2015; Abdullahi et al., 2017) and was published in (Stanojcic et al., 2016a).
Lipid metabolism is another significantly augmented pathway as a result of the hypermetabolic response. Lipolysis, characterized by the reduction of triacylglycerol into glycerol and free fatty acids (FFA), contributes to post-burn morbidity and mortality in part due to their infiltration into organs (Randle et al., 1963). FFAs specifically hamper insulin-stimulated glucose uptake (Boden et al., 1994; Shah et al., 2002) and, through inhibition of glucose transport, induce insulin resistance (Dresner et al., 1999). Increased abundance of FFAs, are predictive not only of the incidence of type 2 diabetes but also of the disease severity (Pankow et al., 2004). Modulation of plasma FFA concentrations can be the result of hypo-albuminemia or elevations in intracellular FFA turnover. This is part of the futile cycle involving the generation of FFA from muscle and adipose triglycerides (TGs). In general, the anabolic effect of insulin is countered by catabolic hormones causing significant lipolysis, proteolysis, and hyperglycemia (Gauglitz et al., 2009a; Jeschke et al., 2004). In an attempt to fulfill unmet metabolic requirements, the body inefficiently utilizes lipids and proteins after critical illness (Gauglitz et al., 2009a; Jeschke et al., 2004). Due to the extensive depletion of net protein, consequential muscle wasting and loss of body mass occur, ostensibly contributing to reduced strength and an inability to completely rehabilitate (Hart et al., 2000a). In addition to changes in tissue loss, protein degradation also interferes with whole-body nitrogen balance (Herndon et al., 2004; Hart et al., 2000a). Hence, protein catabolism is directly associated with elevated metabolic rate (Hart et al., 2000b). The loss of body mass affects other key processes needed for recovery: a reduction of total body mass by 10% induces immune dysfunction, wound healing is compromised with decreases of 20%, severe infections result from loss of 30%, and a 40% loss becomes fatal (Lang et al., 1992).
The hypermetabolic state that is induced post-burn can be more effectively treated if the burned skin is excised and replaced with a graft, thus decreasing the metabolic rate by 40% for a >50% total body surface burn compared to no graft replacement (Herndon et al., 2004). It has also been found that enteral feeding can lower the hypermetabolic response and help prevent the rapid weight loss which is often seen in burn patients; this being an improvement from using both parenteral and enteral feeding in combination (Herndon et al., 2004). It is also relevant to note that even though the patient may be totally healed, a chance of death still exists up to three years after trauma (Jeschke et al., 2011). It has been demonstrated that catecholamine’s and stress hormones remain elevated for up to 36 months and should be monitored (Jeschke et al., 2011). In addition to this, the acute phase that follows burn is very vital due to the increased acute phase proteins and factors being released as well as changes to the hormonal axis between the hypothalamus and anterior pituitary (Jeschke et al., 2011). Previously, recombinant human growth hormone (rhGH) was used to modulate the hepatic acute response but was shown to increase mortality, thus studies have begun considering insulin-like growth factor 1 (IGF-1) administration to improve the inflammatory response (Debroy, 1999). Although numerous advancements are being made in the treatment options for burn patients, these injuries still remain dangerous and lethal today.

Extending this notion of increased hyperinflammatory and hypermetabolic response, the following section will introduce an acute phase immune sensor, the NLRP3 inflammasome, which has established itself as an important mediator driving inflammation.
1.6 NLRP3 Inflammasome

1.6.1 Introduction to NLRP3 Inflammasome

Innate immunity has been traditionally regarded as the first line of defense discriminating between the “self” and “non-self”. The Nod-like receptor (NLR) family belongs to a group of proteins containing nucleotide-binding and oligomerization (NACHT) domains, C-terminus that contains leucine-rich repeats (LRR), and N-terminal caspase-recruitment (CARD) or pyrin (PYY) domains (Schroder and Tschopp, 2010a). One of the distinct subfamilies is the NLRPs and of these the most characterized is the NALP3 or NLRP3 inflammasome, which consists of NLRP3 scaffold, ASC (PYCARD) adaptor and caspase-1. Martinon and Tschopp (Martinon et al., 2002; Martinon and Tschopp, 2007) revolutionized our understanding of the acute phase response with the discovery of the NLRP3 inflammasome scaffold and its function in inflammation. The major function of the NLRP3 is to perform cytoplasmic detection of PAMPs and DAMPs (Schroder and Tschopp, 2010). Upon exposure to an invading pathogen, the NLRP3 inflammasome activates and autocleaves procaspase-1 to activated caspase-1, which subsequently proteolytically matures proforms of pro-IL-1β and pro-IL-18 into their pro-inflammatory forms (IL-1β and IL-18) (Schroder and Tschopp, 2010). The NLRP3 inflammasome can be activated under various circumstances outlined below.
1.6.2 Mechanisms of NLRP3 Inflammasome Activation

DAMPs & PAMPs

PRRs on innate immune cells are responsible for detecting pathogens from a number of sources and in turn result in NLRP3 inflammasome activation (Takeuchi and Akira, 2010). Two of the main sources of PRR activation include pathogen-associated molecular patterns and danger-associated molecular patterns (Lamkanfi and Dixit, 2014). PAMPs are unique microbial structures that activate PRRs and include adenovirus (Muruve et al., 2008), and bacteria such as *Staphylococcus aureus* (Mariathasan et al., 2006; Miao et al., 2008). On the other hand, DAMPs from damaged host cells can also result in activation and include ATP (Mariathasan et al., 2006), heat-shock proteins (Lamkanfi and Dixit, 2014), mitochondrial damage (Shimada et al., 2012) and endoplasmic reticulum (ER) stress (Bronner et al., 2015). Collectively, activation of PRRs by DAMPs and PAMPs promotes an upregulation of NF-κB driven gene transcription and pro-inflammation. As outlined above, one of these mechanisms for increasing pro-inflammatory cytokine release includes activation of the NLRP3 inflammasome.

Endoplasmic Reticulum Stress

The ER is a vital organelle responsible for the synthesis and folding of secreted and membranous proteins. It also functions in lipid biosynthesis and acts as one of the main cellular calcium depots (Hetz et al., 2012). Alterations in ER homeostasis lead to the accumulation of misfolded proteins and a subsequent stress response hallmarked by the activation of the unfolded protein response (UPR). The UPR initiates a series of adaptive mechanisms such as inducing the expression of ER resident chaperones, halting further protein translation, and increasing protein
degradation in order to cope with organelle stress (Harding et al., 2000; Kroemer et al., 2010; Lin et al., 2007). Three trans-membrane proteins mediate these activities: protein kinase RNA-dependent-like ER kinase (PERK), inositol requiring ER-to-nucleus signal kinase 1 (IRE-1) and activating transcription factor 6 (ATF-6) (Hetz et al., 2012). Under normal conditions, these proteins are maintained in an inactive state via the binding of ER luminal regulatory domain to the chaperone binding immunoglobulin protein (BiP) (Walter and Ron, 2011). Upon ER stress, BiP migrates to the lumen of the ER in order to help bind misfolded proteins. Thus, the release of BiP facilitates the activation of transmembrane proteins as described above. If the ER stress response is severe, the UPR initiates apoptotic pathways through the actions of transcription factor Chop and pro-apoptotic caspase-3 and the kinase JNK among others (Tabas and Ron, 2011).

Extending this notion of various pathways of ER stress, ER stress has been shown to activate the NLRP3 inflammasome through multiple mechanisms. A noteworthy study conducted by Bronner and colleagues found that using an infection model, IRE1α regulates mitochondrial dysfunction through NLRP3- and caspase-2 (Bronner et al., 2015). They found that upon activation of ER stress, the NLRP3 was required for caspase-2 cleavage that leads to the subsequent release of mitochondrial contents via reactive oxygen species (ROS), and ultimately promotes inflammation. Further evidence of this association was demonstrated by the pre-treatment of macrophages with ROS inhibitors resulting in significant inhibition of caspase-1 and IL-1β release (Gan et al., 2016). It has also been shown in rodent primary hepatocytes that overexpression of CHOP activates the NLRP3 inflammasome and mediates proinflammatory caspases and cell death, which promoted liver injury (Lebeaupin et al., 2015). Thus, despite numerous mediators of induction, the association between ER stress driving NLRP3 inflammasome activation is not fully elucidated.
In burn patients, it has been shown that the white adipose tissue has increased gene expression of ER stress (Jeschke et al., 2012b). This is a well-documented phenomenon that results in a robust response after thermal injury and has been shown to activate numerous ER stress markers including IRE-1, ATF-6, JNK and eIF2α. Interestingly, ER stress has further shown to augment lipolysis in cultured adipocytes from burn patients and may account for the free fatty acids in circulation and organs (Bogdanovic et al., 2015). In animal models of burn, ER stress has been shown to be significantly upregulated both acutely and much later after injury (Song et al., 2009). Similarly, ER stress induced HepG2 cells exhibit greater apoptosis and mitochondrial dysfunction in vitro (Song et al., 2014). Recent reports have shown that induction of ER stress in liver cells drives alternative activation of macrophages when co-cultured (Xiu et al., 2015).

While it is known that inflammation, such as pro-inflammatory cytokines, can initiate ER stress and thus UPR activation, the converse is true as well (Hotamisligil, 2010). This creates a vicious cycle of inflammation triggering ER stress via metabolic factors, which further disrupts homeostasis and consequently creates more inflammation (Guo et al., 2015). This process is driven via multiple routes including the production of ROS, calcium release from the ER as well as the activation of NF-κB and MAPK signaling pathways (Johnson et al., 2012; Tam et al., 2012; Sano and Reed, 2013). Owning to the fact that XBP-1, which is activated via IRE-1, can increase the amount of the pro-inflammatory cytokine IL-6 (Martinon et al., 2010), this further supports the importance of ER stress response in pathophysiology of burn injury. Thus, the ER stress appears to be integrated within the immune and metabolic responses.
1.6.3 NLRP3 Inflammasome in Disease and Burns

As a major controller of cytosolic surveillance of invading pathogens, the NLRP3 inflammasome has been implicated in various medical conditions including gout, lupus, type II diabetes and obesity. In autoimmune gout, MSU crystals are found in joints and are known to be potent inducers of NLRP3 inflammasome activation (Martinon et al., 2006). In systemic lupus erythematosus, the production of macrophages results in hyper-responsiveness to innate immune stimuli and exacerbated inflammatory response (Kahlenberg and Kaplan, 2014). Recently, in addition to the long-established molecular pathways that regulate insulin signaling and downstream effectors, IL-1 antagonists were identified as a new modulator of insulin sensitivity. Similar to the metabolic function established in gout, IL-1 antagonists also showed success as a treatment for diabetes by reducing the risk of insulin resistance due to elevated IL-1β (Larsen et al., 2007; Spranger et al., 2003). Lastly, in obesity, IL-1β has been shown to inhibit adipocytes differentiation and the absence of NLRP3 inflammasome has been shown to prevent obesity-induced insulin resistance (Jager et al., 2007; Vandanmagsar et al., 2011). The NLRP3 is now known to be an important mediator in the cross talk between inflammation and metabolic regulation (Wen et al., 2012; Stienstra et al., 2012; Lamkanfi et al., 2011; Lukens et al., 2011; Menu and Vince, 2011; De Nardo and Latz, 2011; Mori et al., 2011).

Although a variety of cell types are able to induce NLRP3 inflammasome assembly, macrophages represent the primary cell type in which activation occurs following burn injury (Osuka et al., 2011) and in other models (Multiple Sclerosis, Diabetes) (Gris et al., 2010; Donath and Shoelson, 2011). Originally believed to have a protective role after burn injury in rodents, blocking caspase-1 (NLRP3 inflammasome effector component that cleaves IL-1β and IL-18 into their mature forms) resulted in significantly higher mortality after insult (Osuka et al., 2011).
The inflammasome is assembled by a number of instigators including ER stress (Bronner et al., 2015), mitochondrial damage (Shimada et al., 2012) and lipids (Wen et al., 2011), all of which have been shown to be significantly upregulated after burn injury due to dysfunctional tissue regulation in adipose tissue and liver (Song et al., 2009; Bogdanovic et al., 2015). In polymicrobial sepsis models, blocking ER stress improved lymphocyte homeostasis and overall survival in rodents by reducing lymphocyte apoptosis (Doerflinger et al., 2016). Despite the handful of studies performed involving ER stress and innate immune mediators, only one rodent study was conducted that directly measured the NLRP3 inflammasome in burned mice prior to the experiments performed in the subsequent chapters. Thus, the activation and function of the NLRP3 inflammasome has yet to be elucidated under conditions of septic burn in mice. Due to the limitations of investigating mechanisms in humans, the use of animal models provide the opportunity to overcome this and the following sections will outline the various rodent models of sepsis and introduce the two-hit model of burn plus infection.

1.7 Immunological Rodent Models of Sepsis

1.7.1 Introduction to Immunological Rodent Models of Sepsis

Fervent attempts to study and develop novel clinical therapeutics for sepsis have led to the implementation of mouse-models. Currently, there are three different approaches to induce sepsis in mice that are commonly practiced: exogenous administration of a toxin (such as lipopolysaccharide (LPS), other endotoxins or zymosan); disturbance of the animals’
endogenous protective barrier (introducing colonic leakage to allow migration of bacteria) and exogenous administration of live bacteria. However, despite the definitive progression of our understanding about the physiological and immune responses to sepsis with these models, the practicality of translating this knowledge to clinical applications is controversial. The source of this controversy lies within the inherent discrepancy of these models to recapitulate the course of human disease. Specific types of models (eg. exogenous toxin administration) may depict only a particular type of sepsis or partial pathophysiology of septic patients. Thus, the main challenge researchers face is to select the appropriate model that most accurately reflects the human disease. This continues to be the overall goal in which applying the knowledge generated from these models dictates the mechanistic coarse for therapeutic development. Below is a comprehensive overview of the different mouse models of sepsis and its relevancy to human sepsis and septic burn is presented.

1.7.2 Toxemia Model of Sepsis

The toxemia model is established by the injection of TLR agonists such as LPS (Poltorak et al., 1998) or CpG DNA (Hemmi et al., 2000) into mice via intravenous or intraperitoneal routes. The efficacy of this model in recapitulating clinical sepsis is summarized in Table 1-3. One of the major deviations of this model to clinical sepsis is the differences in the immune response, where a single bolus of endotoxin administration in mice results in a transient and a rapid “spike” of systemic cytokines production; whereas clinical sepsis exhibits a prolonged elevation of systemic cytokine production, and it is typically lower in serum concentration by multiple orders of
magnitude. Furthermore, neutralization of pro-inflammatory mediators ameliorated septic pathophysiology in endotoxicosis mouse models, whereas this approach did not succeed in clinical trials.

In general, the culprit of the numerous discrepancies observed between the toxemia models of sepsis versus clinical pathology is inherently attributed to the differential physiology between human and mice. For example, hemopexin, an iron-binding acute phase protein that is present in mouse serum but absent from human’s, may account for the difference in LPS sensitivity between mice and human (Liang et al., 2009). These soluble factors have been suggested to suppress the production of pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) when challenged by endotoxins such as LPS, or other exogenous danger signals recognized by pattern recognition receptors (Warren et al., 2010).
Table 1-3. Comparison of the toxemia model to clinical sepsis

<table>
<thead>
<tr>
<th>Similarities with Human Disease</th>
<th>Differences with Human Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Endotoxin induces shock state in both mice and humans</td>
<td>• Humans more sensitive to endotoxin compared to mice</td>
</tr>
<tr>
<td></td>
<td>• The dose of LPS resulting in 50% death (LD50) in mice is approximately 1-25mg/kg body weight (Glode et al., 1976; McCuckey et al., 1984; Reynolds et al., 1984; Reynolds et al., 2002); whereas a LPS dosage of 2-4ng/kg body weight in humans can inflict severe sickness (Barber et al., 1995; Suffredini et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>• Model does not recapitulate the clinical hemodynamic phases</td>
</tr>
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1.7.3 Host Barrier Disruption Model of Sepsis

The most credible animal-model of sepsis involves the perturbation of endogenous physical barriers, consequently granting bacterial access to sterile compartments that were originally inaccessible (e.g. peritoneal cavity). Two major host barrier disruption mouse-models are currently implemented in laboratory settings: Cecal Ligation and Puncture (CLP) (Wichterman et al., 1980) and Colon Ascendens Stent Peritonitis (CASP) (Zantl et al., 1998). Due to the extensive amount of information and complexity of these models, a point-by-point depiction of the relevant details is listed below.

Cecal Ligation and Puncture Model

CLP is the most widely used and researched model of sepsis in rodents mainly due to its ability to recapitulate the hemodynamic, metabolic, and immunologic phases of human sepsis. However, it is still very difficult to mimic human sepsis with this model alone (especially the end-stage of sepsis, such as severe sepsis and septic shock), due to the lack of common and regular physiological monitoring in these models, which are available to humans in the clinic. In addition, the infectious bacterial flora in CLP versus natural infections may be different, thus the immunological responses observed in these models in comparison to the clinic can obviously be different. Lastly, the age discrepancy of sepsis mouse-models versus clinical sepsis may suggest a differential composition and efficacy of their immune system in response to bacterial insult; therefore results from adult mouse-models may not match clinical reports, and should be interpreted with caution when extrapolating to pediatric/elderly patients.
<table>
<thead>
<tr>
<th>Similarities with Human Disease</th>
<th>Differences with Human Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Considered the gold standard for sepsis research</td>
<td>• Variable outcomes reported between laboratories where some CLP models using 20-gauge needles for the puncture show higher survival rates than 22-gauge (Baker et al., 1983)</td>
</tr>
<tr>
<td>• Mimics human appendicitis or perforated diverticulitis</td>
<td>• The stain(s) of bacteria that cause the infection in CLP may not represent the flora that are commonly depicted as the culprits of infection which leads to clinical sepsis (e.g. <em>Pseudomonas aeruginosa</em>)</td>
</tr>
<tr>
<td>• This model can mimic both the early and late (irreversible) phases of sepsis, as surgical excision of necrotic tissues beyond certain time points are unable to improve survival</td>
<td>• Most reports of clinical sepsis and patients that exhibit septic shock are paediatric or elderly patients (Angus et al., 2001), however, adult mice are the usual candidates when using mouse models; the age discrepancy of model vs. clinic should not be overlooked</td>
</tr>
<tr>
<td>• Importantly, the CLP model recreates the hemodynamic, metabolic, and immunologic phases of human sepsis (Wichterman et al., 1980)</td>
<td></td>
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</table>

Table 1-4. Comparison of the CLP model to clinical sepsis
Colon Ascendens Stent Peritonitis Model

**CASP: Similarities with human disease**

- A stent is inserted into the colon ascendens of the animal, which causes a continuous leakage of fecal matter into the peritoneal cavity (Buras et al., 2005; Zantl et al., 1998).
- The diameter of the inserted stent determines the severity of the sepsis pathology: the bigger the diameter, the more stool leakage, higher incidence infection and sepsis progression (Zantl et al., 1998).
- Lethality observed in CASP is usually a consequence of sepsis induced multi-organ failure (lung, liver, and kidney), which is highly reminiscent of clinical reports of sepsis induced death.
- CASP mimics the pro-inflammatory profile of clinical sepsis, as demonstrated by the rapid elevation of pro-inflammatory cytokines (eg. TNF-α, IL-1) three hours post stent insertion (Zantl et al., 1998; Maier et al., 2004).

**CASP: Differences with human disease**

- Does not recapitulate the hemodynamic phases of clinical sepsis (Buras et al., 2005).
- The anti-inflammatory cytokine response occurs simultaneously as the pro-inflammatory (three hours post stent insertion), which deviates from two-phase immune-profile [pro-inflammatory (SIRS) followed by anti-inflammatory (CARS)] of clinical sepsis (Zantl et al., 1998; Maier et al., 2004; Emmanuilidis et al., 2001).
- Like CLP, multiple bacterial flora originating from fecal matter of the animal will act in concert to induce sepsis, which may confound when comparing to the culprit bacterial species of clinical sepsis.
1.7.4 Two-Hit Model of Sepsis

As a consequence of thermal injury, untreated sepsis may result in multiple organ failure (MOF). It usually follows the “two-hit” theory of inflammation, which claims that an initial injury, in this case burn, can prime a host for an exaggerated inflammatory response, followed by a second insult, such as infection, leading to MOF (Murphy et al., 2005; O’Riordain et al., 1996). When applying this model to sepsis, burn injury has been shown to prime rodent macrophages and dendritic cells for responses to bacterial products (i.e. LPS or Lipid A) by heightening the sensitivity of bacterial-sensing proteins (i.e. TLR2 or TLR4), leading to an intense cytokine cascade (Murphy et al., 2005). However, animal models demonstrating endotoxemia-related sepsis as well as peritonitis do not represent the pathogenic process of burn-related shock and infection (Fink et al., 2014). On the other hand, topical administration of live bacteria at the site of injury (eg. burns) is an option to mimic post-injury infections observed in clinical settings. When compared to clinical pathology, this method of sepsis induction still possesses several major pitfalls. In mice, low bacterial inoculant leads to clearance, whereas high bacterial load results in complement fixation consequently resulting in rapid bacteria lysis (Cross et al., 1993). Therefore, it is very difficult for this model to recapitulate the physiological consequences of bacterial growth in the host and organ infiltration reported clinically, as the septic-like response observed in the mouse model is mainly inflicted by endotoxicemia.

Nonetheless, the two-hit model was created to conform to the multiple insult process as well as mimic the pathophysiological changes observed in septic-burn patients (Orman et al., 2011). PA infections are prevalent in burn wounds and account for over half of all severe burn infections (Hodle et al., 2006). As an opportunistic, gram-negative bacterium, PA is well adapted to the burn environment. Thermal injury patients who acquire PA infections have a fourfold greater
mortality than those who do not (Nichols et al., 2013). In vivo, the two-hit mouse model has demonstrated similar cytokine responses observed in the human septic response, such as the elevation of cytokines driving the systemic immune response: TNF-α, IL-1β, IL-6 and IL-8 (Restagno et al., 2016). In addition, the double insult model also shows higher cytokine expression than burn alone, which is analogous to the human condition (Przkora et al., 2007; Toliver-Kinsky et al., 2002).

Extending the caveats described above, there have been reports describing differential routes of bacterial inoculation leading to unique cytokine responses. For example, bacterial inoculation into the blood compartment of animal models results in the strong generation of pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-1); whereas peritoneal administration does not lead to such a robust cytokine response (Evans et al., 1989; Zantl et al., 1998). Interestingly, the anti-inflammatory cytokine IL-10 has a protective role in sepsis induced by peritoneal administration of bacteria, in contrast to its worsening effects in the lung infection models (Greenberger et al., 1995; van der Poll et al., 1995; van der Poll et al., 1996). Collectively, these differences suggest that there may be an organ/site-specific immunity that is mounted against microbial infections, and these unique responses may account for the reported differences between mouse models of sepsis and clinical incidents, as outlined in Table 1-5.
Table 1-5. Comparison of the live bacteria infection model and clinical sepsis

<table>
<thead>
<tr>
<th>Similarities with Human Disease</th>
<th>Differences with Human Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Induces a similar shock state that is observed in human bacterial infections</td>
<td>• High inoculating bacterial dose in mice often results in rapid lysis of bacteria by complement (Cross et al., 1993)</td>
</tr>
<tr>
<td>• This model is more accurate in recapitulating a clinical infectious scenerio in contrast to the artificial LPS administration approach</td>
<td>• Pathological phenotype observed in model is likely due to endotoxicemia and not infection</td>
</tr>
<tr>
<td>• Dosage and the viable phases of bacteria inoculant can be manipulated to mimic clinical infection, sepsis, or SIRS.</td>
<td>• Route of bacterial administration (peritoneal cavity, blood, lungs burn site/skin) may have confounding roles in cytokine functions and the mediation/alleviation of sepsis pathology.</td>
</tr>
</tbody>
</table>
Hypotheses and Objectives

Hypotheses

1. Adult burn patients have a hyperinflammatory response after injury that parallels increased NLRP3 inflammasome activation.

2. Septic burn patients have a greater hyperinflammatory response and NLRP3 inflammasome activation than burn alone.

3. Burn plus *pseudomonas aeruginosa* induced sepsis in rodent’s results in NLRP3 inflammasome activation and blocking this activation will result in dampened inflammation, improved bacterial clearance and overall survival.

4. NLRP3 inflammasome is activated in adipose tissue of thermally injured patients and can be used to delineate patients susceptible to sepsis.

Objectives

1. To characterize the hyperinflammatory response and activation of the NLRP3 inflammasome in white adipose tissue of adult burn patients.

2. To characterize the hyperinflammatory response and activation of the NLRP3 inflammasome in white adipose tissue of septic burn patients.

3. Establish the hyperinflammatory response and activation of the NLRP3 inflammasome as a function of burn severity

4. To establish a two-hit model of post-burn *pseudomonas aeruginosa* induced sepsis in rodents.

5. To determine whether NLRP3 inflammasome systemic knockout will result in dampened innate immune activity, reduced inflammation, improved bacterial clearance and survival in rodents during the course of post-burn sepsis.

6. To determine whether the NLRP3 inflammasome can be used as a potential biomarker to determine thermally injured patients susceptible to sepsis.
Establish the hyperinflammatory response and activation of the NLRP3 inflammasome as a function of burn severity.
Chapter 2

Establishing the hyperinflammatory response and activation of the NLRP3 inflammasome during burn and burn-sepsis

The contents of this chapter have been published in Critical Care Medicine and Annals of Surgery:


The Role of NLPR3 Inflammasome in Response to Thermal Injury and Sepsis

Hypothesis

Adult burn and burn-sepsis patients will have a hyperinflammatory response after injury that parallels increased NLPR3 inflammasome activation.

Burn plus *Pseudomonas aeruginosa* induced sepsis in rodents results in NLPR3 inflammasome activation and blocking this activation will result in dampened inflammation, improved bacterial clearance and overall survival.

NLRP3 inflammasome is activated in adipose tissue of thermally injured patients and can be used to delineate patients susceptible to sepsis.

Objectives

To characterize the hyperinflammatory response and activation of the NLPR3 inflammasome in WAT of adult burn patients.

To establish a two-hit model of post-burn *Pseudomonas aeruginosa* induced sepsis in rodents.

To determine whether the NLRP3 inflammasome can be used as a biomarker to identify thermally injured patients susceptible to sepsis.

To characterize the hyperinflammatory response and activation of the NLPR3 inflammasome in WAT of septic burn patients.

To determine whether NLPR3 inflammasome systemic knockout will result in dampened innate immune activity, reduced inflammation, improved bacterial clearance and survival in rodents during the course of post-burn sepsis.
2.1.1 Rationale and Summary

Burn injury leads to a complex response that is associated with hypermetabolism, morbidity and mortality. The underlying pathophysiology and the correlations between humoral changes and organ function has not been well delineated in adult burn patients.

Objective of Study: Comparing the inflammatory trajectories in burn and septic-burn adults to gain insight into the pathophysiological alterations and outcomes after injury.

Summary of Results: Adult burn patients (n=1288) admitted to our centre from 2006 to 2016 were enrolled in this prospective study. Demographics, clinical data, inflammatory markers, organ function, and clinical outcomes were obtained throughout acute hospitalization. We then stratified patients according to burn size (<20%, 20-40%, and >40% TBSA) and compared biomedical profiles and clinical outcomes for these patients. Plasma of burn patients revealed elevated pro-inflammatory cytokines, chemokines and metabolic hormones. White adipose tissue from the site of injury had increased ER stress, mitochondrial damage and inflammasome activity, which was exacerbated with increasing burn severity.

Conclusions: In this large prospective trial, we delineated the complexity of the pathophysiologic responses post-burn and conclude that these profound responses are time and burn size dependent. Sepsis patients also displayed an exacerbated immune and inflammatory response in comparison to non-sepsis burn patients. Patients with medium size (20-40% TBSA) burn demonstrated a very robust response that is similar to large burns.
### 2.1.2 Study Background

Mortality of burn patients has significantly improved over the last decades with the establishment of critical care bundles and protocolized burn care. Despite improvements in mortality, post-burn morbidity is tremendous and remains a challenge for clinicians. Jeschke and colleagues, and others have shown that after a severe thermal injury, patients are hyper-inflammatory and hypermetabolic, disabled and debilitated over a period of at least 24-36 months (Jeschke et al., 2011; Williams et al., 2009b). Thus, it is hypothesized that these long-term alterations are sequela from the pathophysiologic response that occurs during the acute phase after burn. The changes occurring during the acute phase have been delineated in the pediatric and recently in the elderly population (Jeschke et al., 2015a; Kraft et al., 2012; Stanojcic et al., 2016b), but not in the adult burn population. Additionally, little data exists about the divergence in pathologic response that occurs according to burn size.

In pediatric and elderly patients, it has been shown that hypermetabolic responses induced by stress and inflammation start immediately after burn and persist for several months to years, impacting every aspect of the body. Furthermore, trajectories and alterations thereof, determined outcomes and overall survival of burn. These results have been used to design novel treatment regimes and interventions to improve outcomes. For example, burn patients with poor outcomes have substantial alterations in their glucose profile encompassing hyperglycemia as well as hypoglycemia (Finney et al., 2003; Jeschke et al., 2014c; Mecott et al., 2010). Both, hyperglycemia and hypoglycemia are detrimental to burn patients and can lead to infections,
impaired wound healing, and mortality (Rehou et al., 2016). In this case, novel treatment approaches are being investigated in order to control hyperglycemia without the competing risk of hypoglycemia susceptibility. Similarly, the impaired inflammatory and immune responses in elderly are now being studied to determine how to stimulate these dampened responses to improve and alter trajectories so when faced with complications, outcomes will shift from death to survival.

Despite the complex pathophysiological responses being extensively eluded in children and elderly, there is a lack of comprehensive oversight in adults. Therefore, the purpose of the present study was to characterize the pathophysiologic responses post-burn in terms of clinical outcomes, inflammation and organ function in order to understand pathophysiologic mechanisms and allow burn care providers globally to develop new specific treatment options to improve outcome of severely burned adults.
2.1.3 Materials and Methods

Patients

In this prospective study we included patients admitted from 2006 to 2016 (n=1288). In general, burn patients aged 18-65 years that were admitted to our burn center with thermal injuries were eligible for enrollment. Demographic data was collected on all patients. Patients who required surgery were additionally consented for blood and tissue collection. Procedures were approved by the Research Ethics Board of Sunnybrook Health Sciences Centre (Study #194-2010). All patients received standard of care according to our clinical protocols, including early excision and grafting, early nutrition, adequate ventilation, adequate antibiotic coverage, etc., as previously published (Jeschke et al., 2011; Jeschke et al., 2008a; Jeschke et al., 2013; Kraft et al., 2013). Clinical data as well as WAT and plasma was obtained at various time points and processed according to established protocols.

Clinical outcomes

Data was recorded prospectively and entered into our burn registry database. Demographic data included height, weight, burn size, length of stay, heart rate, blood pressure, nutritional intake, presence of inhalation injury, mechanical ventilation, number of surgeries, in-hospital complications and mortality (Jeschke et al., 2011; Jeschke et al., 2008a; Jeschke et al., 2013; Kraft et al., 2013). In-hospital complications included infections, pneumonia and septic episodes as defined by the American Burn Association guidelines (Kraft et al. 2014; Greenhalgh et al.,
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 guidelines as well as new Critical Care Guidelines (Greenhalgh et al., 2007; Singer et al., 2016). Organ function markers were troponin T, lactate, blood urea nitrogen, creatinine, amylase, lipase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and bilirubin.

**Metabolic responses**

Metabolic outcomes were compared for burn patients vs. controls and amongst burn patients of different burn severities (<20%, 20-40, >40% TBSA). Protein lysates from fat obtained from OR (greater than 10 days) was extracted using a RIPA lysis buffer and separated on SDS- PAGE gel prior to immunoblotting. BiP, COXIV and AMPK antibodies were from Cell Signaling Technology (MA, USA) and β-Actin antibody was Thermo Fisher (MA, USA).

Total RNA was extracted from fat tissue using TRIzol-chloroform (Life Technologies) with subsequent purification using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. RNA (2mg) was transcribed to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed using the Applied Biosystems Step One Plus Real-Time PCR System. Primer sequences used are available upon request.
Inflammatory and immunological responses

Inflammatory responses were determined in plasma and adipose tissue. Plasma cytokine profiling was conducted twice weekly until time of discharge. Adipose tissue inflammatory markers, cytokines and NLRP3 inflammasome activity (IL-1β) were determined in white adipose tissue that was collected within the first week after injury as discarded tissue at surgeries. Inflammation from both the site of injury and systemically was determined by standard biochemical techniques (Western blotting; Bio-Rad, Hercules, CA), by the Bio-Plex Suspension Array System (Millipore, MA) measuring 17 different cytokines and by quantitative RT-PCR (refer to above section) (Jeschke et al., 2008a; Gauglitz et al., 2009b; Gauglitz et al., 2008a; Gauglitz et al., 2008b; Gauglitz et al., 2010; Jeschke et al., 2007b).

Statistical analysis

Data is presented as mean ± standard deviation (SD), mean ± standard error of the mean (SEM), or median (IQR) for continuous variables and as number (%) for categorical variables. Statistical analysis was conducted using Student’s t-test, ANOVA with Welch’s correction, Wilcoxon rank-sum test and the χ² test where appropriate. Statistical comparisons were conducted using SPSS 20 and figures were generated using GraphPad Prism 6.0 software or Microsoft Excel. Significance was accepted at a p value less than 0.05.
2.1.4 Results

Clinical outcomes

Clinical outcomes were assessed over a 10-year period, 2006 to 2016; during this time 1288 patients met inclusion criteria (Figure 2-1). Demographics, clinical markers, and incidence of various morbidities are shown in Table 2-1. Five healthy controls were used to compare burn patients to normal tissue, of which 2 were females and the mean age was 28. In terms of clinical outcomes, 34% of patients had burn wound infections, 14% pneumonia, 12% bacteremia, with 8% incidence of sepsis. Morbidities ranged from 2% for renal failure, to 1% for abdominal compartment syndrome. Crude mortality was 1.7% when futile patients were excluded.

We then divided these patients based on burn size: <20%, 20-40% and >40% TBSA. We found that there was a slight but significant difference between these groups for age distribution. Burn size, inhalation injury and burn severity were all significantly different between groups, p<0.0001. In terms of clinical outcomes, we found that the amount of surgeries and escharotomies, number of ORs, LOS, and all morbidities were significantly different, p<0.001. In addition, there were significant differences between groups for clinical complications (e.g. ARDS, pneumonia, sepsis, burn wound infection, and mortality) with burns over 40% TBSA being the worst in terms of adverse outcomes, p<0.001 (Table 2-1). Mortality was also profoundly different between the severity groups with adjusted proportions, which includes the removal of futile patients, manifesting as 1.7% mortality for all burns, 1% for <20% TBSA, 4% for 20-40% TBSA, and 15% for >40% TBSA, p<0.0001 (Table 2-1). This is represented in the Kaplan Meier survival curve (Figure 2-2).
Figure 2-1. Patient flow diagram.
Table 2-1. Demographics and outcomes of patients by injury severity group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>TBSA &lt;20%</th>
<th>TBSA 20-40%</th>
<th>TBSA ≥40%</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>1288</td>
<td>1058</td>
<td>157</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years, mean ± SD</td>
<td>40 ± 13</td>
<td>40 ± 13</td>
<td>43 ± 13</td>
<td>42 ± 12</td>
<td>0.014</td>
</tr>
<tr>
<td>Male, no. (%)</td>
<td>942 (73%)</td>
<td>765 (72%)</td>
<td>118 (75%)</td>
<td>59 (81%)</td>
<td>0.235</td>
</tr>
<tr>
<td>Injury characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBSA, %, mean ± SD</td>
<td>12 ± 13</td>
<td>7 ± 5</td>
<td>27 ± 6</td>
<td>53 ± 10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3rd TBSA, %, mean ± SD</td>
<td>5 ± 11</td>
<td>2 ± 3</td>
<td>12 ± 12</td>
<td>38 ± 20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Inhalation injury, no. (%)</td>
<td>181 (14%)</td>
<td>92 (9%)</td>
<td>52 (33%)</td>
<td>37 (51%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baux score, mean ± SD</td>
<td>55 ± 22</td>
<td>48 ± 15</td>
<td>76 ± 17</td>
<td>104 ± 15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flame, no. (%)</td>
<td>723 (56%)</td>
<td>540 (51%)</td>
<td>118 (75%)</td>
<td>65 (89%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Scald, no. (%)</td>
<td>382 (30%)</td>
<td>349 (33%)</td>
<td>29 (19%)</td>
<td>4 (6%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Electrical, no. (%)</td>
<td>104 (8%)</td>
<td>93 (9%)</td>
<td>8 (5%)</td>
<td>3 (4%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Other, no. (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 (6%)</td>
<td>76 (7%)</td>
<td>2 (1%)</td>
<td>1 (1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Outcomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical ventilation, no. (%)</td>
<td>375 (29%)</td>
<td>199 (22%)</td>
<td>111 (73%)</td>
<td>65 (92%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OR procedure required, no. (%)</td>
<td>855 (66%)</td>
<td>640 (61%)</td>
<td>144 (92%)</td>
<td>71 (97%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OR visits, mean ± SD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 ± 2</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>7 ± 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Complications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS, no. (%)</td>
<td>1 (1%)</td>
<td>0</td>
<td>0</td>
<td>1 (1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARDS, no. (%)</td>
<td>53 (4%)</td>
<td>18 (2%)</td>
<td>17 (11%)</td>
<td>18 (25%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pneumonia, no. (%)</td>
<td>184 (14%)</td>
<td>64 (6%)</td>
<td>74 (47%)</td>
<td>46 (63%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bacteremia, no. (%)</td>
<td>152 (12%)</td>
<td>28 (3%)</td>
<td>64 (41%)</td>
<td>60 (82%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sepsis, no. (%)</td>
<td>106 (8%)</td>
<td>21 (2%)</td>
<td>41 (26%)</td>
<td>44 (60%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Burn wound infections, no. (%)</td>
<td>442 (34%)</td>
<td>317 (30%)</td>
<td>84 (54%)</td>
<td>41 (56%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Renal failure, no. (%)</td>
<td>32 (2%)</td>
<td>9 (1%)</td>
<td>7 (5%)</td>
<td>16 (22%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LOS, days, mean ± SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18 ± 23</td>
<td>12 ± 10</td>
<td>33 ± 23</td>
<td>83 ± 54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LOS/TBSA, days%/mean ± SD&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.6 ± 4.7</td>
<td>2.9 ± 5.1</td>
<td>1.2 ± 0.9</td>
<td>1.5 ± 1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mortality, no. (%)</td>
<td>22 (1.7%)</td>
<td>5 (1%)</td>
<td>6 (4%)</td>
<td>11 (15%)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

ACS, abdominal compartment syndrome; ARDS, acute respiratory distress syndrome; LOS, length of stay; OR, operating room; TBSA, total body surface area

<sup>a</sup> Significant differences among injury severity groups (P<0.05)

<sup>b</sup> Other etiology includes chemical, radiation, and contact burn

<sup>c</sup> Analysis restricted to patients with ≥ 1 OR visit

<sup>d</sup> Analysis excludes mortality ≤ 72 hours post-admission.

<sup>e</sup> Analysis restricted to patients alive until discharge
Figure 2-2. Kaplan-Meier survival curves for adult burn patients and burn patients stratified based on TBSA burn groups. Less than 20% and 20-40% TBSA burn groups had similar survival rates with the lowest (80%) representing the severe burn group (≥40% TBSA).
Systemic inflammatory cytokine profile

Using a multiplex platform, we found that pro-inflammatory biomarkers are substantially increased after burn, with a steady decrease over time, while anti-inflammatory IL-10 was elevated acutely after burn and normalized over time (Figure 2-3). Collectively, the exacerbated and uncoordinated response may be contributing to the susceptibility of infection and sepsis (Lord et al., 2014; Shankar et al., 2007). This notion was further supported when we compared burn size with substantial changes occurring in the first 2 weeks; while smaller burns usually recover and approach normal levels, big burns remain augmented (Figure 2-4). Collectively, these data suggest a consort of augmented immune-metabolic response that worsens with increasing burn severity.

Next we wanted to determine if this augmented hyper-inflammatory response was exacerbated in septic burn patients. Sepsis burn patients had an exacerbated hyper-inflammatory response relative to non-sepsis during the early or acute phase (<14 days after injury, Figure 2-5). These results were consistent for IL-6, TNF-α, IL-1β, IL-1α and IFN-γ. The most pronounced increase occurring within 4 days after injury was for IL-6 (p<0.01). Interestingly, when looking at anti-inflammatory IL-10 the same trend was observed during the acute phase, which may suggest that this uncoordinated inflammatory response may be predisposing patients to septic complications. Similar observations were noted for chemokines MCP-1, GRO, IP-10, MIP-1β and immune mediator G-CSF. Thus, consistent with the hyperinflammatory response with burn patients, patients that succumb to sepsis have an early-exaggerated response that relative to non-septic counterparts.
A significantly augmented inflammatory, chemokine and metabolic response was present in burn patients and persisted throughout hospital course, with greatest alterations during the acute phase (within 14 days post burn). Data is represented as mean ± SEM. *, **, *** = significant difference between controls (grey boxes) vs. burned adults; p<0.05, p<0.01 and p<0.001, respectively.

**Figure 2-3:** Plasma cytokine profiling of burn (n=128) and healthy controls (n=10) during the course of hospital stay (0-3, 4-7, 8-14, 15-21, 22-28, >28 days post burn).
Figure 2-4. Plasma cytokine profiling of burn patients stratified based on burn TBSA groups (<20%, 20-40% and >40%). As expected, severe burn (>40% TBSA) resulted in the modest profound alterations in inflammatory, chemokine, immune and metabolic trajectories. Data is represented as mean ± SEM with healthy controls as grey regions. *, ** & *** = significant difference between <20% and 20-40% TBSA; #, ## & ### = significant difference between <20% and >40% TBSA; ^, ^^ & ^^^ = significant difference between 20-40% and >40%; p<0.05, p<0.01, p<0.001, respectively.
Figure 2-5: Plasma cytokine profiling of non-sepsis (n=93) and sepsis (n=35) burn patients during the course of hospital stay (0-3, 4-7, 8-14, 15-21, 22-28, >28 days post burn). A significantly augmented inflammatory response was present in sepsis relative to non-sepsis burn patients. Specifically, a hyperinflammatory response was present at earlier time or the acute phase (within 14 days post burn). This response was consistent for chemokine and immune mediator cytokines measured. Data is represented as mean ± SEM. *, **, *** = significant difference between non-sepsis (dashed lines) vs. sepsis (solid lines) burned adults; p<0.05, p<0.01 and p<0.001, respectively.
Metabolic alterations and inflammasome activity

As an acute phase sentinel of stress and inflammation, the byproduct of NLRP3 inflammasome assembly results in the production of IL-1β (Gross et al., 2011). Both of which have been shown to be upregulated after burn injury (Jeschke et al., 2015a; Stanojcic et al., 2014). In addition, the NLRP3 inflammasome assembly has shown to be activated by a number of sources including ER stress (Menu et al., 2012) and mitochondrial dysfunction (Iyer et al., 2013). Presently, we hypothesized that increasing burn severity causes cellular alterations that are associated with metabolic changes and hence result in representative inflammasome activity. All burn sizes caused marked upregulation of genetic and protein expression of ER stress marker BiP relative to controls, with elevated proportions in the >40% TBSA group (Figure 2-6A). Similarly, there was also a downregulation of COXIV, a well-established mitochondrial marker, indicating mitochondrial damage and concurrently AMPK activation supporting a compensatory response to stimulate mitochondrial biogenesis (Figure 2-6B-C). As a byproduct of NLRP3 inflammasome activation, IL-1β RNA expression was upregulated in WAT that increased with burn severity (Figure 2-6D).
Figure 2-6. Metabolic markers of augmented response in WAT. (A) ER stress marker BIP was up-regulated in all burn groups relative to controls with highest proportion in >40 TBSA. (B) Mitochondrial dysfunction was present and manifested by decreased expression of COXIV and increased (C) AMPK. As a measure of NLRP3 inflammasome activity, (D) adipose tissue IL-1β was increased in all burn groups and increased with increasing burn severity. All tissues were taken within 7 days post-burn. Data is represented as mean ± SEM. * ** & *** = significant difference between controls (n=5) and burned adults (n=34); ^, ^^ & ^^^ = significant difference between <20% TBSA and 20-40% TBSA; #, ## & ### = significant difference between <20% TBSA and >40%TBSA; ψ, ψψ ψ & ψψ ψ = significant difference between 20-40% TBSA and >40%TBSA; p<0.05, p<0.01 & p<0.001, respectively.
Organ biomarkers

We measured biomarkers to indicate integrity and function of the heart, kidney, pancreas, and liver. We found that burn causes alteration in cardiac biomarkers after 3 weeks post injury and is associated with decreased organ perfusion. Initial lactate was increased which is part of the initial response after burn (Figure 2-7A). Over time with an increasing incidence of organ complications and sepsis, lactate rose and so did troponin (Figure 2-7B). Renal biomarkers BUN and creatinine followed the aforementioned trajectory (Figure 2-7C-D). A similar trend was observed for pancreatic amylase and lipase with early increases and peaks between days 15-21 (Figure 2-7E-F). The liver profile revealed plateaus early and values decreasing over time (Figure 2-7G-J).

When stratified into the three burn groups, patients with larger burns (>40% TBSA) had significantly higher values than smaller burns (<20% TBSA) for these markers indicating impaired organ function. These observations parallel the clinical incidence of organ complications and sepsis where increasing burn injury severity is reflected in cardiac, renal, and liver markers alike (Figure 2-8).
Figure 2-7. Markers of organ function in burn patients. (A) Mean troponin T, (B) lactate, (C) blood urea nitrogen, (D) creatinine, (E) amylase, (F) lipase, (G) AST, (H) ALT, (I) ALP, and (J) bilirubin levels of adult burn patients. Error bars indicate SEM. TBSA, total body surface area; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.
Figure 2-8. Markers of organ function in burn patients stratified by injury severity. (A) Mean troponin T, (B) lactate, (C) blood urea nitrogen, (D) creatinine, (E) amylase, (F) lipase, (G) AST, (H) ALT, (I) ALP, and (J) bilirubin levels of adult burn patients by burn severity. Error bars indicate SEM. TBSA, total body surface area; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. * = significant differences between the severity groups (ANOVA test), p<0.05.
2.1.5 Discussion

In general burn outcomes have significantly improved over the last decades and survival can be expected. However, mortality and substantial morbidities still occur, and lead to debilitation of severely burned patients. The responses after burn are complex, profoundly altered and are very difficult to dissect. Despite the identification and delineation of the numerous components of the post-burn response, no prospective large clinical study has ever fully characterized the acute phase post-burn comprehensively in adult patients. The purpose of the present study was to determine the pathophysiologic response in terms of hypermetabolism, inflammatory stress responses and organ function in a large prospective clinical trial to enable developments of future interventions and treatment options.

This trial included 1288 patients with the vast majority being burn under 20% TBSA. The overall incidence of morbidities and complications was low, but it is interesting to note that 30% of these patients under 20% TBSA burn had wound infections, 2% sepsis and 5 patients deceased. When comparing patients suffering from 20-40% TBSA burns, it revealed that these patients have the shortest LOS and a mortality of 4%. The morbidities within this patient group are similar to the severe burn group (>40% TBSA), with smaller than expected differences; e.g. pneumonia 47% vs. 63%. However, profound differences in incidence proportions was supported when comparing the 20-40% and >40% TBSA groups for sepsis (26% vs. 60%), renal failure (5% vs. 22%) and mortality (4% vs. 15%), respectively. These results indicate the profound and substantial impact of larger burns on systemic morbidities.
The present study yielded both expected and some unexpected results. The expected results included the difference in clinical outcomes, the augmented metabolic response, and the profound inflammatory and stress responses in larger burn patients. However, the underlying signals and mechanisms are not entirely clear, as the majority of inflammatory stress markers such as cytokines are increased to a greater extent in the large burn group when compared to the <40% TBSA burn groups. One possible explanation could be ER stress in adipose tissue. We showed that even in the adipose tissue from smaller burns, ER stress is similarly increased when compared to larger burns. The adipose tissue is an important “organ” and has recently gained attention as two groups independently showed that the adipose tissue undergoes a response called browning (Patsouris et al., 2015; Sidossis et al., 2015). Hence, regardless of the burn size, ER-associated stress was present. These results indicate that even smaller burns undergo hypermetabolic alterations, which can be detrimental. Agonists of the hypermetabolic response include catecholamines and stress hormones such as cortisol (Goodall et al., 1957; Goodall, 1966; Wilmore, 1976; Wilmore et al., 1974). In the present study, we did not determine catecholamines or cortisol, but Jeschke and colleagues have recently shown that in burned children serum and urine cortisol increased 5-7-fold and remained elevated throughout the entire acute hospital stay (Jeschke et al., 2008a). Stress hormones such as glucocorticoids have been described as one of the major hormones responsible for proteolysis and catabolism (Hasselgren, 1995; Hasselgren, 1999; Tiao et al., 1996; Wang et al., 1998). Glucocorticoid levels are markedly increased post-burn, and therefore, a hypothetical approach to attenuate protein breakdown and hypermetabolism would be to block cortisol production. Additionally, Jeschke and colleagues have determined catecholamines (epinephrine and norepinephrine) in serum are elevated during acute hospitalization about 10-20 fold and remain elevated for up to 3-5 years (Jeschke et al., 2011). As catecholamines are mediators of inflammation, it seems therefore
logical for future studies to utilize catecholamine blockers and determine whether these agents can reduce hypermetabolism, stress and inflammation.

When considered as many parts working collectively and contributing to real physical manifestations, the impact of all these augmented biomedical markers is demonstrated by organ function, sepsis, and death. We found impairments in the heart, liver, and most importantly kidney, all of which are relative to burn size. While organ function returned to normal in the majority of patients, subsets of patients remained impaired and had organ dysfunction, further supporting underlying mechanisms dividing these injury groups.

Another point worth emphasizing is that the present study cohort is heterogeneous. We did not eliminate patients based on gender, inhalation injury, sepsis, multiple organ failure or death to smoothen out the trajectory or variables measured. The inclusion criteria facilitated achieving a large adult patient population in order to be able to perform robust statistics. We propose that the development of trajectories or patterns of these detrimental outcomes will be the focus of future studies.

In summary, based on our findings we suggest that a burn injury involving more than 20% of the total body surface can cause hypermetabolism, inflammatory and stress responses that are in some aspects as profound as in patients with over 40% TBSA burns. Despite being descriptive, this study highlights the important elements for all global burn care providers alike to utilize in order to understand the complicated immune and metabolic responses in order to treat their
patients. Collectively, treatments should focus on several aspects of the pathophysiologic events post-burn, such as alleviate hyperglycemia, anti-inflammation and attenuate hypermetabolism.
2.2.1 Rationale and Summary

We previously showed that severe thermal injury is associated with extreme and prolonged inflammatory response and in septic burn patients there is a significant and uncoordinated response during the early phases after insult. The aforementioned response has been shown to drive catabolism that collectively delay recovery or even leads to multiple organ failure and death. Burned patients exhibit many symptoms of stress-induced diabetes, including hyperglycemia, hyperinsulinemia, and hyperlipidemia. Recently, the NLRP3 inflammasome has received much attention as the sensor of endogenous “danger signals” and mediator of “sterile inflammation” in type II diabetes.

Objective of Study: We investigated whether the NLRP3 inflammasome is activated in the adipose tissue of burned patients, as we hypothesize that, similar to the scenario observed in chronic diabetes, the cytokines produced by the inflammasome mediate insulin resistance and metabolic dysfunction.

Summary of Results: Severely burned patients all exhibited burn-induced insulin resistance and hyperglycemia. We examined the adipose tissue of control and burned patients and found, via flow cytometry and gene expression studies, increased infiltration of leukocytes - especially macrophages - and evidence of inflammasome priming and activation. Furthermore, we observed increased levels of IL-1β in the plasma of burned patients when compared to controls.

Conclusions: In summary, our study is the first to show activation of the inflammasome in burned humans, and our results provide impetus for further investigation of the role of the inflammasome in burn-induced hypermetabolism and, potentially, developing novel therapies targeting this protein complex for the treatment of stress-induced diabetes.
2.2.2 Study Background

Severe thermal injuries result in a wide array of stress-associated inflammatory and metabolic changes aimed at restoring homeostasis of the body (Xiao et al., 2008; Williams et al., 2009b). Unfortunately, when these changes become uncontrolled, persisting far past the initial trauma, they lead to a state of severe metabolic dysfunction (Jeschke et al., 2011). Accordingly, trauma, critically ill, and burned patients often develop a form of stress-induced diabetes (with hyperglycemia, insulin resistance, and hyperlipidemia) (Jeschke et al., 2012a), which is linked to marked increases in morbidity and mortality (Jeschke et al., 2007b). In burned patients, studies have demonstrated that the significant pathophysiological changes and extreme inflammatory responses are not only present during acute hospitalization, but persist for a prolonged period and lead to severe catabolism, subsequently causing delays in their rehabilitation and reintegration (Gauglitz et al., 2009a). Although intensive efforts have long focused on identifying the underlying mechanisms of these extreme metabolic alterations, few studies have manage to elucidate how thermal injury induces hypermetabolism, prolonged inflammation and stress responses, and insulin resistance, and whether these alterations are responsible for the increased morbidity and mortality.

Due to the previously established increases in IL-1β in plasma of burn and septic patients, in the current study we hypothesized that the NLRP3 inflammasome is activated in the white adipose tissue of burned patients, resulting in concomitant elevation of serum IL-1β, and that this may be one mechanism of insulin resistance/metabolic alterations in patients with thermal injuries and stress-induced diabetes. We therefore designed a study to examine the subcutaneous fat of burned and non-burned patients for relevant parameters such as leukocyte infiltration, expression of macrophage markers, and elevated inflammasome activity.
2.2.3 Materials and Methods

Patients

Patients that were admitted to our burn centre with thermal injuries, required surgery, and were eligible for enrollment were consented for blood and tissue collection. These procedures were approved by the Research Ethics Board of Sunnybrook Health Sciences Centre (Study #194-2010). All patients received standard of care according to our clinical protocols, including early excision and grafting, early nutrition, adequate ventilation, adequate antibiotic coverage, etc. All patients studied suffered hyperglycemia and required insulin treatment during their stay in the burn unit. Insulin dosage was titrated on a sliding scale pursuant to the patient’s blood glucose levels and corresponding needs.

For analyses conducted in this study, patients are classified as burned/non-burned, or as acute moderate burns (burns that covered a total body surface area (TBSA) of <30%), acute severe burns (TBSA of ≥30%), and controls (patients suffering a thermal injury >3 years ago, admitted into our burn centre for wound management, or for reconstructive surgeries unrelated to burns). Excised fat was removed until the level of healthy tissue. Skin containing the upper layer of the adipose tissue that was injured was not included in this examination. Upon excision, all adipose tissue samples were immediately prepared for flow cytometry staining. The amount of patients for each of the measured variables differed due to the laborious flow analysis procedure which takes 2-3 days and requiring a large amount of adipose tissue which was not available in every patient. Moreover, obese patients (BMI>30) or those having a known medical history of diabetes mellitus were excluded, as these patients would be expected to exhibit leukocyte infiltration and inflammasome activation in the adipose tissue, independent of burn injury.
Harvesting of stromal vascular fractions from white adipose tissue (WAT)

Collected specimens were immediately transferred to the laboratory, where they were digested with collagenase (Sigma, St. Louis, MO) at 1 mg/ml in 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), resuspended 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.

Flow cytometry analysis

The percentage of leukocytes, monocytes, and T lymphocytes in the stromal vascular fraction (SVF) was determined by conducting flow cytometric analysis of cell surface markers. The following fluorochrome-conjugated antibodies were used: anti-CD45 (FITC, BD Biosciences, Mississauga, ON), anti-CD14 (PE, eBiosciences, San Diego, CA), anti-CD3 (APC, BD Biosciences, Mississauga, ON). Activity of caspase-1 was determined using the Green FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN), following the manufacturer’s flow cytometry protocol.
Gene expression studies

Total RNA was harvested from fresh or snap-frozen adipose tissue using the RNeasy Mini Kit (Qiagen, Germantown, MD). After the yield and quality of RNA were determined via Nanodrop, RT-PCR was conducted (Invitrogen/Life Technologies, Carlsbad, CA), and the cDNA was used in real-time gene expression studies (ABI/Life Technologies, Carlsbad, CA). The sequences of the primers for each target gene are listed below. Expression of 18s rRNA was used as a “loading” control for each sample. Gene expression levels were determined using the following formula: $2^{\Delta \Delta Ct}$

Primer sequences:

EMR-1; F-GATGAAGATCGGGTGTTCCACAA, R-CCATGCCCACAAAGGAGACAA

CD11b; F-AGATTGTGTGTTTGAGGTTTC, R-TGTGTATGTGTTGTTGTGT

NLRP3; F-TGAAGAAAGATTACCGTAAG, R-GCGTTTGTTGAGGCTCACACT

IL-1β; F-ATGATGGCTATTACAGTG, R-AGAGGTCCAGGTCCTGGAA

18s; F-GGCCCTGTAATTGGAATGAGTC, R-CCAAGATCCAAACTACGAGCTT

Circulating IL-1β levels

The cytokine profile in the plasma of patients was analysed using the Luminex Multiplex system with the Milliplex (Millipore, Billerica, MA) human cytokine/chemokine 39-plex (cat. HCYTOMAG-60K-PX39), following the manufacturer’s protocol. However, for the purposes of this study, only the level of IL-1β, TNF-α, and IL-6 are presented.
Statistical analysis

Student’s unpaired t-test was used to compare all results, with Welch’s correction where appropriate. Data is presented as means and standard error of the mean (SEM) for continuous variables, frequency and percentages for categorical variables. Comparisons between smaller sample sizes were analysed using Wilcoxon rank sum test. Statistical comparisons were conducted using SPSS 20 and figures were generated using GraphPad Prism 5.0 software. Significance was accepted at a p value less than 0.05.
### 2.2.4 Results

Including 64 burns and 12 controls, a total of 76 patients were enrolled in this study. Demographics and outcomes are presented in Table 2-2. The average age of our patients was between 40 (control) and 50 (burn) years, average length of stay was 34 days, and average TBSA was 25%. Of the 64 burned patients, 56 survived and were released from the burn unit, while 8 of the patients expired during their stay. All burned patients demonstrated some degree of insulin resistance, associated with hyperglycemia (n=38, Figure 2-9).

**Infiltration of leukocytes in white adipose tissue, post-burn injury**

We first investigated whether there are increased numbers of leukocytes, particularly macrophages, in the white adipose tissue of burned patients. Subcutaneous adipose tissue was collected from patients that required surgery to excise thermally injured tissue. Following processing, the SVF was harvested and labelled with anti-CD45, a common leukocyte marker (Figure 2-10A-B). To further characterize the CD45+ population, we studied the fraction of CD14+ (monocytes/macrophages) and CD3+ (T lymphocytes) cells in the SVF. Though we saw elevated levels of CD14 and CD3+ cells in the SVF of burned patients, without fail (data not shown), the observed levels of CD14+ cells and T cells in patients, post-burn, varied greatly (Fig. 2-10C).

Using flow cytometry, we found that the percentage of CD45+ cells within the isolated SVF was elevated more than 3-fold in patients suffering from acute thermal injury when compared to reconstructive surgical patients, (p=0.009, Fig. 2-10D).
Table 2-2. Patient Demographics

<table>
<thead>
<tr>
<th></th>
<th>Burned Patients</th>
<th>Control Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>Age, years, mean ± SD</td>
<td>51 ± 18</td>
<td>40 ± 13</td>
</tr>
<tr>
<td>Sex, Male/Female</td>
<td>21/43</td>
<td>8/4</td>
</tr>
<tr>
<td>Length of Stay (days)</td>
<td>34 ± 28</td>
<td></td>
</tr>
<tr>
<td>TBSA, %, mean ± SD</td>
<td>25 ± 18</td>
<td></td>
</tr>
<tr>
<td>Mortality, Survivor/Nonsurvivor</td>
<td>56/8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2-9. Hyperglycemia, hyperinsulinenia, and insulin resistance in burned patients. 
(Black line) Average daily glucose is shown for a portion of the patients included in this study. 
Burned patients exhibit sustained hyperglycemia following thermal injury. (Grey line) Average 
exogenous insulin units administered during the course of standard burn care per day are shown 
for a subset of the patients included in this study. Dashed lines represent normal glucose range.
Figure 2-10. Increased leukocyte infiltrates in white adipose tissue of patients with acute burn injuries. (A) A representative forward and side scatter profile of flow cytometric analysis of the stromal vascular fraction isolated from human white adipose tissue. (B) A histogram analysis to measure the percentage of CD45+ cells within the SVF. (C) A representative profile of CD14 and CD3 surface expression within the CD45+ population of the SVF. (D) Mean and SEM for the percentage of CD45+ cells in the SVF isolated from control (n=3) or burned patients (n=28). *p<0.05 vs. control.
Myeloid markers and NLRP3 inflammasome-associated transcripts are increased in adipose tissue post-burn.

In order to corroborate our flow cytometry findings, we then studied the transcript levels of various myeloid and macrophage markers in adipose tissue, post-burn. Real-time gene expression studies of myeloid marker CD11b and macrophage marker EMR-1 indicated that there are increased levels of these transcripts in patients with severe acute burns when compared to controls (Figure 2-11C & D), EMR-1 significantly so (~10-fold, p<0.01). Furthermore, analysis of genes indicating NLRP3 inflammasome priming, namely NLRP3 and IL-1β, revealed a significant increase (~4-fold and 15-fold, respectively, p<0.05 for both) in transcript levels in the adipose tissue of burned patients (Fig. 2-11A & B).
Figure 2-11. Elevated expression of inflammasome priming and myeloid/macrophage markers in white adipose tissue, post-burn. (A) and (B) illustrate gene expression of inflammasome priming markers. Steady-state transcript levels for both NLRP3 (control, n=6 and burn, n=25) and IL-1β (control, n=10 and burn, n=34) are significantly increased in burned patients. (C) Expression of CD11b (control, n=3 and burn, n=9), a myeloid and activated lymphocyte marker was not significantly increased in burned patients, but (D) levels of EMR1 mRNA (control, n=3 and burn, n=9), a specific macrophage marker, were significantly higher in the WAT tissue of the burn group when compared with controls. All data is presented as mean values with error bars as SEM, *p<0.05 and **p<0.01. rRNA = ribosomal RNA.
Caspase-1 is activated in CD14+ leukocytes of the SVF post-burn

Next, we sought to determine if the cells of the SVF demonstrated evidence of not only inflammasome priming, but also inflammasome activity. Assembly and activation of the NLRP3-inflammasome ultimately leads to caspase-1 activity (the “active” portion of the protein complex). Caspase-1 then processes pro-IL-1β into the mature form. Mature IL-1β can then act upon metabolic tissues, subsequently leading to altered insulin signaling and insulin resistance. To investigate the activity of caspase-1 in the CD14+ cells of the SVF, post-burn, we utilized the flow cytometry-based FLICA assay. Our results showed that patients with a burn size of less than or equal to 30% TBSA exhibited increased caspase-1 activity in the CD14+ fraction relative to controls. The same population of cells derived from patients suffering a burn greater than 30% TBSA demonstrated even greater activity (nearly double) when compared to smaller burns (p<0.0001) or controls (Figure 2-12).
Figure 2-12. Inflammasome activity is increased in the CD14+ portion of the stromal vascular fraction in white adipose tissue post-burn. Percentage of monocytes staining positive for inflammasome activity in the SVF isolated from WAT of control (n=2), <30% TBSA burns (n=5), and ≥30% TBSA burns (n=4) patients. Results were first gated to exclude CD14- cells, then analyzed for FLICA+ staining in order to ascertain caspase 1 activity (refer to methods). Inflammasome activity was significantly greater in monocytes of the severe burn group (***p<0.001) when compared to the moderate burn group. Control patients were unburned and donated subcutaneous fat tissue after undergoing liposuction or other surgical procedures for reconstructive purposes. All data is presented as mean values with error bars as SEM.
Circulating levels of IL-1β are increased post-burn

Assuming that increased NLRP3 inflammasome activity should lead to higher serum levels of IL-1β, we next investigated circulating levels of cytokines in patients following acute burn injuries. Indeed, we found that serum IL-1β was increased for minor burns (approximately 4-fold, p=0.004) and in the major burn group (approximately 6-fold, p<0.0001) when compared with controls (Figure 2-13). In addition, the increased IL-1β in the major burn group was significantly greater (p=0.005) relative to minor burns (Figure 2-13). Furthermore, several other inflammatory cytokines (TNF-α, IL-6 and IL-8) known to be involved in metabolic regulation were elevated in the burned group, as expected and demonstrated in previous publications from our laboratory (Figure 2-14). Despite the greater proportion of males with burn injuries, there were no significant sex differences between the groups for cytokine expression and inflammasome activity (data not shown).
Figure 2-13. Elevated circulating protein levels of IL-1β post-burn. Patients with acute moderate burns (<30% TBSA, n=42) and acute severe burns (≥ 30% TBSA, n=34) exhibit significantly elevated levels of plasma IL-1β (***p<0.0001) when compared to controls (n=5), as measured by luminex technology. Comparing severity of burn injury, the severe burn group demonstrated significantly greater concentration of IL-1β (#p<0.05). All data is presented as mean values with error bars as SEM.
Figure 2-14. Elevated circulating inflammatory cytokines post-burn.
Burned patients (n=31) exhibit significantly elevated levels of plasma (A) TNFα, (B) IL-6, and (C) IL-8 (*p<0.05, ***p<0.001) when compared to controls (n=2), as measured by luminex technology. Each of these pro-inflammatory cytokines has been shown to contribute to both the acute phase response and insulin resistance in various settings. All data is presented as mean values with error bars as SEM.
2.2.5 Discussion

To our knowledge, this is the first report of increased inflammasome activity in the adipose tissue of burned patients. Burned patients not only endure catastrophic surface wounds, but also body-wide deleterious effects resulting from metabolic perturbations such as hyperlipidemia, hyperinsulinemia, and hyperglycemia, collectively referred to as “hypermetabolism” (Jeschke et al., 2008a). Specifically, metabolic dysfunction in burned patients is more severe than that seen in any other trauma populations and has been linked to increased incidence of several poor outcomes, including infections, sepsis, delayed wound healing, multi-organ failure, and most importantly mortality (Gore et al., 2001; Gore et al., 2002; Hemmila et al., 2008; Jeschke et al., 2010). Discovering the mechanisms that lead to post-burn hypermetabolism is of the utmost importance, as little progress has been made in the effort to improve outcomes over the past several decades, and a dearth of novel therapies remains. Thus, the significance of the present study lies in the translational nature – by analyzing samples derived from burned patients, our results provide direct impetus for targeting burn-induced inflammasome activity, ultimately making the goal of novel therapies more attainable.

Our initial efforts to investigate activity of the NLRP3 inflammasome in the subcutaneous fat of our burned patients stemmed from both: 1) our observation that burned patients suffer extreme inflammatory responses (even in the absence of complicating infections) (Jeschke et al., 2011; Jeschke et al., 2004) and 2) the attention this protein complex is receiving in the type II diabetes literature (mainly regarding macrophages and their contribution to “sterile” inflammation and
adipose tissue dysfunction), as our patients suffer similar symptoms, though stress-induced diabetes is of a more acute nature. Presently, we established that there is a 3-fold increase in leukocyte levels in the adipose tissue harvested from our patients (Figure 2-10D), indicating increased infiltration and inflammation, as we expected. According to work published by Koenen, in the context of adipose tissue and metabolic syndrome, the majority of these leukocytes should be granulocytes, monocytes/macrophages, and T cells (Koenen et al., 2011). Our flow cytometry findings confirm the presence of monocytes and T cells (Figure 2-10C), which we have found to be consistently higher in the burned group. However, the levels of monocytes/macrophages and T cells seem to vary greatly with time – and perhaps other factors – and the significance of this finding is currently under investigation.

As such, we went on to corroborate our flow cytometry findings with gene expression studies. Figure 2-11A and B show a significant increase in NLRP3 and IL-1β transcripts, indicating greater priming of the NLRP3 inflammasome in the burned group. EMR1 levels were also significantly increased in the RNA isolated from adipose tissue of burned patients (Figure 2-11D), indicating an increased presence of macrophages, the most-studied leukocyte with regard to adipose inflammation and metabolic disorders. The CD11b transcript level was not significantly increased (Figure 2-11C), but we did not find this data particularly discomfiting, as CD11b is not a marker specific to macrophages and monocytes; it can also be found on granulocytes, neutrophils, and activated T cells. Furthermore, the “control” patients also underwent surgical procedures, which we would expect to cause some local inflammatory responses. Therefore, we had no reason to necessarily expect the expression to be significantly higher in the burn group.
However, the key question posed in this study was: is there greater activation of the inflammasome in the adipose tissue of burned patients? If so, is this activity greater with increasing burn size? If inflammasome activity could be contributing to metabolic dysfunction in burn and, hence, poor outcomes, we would expect to see the results obtained in Figure 2-12, where activity was significantly increased in the severe burn group compared to the moderate burn group. We were also surprised to find that, though detectable, inflammasome activity in the control samples was half that observed in the small burn group; liposuction and other surgical procedures involve extensive local tissue destruction, which should generate many DAMPs. Importantly, the observation that inflammasome activation was present, but not as great, in the control group gave us greater confidence that our data is not simply the artificial result of the surgical procedure preceding processing of the tissues.

Furthermore, the data presented in Figure 2-13 illustrates that the observed increase in inflammasome activity results in the expected significant elevation of plasma IL-1β for both acute moderate and severe burns. As previously mentioned, (adipose-derived) increased circulating levels of IL-1β are now thought to be strongly associated with diabetes and insulin resistance in the context of metabolic syndrome. We believe the same sort of mechanism could be playing a role in the metabolic perturbations observed in our burned patients. Currently, we are investigating whether the IL-1β levels are most significantly increased in the fat tissue, itself, as it’s very possible that the paracrine inflammatory and metabolic effects of this cytokine are much greater than the endocrine.
Metabolic regulation is an extremely complicated “symphony” of integrated hormonal and nutritional cues of several metabolic tissues. However, our focus on the adipose tissue in the current study stems from the concept of the “primacy of fat”. According to Osburn and Olefsky, who elegantly reviewed the current state of knowledge regarding inflammation and metabolic disorders recently (Osborn and Olefsky, 2012), tissue-specific knockout studies have shown that restoring metabolic homeostasis in the fat tissue leads to improved metabolic function in other tissues, namely the liver and muscle. Conversely, the opposite relationships do not hold true, indicating that adipose is more important in the “hierarchy” of communication and cross-regulation between these metabolic tissues.

We should mention a few cautionary notes with respect to our study. For instance, the authors believe that the abdominal adipose tissue is probably different from peripheral because the amount is also different. By using adipose tissue that is always excised from the burn wound, it is a medium between the skin that is burned and the superficial adipose that appears unhealthy. Again, this is healthy viable adipose tissue. However it is close to the burn site. Hence, we cannot assume that leukocyte infiltration is uniform within the affected burn tissue and adjacent regions. In addition, there are several types of inflammasomes that lead to caspase-1 activity and generation of IL-1β. Similarly, there are other inflammatory cytokines with established roles in metabolic regulation and insulin resistance (Figure 2-14). We cannot, therefore, conclude that the NLRP3 inflammasome is the only complex, or that IL-1β is the only cytokine, responsible for the results observed. Furthermore, we have focused on macrophages and monocytes in this report, but many other publications have shown that other types of leukocytes play a role in chronic adipose inflammation with regard to metabolic dysfunction, and future studies will take
this into account and be more inclusive. It would also be interesting to investigate the phenotype of the macrophages we are studying here, as there is much evidence that polarization of macrophages is key, with some subtypes being beneficial and some being detrimental in metabolic regulation (Osborn and Olefsky, 2012). Finally, most of the literature pertaining to fat and inflammation focuses on the deleterious effects of visceral adipose tissue, and we are assessing subcutaneous fat in our studies. There are several reasons for this, mainly the ease of accessibility (this fat tissue is removed during debridement, regardless of our studies) and our belief that subcutaneous fat is perhaps of equal or greater importance in the specific context of burns (Cree and Wolfe, 2008).

In summary, we have found increased leukocyte infiltration in the subcutaneous fat tissue of burned patients. At least a portion of these infiltrating leukocytes are of the monocyte lineage, a cell type that is well-characterized in the context of metabolic dysfunction. These monocytes demonstrate increased inflammasome activity that is associated with greater levels of circulating IL-1β. Our data is in agreement with many studies already published in the metabolism literature and indicate a potentially significant role for the NLRP3 inflammasome in the hypermetabolic state that contributes so greatly to poor outcomes in our patient population. Future studies will continue to strengthen the relationship between the NLRP3 inflammasome and stress-induced diabetes and delineate mechanistic details with the goal of developing novel therapies and improving outcomes for burned patients.
In this chapter, we have demonstrated that burn patients have a hyperinflammatory and chemokine response after injury and that this increased expression occurs as a function of increasing injury severity. In septic patients, relative to their non-septic counterparts, they showed an exacerbated hyperinflammatory response that occurs during the acute or early time points after injury. Notably, one of these pro-inflammatory cytokines that is increased in plasma is IL-1β, which is the major byproduct of NLRP3 activation. When looking at white adipose tissue, we found increased gene and protein expression of the NLRP3 inflammasome complex, and its major byproduct. This also occurred as a function of increasing injury severity with more severe burns resulting in greater activity. With this in mind, the next section will establish the rodent model of sepsis using burn plus *pseudomonas aeruginosa* infection and determine if knocking out the NLRP3 inflammasome during sepsis-burn will mitigate or abolish the hyperinflammatory response, decrease immune activity, improve bacterial clearance and overall survival.
Chapter 3

NLRP3 Inflammasome knockout and its effects on innate immune response, inflammation and survival during post-burn sepsis
**The Role of NLPR3 Inflammasome in Response to Thermal Injury and Sepsis**

**Hypothesis**
- Adult burn and burn-sepsis patients will have a hyperinflammatory response after injury that parallels increased NLPR3 inflammasome activation.
- Burn plus *Pseudomonas aeruginosa* induced sepsis in rodents results in NLPR3 inflammasome activation and blocking this activation will result in dampened inflammation, improved bacterial clearance and overall survival.
- NLPR3 inflammasome is activated in adipose tissue of thermally injured patients and can be used to delineate patients susceptible to sepsis.

**Objectives**
- To characterize the hyperinflammatory response and activation of the NLPR3 inflammasome in WAT of adult burn patients.
- To characterize the hyperinflammatory response and activation of the NLPR3 inflammasome in WAT of septic burn patients.
- To determine whether the NLPR3 inflammasome can be used as a biomarker to identify thermally injured patients susceptible to sepsis.
- To determine whether NLPR3 inflammasome systemic knockout will result in dampened innate immune activity, reduced inflammation, improved bacterial clearance and survival in rodents during the course of post-burn sepsis.
3.1.1 Rationale and Summary

Although sepsis in thermally injured patients represents the main contributor to post-burn mortality, effective treatments are presently absent and underlying mechanisms are essentially unknown. Recently, the NLRP3 inflammasome was shown to orchestrate burn-induced inflammatory driven pathophysiologic processes.

Objective of Study: As the NLRP3 inflammasome is activated in the white adipose tissue of burn patients, we hypothesized that NLRP3 activation contributes to adverse outcomes in burn patients who become septic. To test our hypothesis, we utilized the two-hit model of burn plus Pseudomonas aeruginosa wound infection/sepsis in NLRP3 knockout mice.

Summary of Results: Confirming our hypothesis, we found that NLRP3−/− mice had improved survival. In contrast to our hypotheses, we showed that improved survival in NLRP3 k.o. mice was characterized by greater infiltration of immune cells at the site of injury and better bacterial clearance. These robust populations were present acutely after infection and a better mobilization of immune response was attributed to this survival. Lymphoid organs and liver all had increased macrophage and neutrophil expansions beyond the acute phase that occurred secondary to adipose tissue, suggesting its critical role in post-burn sepsis. Interestingly, ablation of NLRP3 in mice resulted in increased acute systemic inflammation (IL-6, TNF-α, IL-1β) and greater ER stress, apoptosis/pyroptosis in adipose tissue. In burn patients, we found that increased NLRP3 gene expression in adipose tissue beyond the acute phase determined sepsis and had higher mortality.
**Conclusion:** Our findings suggest that NLRP3 directly contributes to mortality in post-burn sepsis and ablation results in a tissue-specific responsiveness that improves survival by increasing immune mediators of inflammation in a paradoxical and compensatory non-persistent response.
3.1.2 Study Background

Nowadays, sepsis in burn patients is the leading cause of mortality (Williams et al., 2009a; Church et al., 2006). Burn patients are more prone to sepsis due to loss of skin, the primary barrier against pathogens and bacterial infections. Furthermore, immune dysfunction coupled with protein degradation and catabolism (hallmarks of the hypermetabolic response), collectively contribute to organ dysfunction (Hart et al., 2000a; Williams et al., 2009b). Although the immune-metabolic and inflammatory responses are intended as healing or protective mechanisms, however, when these processes become prolonged beyond the acute phase, patients are at an increased risk of immune-exhaustion making them susceptible to infection and sepsis, and ultimately mortality. The inflammatory response that is initiated immediately post-burn can last for several weeks to months after injury with distinct immune trajectories in these patients (Finnerty et al., 2008; Jeschke et al., 2007b). Cytokines including IL-6, IL-1β, IL-10, MCP-1 and TNF-α are important mediators of the immune response and play a crucial role in the complex pathophysiology underlying post-burn sepsis (Jeschke et al., 2007b). In fact, elevations in pro-inflammatory cytokines have been associated with greater injury severity and poor outcomes in burn patients (Jeschke et al., 2007b).

The pattern of the inflammatory response follows the “two-hit” theory of inflammation, which suggests that the initial injury, in this case burn, primes the host immune system for an exaggerated inflammatory response upon a second insult, such as infections usually involving an opportunistic pathogen (Murphy et al., 2005; O’Riordain et al., 1996). Pathogens that frequently cause sepsis in burn patients, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are opportunistic organisms that thrive in an immunosuppressed host (Lavrentieva et al., 2017;
Mann-Salinas et al., 2013; Hotchkiss et al., 2013; Glik et al., 2012). PA infections are the most prevalent in burn wounds and account for over half of all severe burn infections (Hodle et al., 2006; Nichols et al., 2013). The clinical relevance of the infection is that thermally injured patients who acquire PA infections have a four-fold greater mortality than those who do not (Armour et al., 2007). Although sepsis in thermally injured patients represents the main contributor to post-burn mortality, effective treatment is absent and underlying mechanisms are essentially unknown.

Recently, the NLRP3 inflammasome was shown to orchestrate sepsis induced inflammatory driven pathophysiologic processes. The NLRP3 inflammasome is a protein complex that mediates inflammation and metabolic regulation by cleaving IL-1β and IL-18 into their bioactive forms in response to pathogens or stress (Martinon et al., 2002). The inflammasome is assembled by a number of instigators including ER stress (Bronner et al., 2015), mitochondrial damage (Shimada et al., 2012) and lipids (Wen et al., 2011), all of which have been shown to be significantly upregulated after burn injury due to dysfunctional tissue regulation in adipose tissue and liver (Song et al., 2009; Bogdanovic et al., 2015). We have shown that the NLRP3 inflammasome is activated at the site of injury in white adipose tissue of adult burn patients (Stanojcic et al., 2014). More recently, our group further delineated that NLRP3 inflammasome activation was also increased in adult burn patients relative to injury severity and occurred in the presence of greater ER stress and mitochondrial dysfunction (Stanojcic et al., 2017). Originally believed to have a protective role after burn injury in rodents, blocking caspase-1 (NLRP3 inflammasome effector component that cleaves IL-1β and IL-18 into their mature forms) resulted in significantly higher mortality after insult (Osuka et al., 2012). However, the activation and
function of the NLRP3 inflammasome during sepsis has yet to be elucidated. While numerous animal models of sepsis have been established (Chen et al., 2014), none are truly able to recapture the complexity and heterogeneity of sepsis in humans (Seok et al., 2013; Fink, 2014). To reflect the clinical scenario in burned patients, presently we utilized the two-hit model of sepsis that is characterized by burn plus inoculation of the wound with PA bacteria (Huang et al., 2006). As the NLRP3 inflammasome is activated in the white adipose tissue of burn patients we hypothesized that activation of the NLRP3 complex is detrimental and hence knockout of the NLRP3 inflammasome will improve bacterial clearance and survival in thermally injured septic mice by dampening innate immune activity and overall inflammation over time. To translate our animal data into a clinical setting, we then aimed to determine if NLRP3 inflammasome activation is augmented in severely burned patients who had sepsis during acute hospitalization.
3.1.3 Materials and Methods

Animals and model

Wild-type C57/B6 (WT) and NLRP3 knockout (NLRP3−/−) male mice (6-8 weeks old, n=5 per group) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at ambient temperature and cared in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Sunnybrook Research Institute Animal Care Committee (Toronto, Ontario, Canada). All mice were anesthetized with 2.5% isoflurane and shaved along the dorsal spine region. Ringers lactate (2-3 mL) was injected subcutaneously in all treatment mice to protect the spine and buprenorphine (0.05-0.1 mg/kg body weight) was injected for pain management. A full-thickness, third degree dorsal scald burn encompassing 25% total body surface area (TBSA) was induced by immersing mice in 98°C water for 10 seconds. Control mice received similar conditions excluding the burn and infection insults. All tissues were harvested upon sacrifice and stored in -80°C until analysis. Body and spleen weights were measured prior to infection and at the time of sacrifice for each group.

Pseudomonas infection

Pseudomonas aeruginosa (ATCC, Rockville, MD) was grown in tryptic soy agar (Sigma-Aldrich) overnight than prepared in saline to a concentration of 1.0-1.2 x 10⁷ colony-forming units (CFU). WT and NLRP3−/− burned mice (burn+PA) received a topical infection 72-hours after burn injury, by applying 100uL on the burn area and than subsequently housed individually. All mice were sacrificed at 12- (topical infection is present systemically), 24- and 72- (sepsis onset)
conditions in WT) hours after infection.

**Bacterial counts**

Upon sacrifice, a small portion of blood, lung and injury site were manually homogenized in 1mL saline and plated onto tryptic soy agar. Plates were grown overnight at 37°C and CFU was quantified for PA populations.

**Tissue staining and flow cytometry**

Bone marrow, spleen, site of injury, lung, liver and epididymal adipose tissue cells were digested in collagenase (Life Technologies), Fc blocked (anti-mouse CD16/CD32, BD Pharmeden) on ice for 15 minutes and stained with monoclonal antibodies on ice for 30 minutes. Samples were then washed 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 x FSC-H, SSC-W x SSC-H). The total percentage of monocytes/macrophages and neutrophils were identified using the following fluochrome-conjugated antibodies: anti-CD45 (anti-mouse PE-Cyanine7, eBioscience), anti-CD11b (anti-mouse Alexa APC-eFluor® 780, eBioscience), anti-F4/80 (anti-mouse FITC, eBioscience), anti-CD11c (anti-mouse PerCP-Cyanine5.5, eBioscience) and anti-GR-1 (anti-mouse PE, eBioscience) in accordance with the manufacturer’s flow cytometry protocol. The gating strategy for assessing innate immune cell distributions included leukocytes initially gated based on granularity and CD45 (side scatter x CD45), followed by size (forward scatter). Gated cells were stained for cell surface markers for
monocytes and macrophages (CD11b+/F4/80+) or neutrophils (CD11b+/GR-1+).
Monocytes/macrophages were also gated on CD45+/CD11b+/F4/80+/Ly6C+ populations and showed similar cell proportions and trajectories so all subsequent analysis was continued using CD45+/CD11b+/F4/80+ gating for this population.

**Gene expression using RT-PCR**

RNA was extracted from rodent liver and adipose tissue and excised human adipose tissue from burn patients using Trizol (Invitrogen, CA, USA). Reverse transcription were performed with high-capacity cDNA reverse transcription kit (ABI, MA, USA). RT-PCR was performed using TaqMan® Fast Advanced Master Mix with the following primers il1b (Mm00434228_m1, Casp1 (Mm00438023_m1, ThermoFisher), il18 (Mm00434226_m1, ThermoFisher), Casp3 (Mm01195085_m1, ThermoFisher), Casp8 (Mm01255716_m1, ThermoFisher), Actb (Mm02619580_g1, bip (Mm00517691_m1, ThermoFisher), in accordance with manufacturers protocol. Gene expression was expressed relative to β-actin. Due to inter-species variability between WT and NLRP3−/− control concentrations, all values are presented as a ratio relative to the mean concentration of species-specific controls for a given primer.

**Western blotting**

Proteins from rodent liver and adipose tissues were extracted in RIPA buffer containing phosphatases and proteases inhibitor cocktails (Roche). Protein concentrations were determined by the BCA protein assay kit (Pierce, Mississauga, ON, Canada). Proteins were resolved by
SDS-PAGE followed by western blotting using the following antibodies at 1:500-1:1000 concentration: IL-1β (Cell Signaling, MA, USA), Caspase-1 (Abcam, MA USA), IL-18 (Abcam, MA USA), AIM2 (eBioscience, USA), ASC (Adipogen, CA, USA), NF-κB (Cell Signaling, MA, USA), phospho-eIF2α (Cell Signaling, MA, USA), phospho-JNK (Cell Signaling, MA, USA), BiP (Cell Signaling, MA, USA) and GAPDH (Cell Signaling, MA, USA). Species appropriate secondary antibodies conjugated to horse radish peroxidise (BioRad, Mississauga, ON, Canada) were used and proteins visualized by enhanced chemiluminescence using the BioRad ChemiDoc MP Imaging System. Band intensities were detected, normalized and quantified with the Chemidoc and Image Lab 5.0 software (BioRad Laboratories, Hercules, CA). Antibody concentrations are expressed relative to GAPDH. Due to inter-species variability between WT and NLRP3−/− control concentrations, all values are presented as a ratio relative to the mean concentration of species-specific controls for a given antibody.

**Cytokine profiling**

EDTA-anticoagulated blood samples were collected from all mice at the time of sacrifice and stored in -80°C until analysis. Plasma samples were used to compare inflammatory, chemokine and immune mediators between groups 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 ± SEM and expressed in pg/ml.
**Patient samples and gene expression in adipose tissue**

Adult burn patients (≥ 18 years of age) admitted to the Ross Tilley Burn Centre at Sunnybrook Hospital (Toronto, Canada) or patients undergoing elective surgery were consented pre-operatively for tissue collection and inclusion. Approval for our study was obtained from the Research Ethics Board at Sunnybrook Hospital (REB#: 194-2010). Patients were excluded if the admission was elective or if it was a readmission. We analyzed 34 burned patients WAT obtained from both early (0-11 days post burn) and later (≥12 days post burn) surgical time points (refer to Table 3-1 for detailed patient demographics). 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 (Greenhalgh et al., 2007; Singer et al., 2016). Ten healthy controls were used to compare burn patients to normal tissue. Adipose tissue was immediately transferred to the laboratory and frozen (-80°C) until time of analysis. Specific primers yielding single specific amplicon were chosen for Nlrp3, il1b, Caspase1 and il18. RT-PCR was performed with sybr green Supermix (Biorad, CA, USA).
Table 3-1. Clinical Demographics of Adult Burn Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-Sepsis</th>
<th>Sepsis</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>15</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years, mean ± SD</td>
<td>43 ± 12</td>
<td>50 ± 18</td>
<td>0.184</td>
</tr>
<tr>
<td>Male, no. (%)</td>
<td>11 (73%)</td>
<td>14 (74%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Injury characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBSA, %, mean ± SD</td>
<td>33 ± 14</td>
<td>40 ± 14</td>
<td>0.163</td>
</tr>
<tr>
<td>Inhalation injury, no. (%)</td>
<td>5 (33%)</td>
<td>12 (63%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Baux score, mean ± SD</td>
<td>81 ± 22</td>
<td>98 ± 29</td>
<td>0.059</td>
</tr>
<tr>
<td>Complications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia, no. (%)</td>
<td>3 (20%)</td>
<td>17 (89%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart failure, no. (%)</td>
<td>0</td>
<td>1 (6%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Respiratory failure, no. (%)</td>
<td>0</td>
<td>6 (32%)</td>
<td>0.024</td>
</tr>
<tr>
<td>Liver failure, no. (%)</td>
<td>0</td>
<td>1 (6%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Renal failure, no. (%)</td>
<td>0</td>
<td>7 (37%)</td>
<td>0.01</td>
</tr>
<tr>
<td>MOD, no. (%)</td>
<td>0</td>
<td>3 (16%)</td>
<td>0.23</td>
</tr>
<tr>
<td>LOS, days. mean ± SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37 ± 4.9</td>
<td>61 ± 7.8</td>
<td>0.014</td>
</tr>
<tr>
<td>LOS/TBSA. days/%, mean ± SD&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.2 ± 0.5</td>
<td>1.6 ± 0.9</td>
<td>0.078</td>
</tr>
<tr>
<td>Mortality, no. (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>5 (26%)</td>
<td>0.053</td>
</tr>
</tbody>
</table>

MOD, multiple organ dysfunction; LOS, length of stay; TBSA, total body surface area

<sup>a</sup> Significant differences between non-sepsis and sepsis groups
Statistical analysis

All data are represented as mean ± SEM. Survival curves were analyzed using the log-rank (Mantel–Cox) test. Statistical analysis was performed using student’s t-test, one and two-way ANOVA and Mann–Whitney U test to compare groups, where appropriate. 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 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.05 (#), p < 0.01 (##), p < 0.001 (###), where appropriate.
3.1.4 Results

Post-burn septic NLRP3\(^{-/-}\) have improved bacterial clearance and survivability compared to WT mice

Our model of burn plus sepsis demonstrated a mortality in WT burn+PA of 40% with mortality occurring as early as 3-4 days post infection and was significantly greater than controls (Mantel-Cox = 5.3, \(p=0.021\), Figure 3-1A). This is comparable to the clinical scenario; burn patients with sepsis have a 30-40% mortality rate (D’Avignon et al., 2010). Ablation of the NLRP3 inflammasome resulted in improved overall survival (90%) relative to WT burn+PA (Mantel-Cox = 3.9, \(p=0.047\); Figure 3-1A). Unlike septic wildtypes, NLRP3\(^{-/-}\) burn+PA mice reached their end point significantly later (8 days) after infection (\(p<0.05\)), indicating not only improved but also prolonged survival.

When comparing bacterial counts at 72-hours after infection (Figure 3-1B-D), NLRP3\(^{-/-}\) burn+PA had significantly lower proportions at the site of injury (\(p<0.05\)) and less infiltration in the lungs (\(p<0.05\)) relative to WT burn+PA mice, suggesting that sepsis was exclusive to WT. Thus, despite scarce colonies of PA bacteria, absence of NLRP3 prevented the development of wound infection to systemic sepsis in nearly all mice (\(p<0.05\)).

We next wanted to determine if these improvements were attributed to improved innate immune cell populations at the site of injury. Flow cytometric analysis acutely after infection (12-hours) revealed NLRP3\(^{-/-}\) had increased monocyte/macrophage proportions at the site of injury relative to controls (\(p<0.01\)) and WT burn+PA (\(p<0.05\), Figure 3-1E-F). This was also observed at 24-hours.
Figure 3-1. NLRP3 knockout improves survival via immune infiltration and bacterial clearance at the site of injury. (A) Survival curve of WT (control and burn+PA) and NLRP3−/− (control and burn+PA) mice relative to days post infection (n=10/group). (B-D) Analysis of bacterial counts during sepsis (72-hours post infection) in WT and NLRP3−/− mice for site of injury, lung and plasma, expressed as colony-forming units per gram of tissue. (E) Representative images of monocyte/macrophage (CD45+/CD11b+/F4/80+) populations at the site of injury in WT burn+PA and NLRP3−/− burn+PA, 12-hours after infection. (F, G) Temporal distribution percent of total population of macrophage and neutrophils (CD45+/CD11b+/GR-1+) for WT and NLRP3−/− controls and burn+PA groups at 12, 24 and 72-hours after infection. Dotted lines represent controls (WT and NLRP3−/−) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3−/− burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3−/− burn+PA, (n=5/group). *# p<0.05, **/ ### p<0.01 and ###### p<0.001.
During sepsis (72-hours), increased macrophages at the site of injury were common to both WT and NLRP3−/− relative to inherent controls (p<0.01, Figure 3-1F), despite no significant difference between infection groups. Notably, the increases in macrophage population during sepsis in wildtypes were comparable to that of NLRP3 knockouts during the early time point (12-hours), suggesting a delayed response to infection. A similar trajectory to macrophages was observed in neutrophils of the infectious knockouts who had a greater proportion relative to WT at 24-hours (14.3% vs. 2.5%; p<0.01, Figure 3-1G). At 72-hours after infection, WT burn+PA group had a late surge in neutrophils relative to controls (p<0.001) and NLRP3−/− burn+PA (p<0.01). The absence of an abundant neutrophil population in knockouts during sepsis suggests a decreased immunological demand from pathogenic intrusion. Collectively, these findings suggest that NLRP3 knockout mice survive post-burn sepsis better than WT counterparts in part due to improved macrophage and neutrophil recruitment to the site of injury and containment of bacterial spread than the WT group.

**NLRP3−/− mice have increased macrophage and neutrophil mobilization after infection independent of lymphoid organs.**

Taking into consideration the improved bacterial clearance from the site of injury occurred as a result of greater proportion of macrophage and neutrophils, we next investigated the possible origins of these infiltrated immune cells. When comparing immune cell production in the peripheral lymphoid organ (spleen), WT burn+PA mice show an overall increase in proportions from infection (12-hours) to sepsis (72-hours) progression. Macrophages in the spleen demonstrated an exacerbated response in WT burn+PA group at 24-hours relative to NLRP3−/− burn+PA and controls (p<0.05 & p<0.001, Figure 3-2A-C). A similar trend was observed in
neutrophils with the greatest proportions occurring at 24-hours between the infectious groups (p<0.05, Figure 3-2C). When comparing spleen size, both infection groups had enlarged spleens relative to controls (Figure 3-3A). When comparing the infection groups, WT had significantly greater spleens at 24-hours (p<0.001), whereas at 72-hours an inverse relationship was apparent with NLRP3−/− having greater splenomegaly (WT burn+PA: 99.1mg vs. NLRP3−/− burn+PA: 133.3mg, p<0.05, Figure 3-3B). When comparing the bone marrow monocyte/macrophage population in WT burn+PA, there was a gradual increase that was significantly higher at 24 (p<0.05) and 72-hours (p<0.05), relative to NLRP3−/− burn+PA (Figure 3-2D-E). Interestingly, neutrophils in the bone marrow of NLRP3 knockouts had a greater proportion early after infection (p<0.05) and showed a late peak during sepsis at 72-hours after insult (p<0001, Figure 3-2F). Thus, acute infiltration at the site of injury was most likely not attributed to immune expansion in the lymphoid organs. Extending this analysis to the liver and adipose tissue, the lack of NLRP3 inflammasome resulted in increasingly greater macrophages and neutrophils in the liver over time, peaking at 72-hours after infection (Figure 3-2G). At 24-hours after infection, NLRP3−/− burn+PA had approximately 2-times more macrophages (p<0.01) and neutrophils (p<0.01) than WT counterparts (Figure 3-2H-I). At 72-hours, this trend persisted (macrophage: 14% vs. 5%; neutrophils: 7% vs. 2.5%, p<0.01).
CD11b

Figure 3-2. Flow cytometry analysis of innate immune cells in lymphoid organs and infection infiltration tissues. Representative images of monocyte/macrophage (CD45+/CD11b+/F4/80+) population in the (A) spleen, (D) bone marrow, (G) liver and (J) adipose tissue of WT burn+PA and NLRP3-/- burn+PA groups at 24- and 72-hours after infection. (B, C, E, F) Temporal analysis of lymphoid organs (bone marrow and spleen) for monocyte/macrophage and neutrophils (CD45+/CD11b+/GR-1) of control and burn+PA for each WT and NLRP3-/- strains. (H, I) Hepatic macrophages and neutrophils in NLRP3-/- burn+PA revealed a progressively increased infiltration of immune cells beyond the acute time point (12-hours). (K, L) Adipose tissue of NLRP3-/- burn+PA group exclusively supported an acute elevation in proportion of macrophage and neutrophils acutely after infection (12-hours) and returned back to baseline at later time points relative to WT infectious counterparts. Dotted lines represent controls (WT and NLRP3-/-) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3-/- burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3-/- burn+PA, (n=5/group). */# p<0.05, **/### p<0.01 and ***/#### p<0.001.
Figure 3-3. Spleen Morphology. (A) Representative images of spleens from control and burn+PA mice from both WT and NLRP3⁻⁻ genotypes. (B) Temporal distribution of spleen weight over time. Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3⁻⁻ burn+PA (n=5/group), */# p<0.05, */## p<0.01 and ***/### p<0.001.
Unlike the gradual increases in hepatic innate immune cell populations in NLRP3−/− septic mice, the adipose tissue supported a unique, acute responsiveness to insult relative to WT. At 12-hours after infection, NLRP3−/− burn+PA had a 4-fold increase in macrophages (p<0.05) and nearly 6-fold increase in neutrophils (p<0.05), relative to WT burn+PA. To a lesser extent, WT burn+PA demonstrated macrophage and neutrophil increases taking place much later at 72-hours after infection (Figure 3-2J-L), supporting an abundance of pathogen combatants acutely after insult and exclusive to knockouts. Taken together, these findings suggest that the adipose tissue of NLRP3 knockouts exclusively elicits an immediate response to combat pathogenic invasion and prevent bacterial spread. These observations occurred secondary or later in liver and lymphoid organs. As the main immune modulators after burn trauma (Xiu et. al., 2014), we next wanted to determine the effects increased innate immune cell populations would have on ER stress and inflammasome-related activity in adipose tissue and liver.

**Increased adipose tissue inflammasome activation, ER stress and cell death early after infection in NLRP3−/− burn+PA mice**

The role of adipose tissue has largely been neglected in the context of infectious diseases. Its strong pro-inflammatory and metabolic potential suggests an important role in the systemic innate immune response specifically during septic states. When comparing inflammasome activity in infectious groups early (12-hours) after insult, NLRP3−/− burn+PA showed increased expression in ASC (p<0.05), IL-1β (p<0.05) and IL-18 (p<0.05) relative to WT’s (Figure 3-4A-C). The increased expression in NLRP3−/− persisted at 24-hours for ASC (p<0.01) and IL-18 (p<0.05).
Figure 3-4. Increased inflammation, ER stress and cell death acutely after infection in adipose tissue of NLRP3−/− mice. Adipose tissue western blot analysis at 12, 24 and 72-hour time points and representative images of control and burn+PA treatment groups in WT and NLRP3−/− strains. This includes NLRP3 inflammasome components: (A) ASC (representative images = 12-hours after infection), (B) IL-1β (12-hours after infection) and (C) IL-18 (12-hours after infection). For markers of apoptosis/pyroptosis included (D) Caspase-3 (12-hours after infection) and (E) Caspase-1 (24-hours after infection). ER stress protein expression was measured for (F) p-eIF2α (24-hours after infection), (G) p-JNK (24-hours after infection), (H) BiP (24-hours after infection). Lastly, Alternative inflammasome activation pathways were determined for (I) NF-κB (24-hours after infection) and (J) AIM2 (72-hours after infection). All antibody concentrations are expressed relative to GAPDH. Due to inherent differences between WT and NLRP3−/− control concentrations, all values are presented as a ratio relative to the mean concentration of species-specific controls for a given antibody. Dotted lines represent controls (WT and NLRP3−/−) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3−/− burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3−/− burn+PA, (n=5/group). **p<0.05, ***p<0.01 and ****p<0.001.
Consistent with increases in inflammasome, NLRP3 knockouts also manifested greater apoptosis (Caspase-3; p<0.05) and pyroptosis (Caspase-1; p<0.01) early after infection relative to WT burn+PA (12 & 24-hours, respectively, Figure 3-4D-E). Equivocal peaks in apoptotic protein expression in WT burn+PA mice occurred later at 72-hours after infection relative to knockout counterparts (p<0.05), suggesting a delayed cell death response to infection in the presence of NLRP3 inflammasome. ER stress markers did not show significantly greater expression in NLRP3 knockouts relative to controls at 12 hours (Figure 3-4F-H). Peak expression distinguished the groups at 24-hours with significantly greater ER stress in the NLRP3⁻/⁻ burn+PA relative to WT burn+PA for p-eIF2α (p<0.05), p-JNK (p<0.05) and BiP (p<0.05). In addition to inflammasome components, we found that NF-κB protein expression was increased earlier in NLRP3⁻/⁻ burn+PA group at 12 (p<0.05) and 24-hours (p<0.01) relative to WT burn+PA. Similar to the delayed increase in apoptosis, septic WT mice had greater NF-κB expression at 72-hours after insult (p<0.05, Figure 3-4I). Interestingly, there were no distinguishable differences between the infection groups for AIM2 protein expression in adipose (Figure 3-4J). Similar effects were observed in adipose tissue gene expression with increases in the NLRP3⁻/⁻ infectious group relative to WT counterparts, however it was most pronounced at the 72-hour time point (Figure 3-5).
Figure 3-5. Gene expression of inflammasome, ER stress and apoptosis in adipose tissue. Quantitative RT-PCR analysis in the adipose tissue for (A) IL-1β, (B) Caspase-1, (C) IL-18, (D) Caspase-3, (E) Caspase-8 and (F) BiP. All data compared genetic trajectories in tissue for control and burn+PA groups in both WT and NLRP3−/−, expressed at 12, 24 and 72-hours after infection. Gene expression was expressed relative to β-actin. Due to inter-species variability between WT and NLRP3−/− control concentrations, all values are presented as a ratio relative to the mean concentration of species-specific controls for a given primer. Dotted lines represent controls (WT and NLRP3−/−) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3−/− burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3−/− burn+PA, (n=5/group). */# p<0.05, **/### p<0.01 and ***/#### p<0.001.
We next compared protein expression in the liver and unexpectedly we did not observe the same acute responsiveness to infectious insult in NLRP3 knockouts as in adipose tissue. Overall, the WT burn+PA group had increased inflammasome activation, cell death, ER stress and alternative inflammasome activation pathways. Specifically, inflammasome activity in WT showed the greatest difference at 24-hours after infection relative to NLRP3⁻/⁻ burn+PA for ASC (p<0.05, Figure 3-6A) and IL-1β (p<0.05, Figure 3-6B). Caspase-8 and Caspase-1 also revealed significantly greater cell death in the WT burn+PA group relative to knockouts for nearly all time points (Figure 3-6D-E). Interestingly, when comparing alternative inflammasome activation pathways at 12 and 24-hours, both the NF-κB (p<0.05) and AIM2 (p<0.01) pathways were significantly greater in WT burn+PA relative to knockout counterparts (Figure 3-6H-I). To a lesser extent, hepatic gene expression revealed a similar trajectory for the aforementioned data (refer to Figure 3-6J-O). In summary, consistent with immune cell infiltration previously shown, the absence of NLRP3 inflammasome resulted in acute responsiveness of the adipose tissue to infection characterized by greater ER stress, inflammasome activity, cell death and alternative pathway activation. The liver showed a near inverse relationship with greater protein expression in the wildtype insult group. With this in mind, in the absence of increases in macrophage proportion in lymphoid organs we next wanted to determine whether systemic mediators are driving these increases in tissue.
Figure 3-6. Protein and gene expression of inflammasome, ER stress and apoptosis in liver of NLRP3−/− burn+PA mice. Western blot analysis and representative blots in the liver of control and burn+PA treatment groups in WT and NLRP3−/− strains for (A) ASC (representative images = 24-hours after infection), (B) IL-1β (24-hours after infection), (C) IL-18 (24-hours after infection). Cell death mediators analyzed include (D) Caspase-8 (24-hours after infection) and (E) Caspase-1 (12-hours after infection). ER stress was determined for the following markers: (F) p-eIF2α (12-hours after infection) and (G) p-JNK (24-hours after infection). Lastly, protein expression of alternative inflammasome activation pathways were assessed for (H) NF-κB (12-hours after infection) and (I) AIM2 (12-hours after infection). (J-O) Gene expression of hepatic tissue was determined using quantitative PCR for IL-1β, Caspase-1, IL-18, Caspase-3, Caspase-8 and BiP. All antibody concentrations are expressed relative to GAPDH for western blot and β-actin for PCR. Due to inherent differences between WT and NLRP3−/− control concentrations, all values are presented as a ratio relative to the mean concentration of species-specific controls for a given antibody or primer. Dotted lines represent controls (WT and NLRP3−/−) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3−/− burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3−/− burn+PA, (n=5/group). */# p<0.05, **/### p<0.01 and ***/#### p<0.001.
**Acute and non-persistent systemic inflammation and chemokine response in NLRP3−/− burn+PA mice**

Taking into consideration the increased immune mobilization and inflammation in tissue, we hypothesized that this would be a result of a dampened systemic inflammatory process. Much to our surprise, this was not the case and in fact NLRP3 knockout resulted in a greater acute response for inflammatory, chemokine and immune mediating cytokines. All integral pro-inflammatory cytokines were increased in the NLRP3−/− burn+PA group relative to WT burn+PA acutely (12-hours), including IL-6 (p<0.01, Figure 3-7A), TNF-α (p<0.001, Figure 3-7B), and IL-1β (p<0.05, Figure 3-7C). Notably, IL-6 had the most pronounced elevation in acute phase response (WT burn+PA = 183 pg/ml vs. NLRP3−/− = 1128 pg/ml) and was the only pro-inflammatory cytokine to gradually return to baseline (24-hours: p<0.05 relative WT burn+PA).

Beyond the early time point, 24 and 72-hours after infection revealed greater systemic inflammation of WT burn+PA mice for IL-13 (Figure 3-7D), IL-1α (Figure 3-7E). This in part may be attributed to the increased anti-inflammatory IL-4 expression 12-hours after infection in WT burn+PA group (p<0.05, Figure 3-7F), thus supporting acute phase dampening of inflammation in circulation paralleled by delayed innate immune response at the site of injury.

Consistent with the observed increase in inflammatory cytokines, chemokine analysis in NLRP3−/− revealed greater concentrations at 12-hours after infection relative to WT burn+PA for MIP-1α (p<0.01, Figure 3-7G), MIP-2 (p<0.05, Figure 3-7H) and MCP-1 (p<0.001, Figure 3-7I). There were no significant differences between the insult groups at either 24 or 72-hour time points.

Lastly, these observations were extended to immune mediators G-CSF (Figure 3-7J), GM-CSF (Figure 3-7K) and IL-9 (Figure 3-7L). Additional cytokines that were analyzed can be found in Figure 3-8. Thus, NLRP3 knockouts have greater chemokine and inflammatory response characterized by acute elevation after PA infection than returning to baseline. This suggests a
better mobilization of immune response occurring in tissue that may be mediated by inflammatory cytokines systemically. By doing so, this avoids an aberrant and prolonged inflammatory response in the presence of bacterial challenge.
Figure 3-7. Greater acute and short-lived inflammation and chemokine concentrations in systemic circulation of NLRP3Δ/Δ mice. Plasma cytokine profiling of control and burn+PA mice for WT and NLRP3Δ/Δ strains at 12, 24 and 72-hours after infection. (A-F) Cytokines that were used in the analysis included pro- and anti-inflammatory, (G-I) chemokines, (J-L) and immune mediators. Dotted lines represent controls (WT and NLRP3Δ/Δ) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3Δ/Δ burn+PA). Data presented as mean ± SEM, * = significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3Δ/Δ burn+PA, (n=5/group). *## p<0.05, **/### p<0.01 and ***/##### p<0.001.
Figure 3-8. Plasma cytokine profiling in WT and NLRP3^{-/-} control and burn plus infection mice. Plasma cytokine profiling of control and burn+PA mice for WT and NLRP3^{-/-} strains at 12, 24 and 72-hours after infection. Dotted lines represent controls (WT and NLRP3^{-/-}) and solid lines are burn plus PA infection groups (WT burn+PA and NLRP3^{-/-} burn+PA). Data presented as mean ± SEM, *= significant difference between strain-specific controls vs. burn+PA, # = significant difference between WT burn+PA vs. NLRP3^{-/-} burn+PA, (n=5/group). */# p<0.05, **/*** p<0.01 and ***/*** p<0.001.
Adult septic burn patients have prolonged increases in NLRP3 inflammasome gene expression in white adipose tissue beyond the acute phase

In order to determine if our present findings in rodents are translatable to patients and whether the adipose tissue is in fact a critical early mediator of insult, we assessed NLRP3 inflammasome gene expression in white adipose tissue of controls, non-septic and septic burn patients. Our findings revealed a temporal trend that was consistent with WT mice during the course of infection and sepsis. Early after burn (0-11 days), the non-sepsis patient group had a fairly consistent increase in gene expression after burn relative to controls for IL-1β (p<0.01, Fig. 3-9A), IL-18 (p<0.01, Fig. 3-9C) and NLRP3 (p<0.01, Fig. 3-9D). At later time points (≥12 days), inflammasome expression returned to baseline or control concentrations. Interestingly, septic burn patients had significantly greater gene expression at later time points relative to controls (IL-1β, Caspase-1 and NLRP3, p<0.05) and non-septic burn patients (IL-1β, IL-18 and NLRP3, p<0.05). When exclusively looking at septic burn patients, late-onset sepsis (≥12 days post-burn) had greater gene expression relative to controls (IL-1β, Caspase-1, IL-18 and NLRP3, p<0.05, Fig. 3-9E-H) and non-septic burns (IL-1β, Capsase-1 and NLRP3, p<0.05). Collectively, this data suggests that septic burn patients have increased NLRP3 inflammasome gene expression in white adipose tissue at later time points and that the persistence of acute phase inflammation is associated with increased sepsis, poor outcomes and death (Table 3-1).
**Figure 3-9. Prolonged elevation of acute phase NLRP3 inflammasome gene expression in adult septic burn patients.** Gene expression in white adipose tissue of healthy controls (n=10), non-sepsis (n=15) and sepsis (n=19) adult burn patients during the course of hospital stay (0-11 and 12+ days post burn). Primers included in the analysis were (A) IL-1β, (B) Caspase-1, (C) IL-18 and (D) NLRP3. Septic burn patients were further stratified into early (0-11 days) and late (≥12 days post burn) sepsis for (E) IL-1β, (F) Caspase-1, (G) IL-18 and (H) NLRP3. Dotted lines represent healthy controls, dashed lines are burn patients and solid lines are septic burn patients. Data presented as mean ± SEM, * = significant difference between healthy controls vs. burn patient group, # = significant difference between non-septic and septic burn patients. */# p<0.05, */*#/*### p<0.01 and *##/*###/*#### p<0.001.
3.1.5 Discussion

Classically, the activation of caspase-1 leading to the production of IL-1β is the result of NLRP3 inflammasome assembly and in its absence one would expect decreased inflammation and immune reactivity. Therefore, it is rather surprising that we observed greater macrophages and neutrophils at the site of injury followed by improved bacterial clearance and overall survival. This occurred in the presence of an acute (12-hours after infection) response in adipose tissue that was hallmarked by greater inflammasome activity, ER stress, cell death and alternative pathway activation. This immediate compensatory response was unique to the adipose tissue and was not observed in the liver or other lymphoid organs, thus supporting the imperative role that the adipose tissue plays in responding to post-burn infection and sepsis. Systemic inflammation appeared to be a major driving force in this response in NLRP3 knockouts due to the acute responsiveness of pro-inflammation and chemokines and likely accelerated the recruitment of neutrophils and macrophages to the WAT. Clinically, thermally injured patients that did not become septic exhibited gradual decrease back to baseline of inflammasome gene expression in WAT at both acute and later time points. In summary, these findings suggest that the lack of NLRP3 improves innate cell infiltration, inflammasome activation and apoptosis acutely after infection and implicates the adipose tissue as a critical mediator of this response to combat bacterial insult.

Numerous reports investigating the role of NLRP3 inflammasome have supported its activation being involved in multiple organ pathology during sepsis. NLRP3 inhibition was shown to prevent sepsis-induced kidney injury by protecting mitochondrial damage via attenuation of
oxidative stress and downregulating IL-1β and IL-18 (Zhao et al., 2016). In the heart, blocking cardiac NLRP3 inflammasome activation during sepsis also prevented cardiomyopathy characterized by ROS production and IL-1β release (Kalbitz et al., 2016). Using the cecal ligation puncture model of sepsis, NLRP3−/− mice have shown lower bacterial load by modulating autophagy and phagocytosis in neutrophils without any impairments in recruitment (Jin et al., 2017). Similarly, conditional knockout of PKM2 (involved in aerobic glycolysis) in myeloid cells resulted in protection from septic-induced death via activation of NLRP3 and AIM2, suggesting the possible immunometabolic therapeutic strategy that may be required to treat sepsis (Xie et al., 2016). Lastly, it has been shown that necroptosis promotes Staphylococcus aureus bacterial clearance by reducing excessive inflammation via RIP3K in skin (Kitur et al., 2016). Considering the acute phase function of the NLRP3 inflammasome, our findings support immune responsiveness as early as 12-hours at the site of injury and adipose tissue. These findings suggest that these tissues are involved in the earliest response to infection whereas in contrast the liver supported a gradual increase and appears to be impacted secondary. This notion is consistent with reports that have shown hepatic lipid infiltration in the liver after burn injury (Barret et al., 2001; Bogdanovic et al., 2015). Collectively these reports propose possible mechanisms and support the interplay in cell death pathways during bacterial sepsis and propose differential organ function in response in septic burn.

As previously described, the NLRP3 inflammasome can be activated by numerous sources resulting in cell death and pyroptosis, however in the context of sepsis, its activity is yet to be fully delineated. As an integral pro-inflammatory caspase that is activated by the NLRP3 inflammasome and inducer of pyroptotic cell death (Miao et al., 2011), Caspase-1 was higher in
the liver of WT burn+PA mice relative to knockouts suggesting pyroptosis may be taking place early after infection. However, apoptosis in this tissue reaches comparable proportions during sepsis. Interestingly, in the adipose tissue there was a different effect-taking place. NLRP3 knockout infectious group had a 8-fold increase in Caspase-1 at 24-hours after insult, but IL-1β and IL-18 had significantly more protein expression as early as 12-hours. This suggests that alternative sources of activation are accounting for the increase in pro-inflammation. Recent reports have suggested the role of AIM2 as an alternative source of activation after pathogenic insult (Man et al., 2015; Alnemri, 2010; Franchi et al., 2009). AIM2 was significantly higher in WT at 12 and 24-hours in liver consistent with a possible source that is driving the heightened caspase-1, independent of traditional NLRP3 pathway. However, in the adipose tissue of NLRP3 knockouts, AIM2 showed no major differences and was likely not responsible for the observed acute response. In adipose tissue, it has been shown in conditions where caspase-8 mediated apoptosis is not present that RIPK1 and RIPK3 are mutually activated to promote necroptosis (He et al., 2009; Cho et al., 2009; Zhang et al., 2009). This discrepancy may be explained by TNF-α. Using Mycobacterium-induced regulated cell death it has been shown that with increasing bacterial challenge there is greater TNF-α signaling (Roca and Ramakrishnan, 2013). This includes necrosis (under conditions of low bacteria), NF-κB mediated cell survival (moderate bacterial challenge) and RIPK mediated necrosis (abundance of bacteria). Presently, the NLRP3 knockouts have increased TNF-α in plasma, NF-κB, Caspase-8 and inflammation (IL-1β) in adipose tissue at 12-hours after infection. Taken together, this supports the notion that the knockouts are utilizing a multitude of processes that are ultimately culminating to achieve the same goal: pathogen clearance and overall survival. In summary, this sheds light on a tissue-specific or differential response taking place and the ability for multiple cell survival and death
pathways to become alternatively activated acutely in the absence of NLRP3 in order to overcome pathogenic conditions and improve survival.

To our knowledge, our present findings are the first to demonstrate in post-burn sepsis in the absence of NLRP3 inflammasome induces greater immune mediators of inflammation function and an acute compensatory upregulation both systemically and in adipose tissue improving overall survival. The unexpected onset of an exacerbated acute pro-inflammatory state post burn and infection due to the absence of the pro-inflammatory NLRP3 inflammasome is rather paradoxical. It induces greater inflammation during the acute phase to contain the bacterial challenge and these effects returns back to baseline and do not persistent chronically. Hence, for the purposes of sepsis, acute inflammation is required and imperative for overall survival. Therefore, instead of perceiving the inflammasome as an inducer of inflammation, in the context of sepsis we should redefine the inflammasome as a “gauge” moderating inflammation to ensure bacterial challenges are mitigated acutely and that these responses recover quickly to avoid inflammatory persistence. The heightened pro-inflammatory response illustrated by both the cellular recruitment to the WAT and site of injury, and the elevated effector molecule expression underscores an important role of the NLRP3 inflammasome in determining sepsis outcomes for burned subjects under both a metabolic and immunologic context.

The aforementioned results have demonstrated that the mechanism of NLRP3 inflammasome activity during sepsis-burn is extremely complex and that by simply blocking its activation does not reduce the immune/inflammatory response. At this time there is not conclusive data to suggest its function as a viable treatment option. Thus, in the following section we transcended our thinking from sepsis treatment to sepsis identification. In other words, can we utilize the NLRP3 inflammasome to determine patients susceptible to septicemia?
Chapter 4

NLRP3 Inflammasome as a biomarker for identifying burn patients susceptible to sepsis

Establish the hyperinflammatory response and activation of the NLRP3 inflammasome as a function of burn severity.

Hypothesis

Objectives

The Role of NLPR3 Inflammasome in Response to Thermal Injury and Sepsis

- Adult burn and burn-sepsis patients will have a hyperinflammatory response after injury that parallels increased NLRP3 inflammasome activation.
- Burn plus *Pseudomonas aeruginosa* induced sepsis in rodents results in NLRP3 inflammasome activation and blocking this activation will result in dampened inflammation, improved bacterial clearance and overall survival.
- NLRP3 inflammasome is activated in adipose tissue of thermally injured patients and can be used to delineate patients susceptible to sepsis.

- To determine whether the NLPR3 inflammasome can be used as a biomarker to identify thermally injured patients susceptible to sepsis.
- To determine whether the NLRP3 inflammasome systemic knockout will result in dampened innate immune activity, reduced inflammation, improved bacterial clearance and survival in rodents during the course of post-burn sepsis.
- To establish a two-hit model of post-burn *Pseudomonas aeruginosa* induced sepsis in rodents.
- To characterize the hyperinflammatory response and activation of the NLRP3 inflammasome in WAT of adult burn patients.
- To characterize the hyperinflammatory response and activation of the NLRP3 inflammasome in WAT of septic burn patients.
4.1.1 Rationale and Summary

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. We have previously demonstrated burn patients have increased NLRP3 inflammasome activation in white adipose tissue.

**Objective of Study:** The aim of the current study was to develop a novel platform to delineate a unique immune profile that can be used to identify septic outcomes in severely burned patients.

**Summary of Results:** We found in 37 patients that within 96-hours post injury those exhibiting aberrantly high levels of pro-inflammatory IL-1β 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-1β 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).

**Conclusions:** The SPI can accurately predict sepsis onset in thermally injured patients *a priori* and further enables clinicians to develop clinical studies and focused therapies specifically designed for septic cohorts.
4.1.2 Study Background

Despite their catastrophic origins, nowadays patients usually survive burn injuries. This is a result in part due to early excision and grafting, implementing critical care protocols and providing nutritional and metabolic support (Ong et al., 2006; Pereira et al., 2006; Kudsk et al., 1992; Snell et al., 2013). 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 (Jeschke et al., 2015c; Kraft et al., 2014). Additionally, if survived, sepsis delays rehabilitation, patient recovery, substantially increases metabolic demands and results in prolonged hospitalization (Kayambu et al., 2011). With this in mind, others and we hypothesize that in order to improve outcomes of burn patients it would be beneficial to determine which patients are at risk to develop sepsis and to initiate various treatment modalities to alter the sepsis trajectory and possible death towards non-sepsis and survival. We believe that the ability to predict sepsis would most likely enable focused therapies that would include faster wound coverage, early initiation of antibiotics or more patient vigilance.

Attempts to diagnose and predict sepsis in non-burn patients have included quantification of systemic biomarkers such as cytokines and chemokines (Faix, 2013). In general, 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, but are not limited to tumor necrosis factors and various interleukins. However, these
approaches have proven ineffective (Cannon et al., 1990), as the window of time between the detection of these biomarkers and sepsis onset is extremely short. 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 targeted 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 (Stanojcic et al., 2014). As NLRP3 expression is predominately observed in the myeloid population of immune cells (Martinon et al., 2002), we surveyed the most abundant tissue-derived myeloid population, macrophages, for NLRP3 inflammasome effector function. Specifically, we assessed white adipose derived macrophage-specific IL-1β secretion via flow cytometry, as IL-1β is the effector cytokine produced by NLRP3 inflammasome assembly (Martinon et al., 2002). 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 representation of depiction of immune status in comparison to short-lived blood-based markers.
4.1.3 Materials and Methods

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.

Study design and patient enrolment criteria
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, indiscriminate of age or gender (Figure 4-1). 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 4-1). 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) (Greenhalgh et al., 2007; Singer et al., 2016).
Figure 4-1. Flow chart of patient enrolment and inclusion in the prospective clinical trial.
Table 4-1. Demographics, burn injury characteristics, and outcomes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-Sepsis</th>
<th>Sepsis</th>
<th>P</th>
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<tbody>
<tr>
<td>No. of patients</td>
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<td>Age, mean ± SD</td>
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<td>Male, No. (%)</td>
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<td>TBSA, mean ± SD</td>
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<td>Etiology, No. (%)</td>
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<td>Flame, no. (%)</td>
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<td>19 (95%)</td>
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<tr>
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<td></td>
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<tr>
<td>Other (chemical, contact, electrical)</td>
<td>-</td>
<td>1 (5%)</td>
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<tr>
<td>Complications, No. (%)</td>
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<td></td>
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<tr>
<td>Pneumonia</td>
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<td>Respiratory failure</td>
<td>1 (6%)</td>
<td>3 (15%)</td>
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<td>Renal failure</td>
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<td>8 (40%)</td>
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<td>MODS</td>
<td>2 (12%)</td>
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<td>LOS(^a). median (IQR)</td>
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<td>33 (23-59)</td>
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<td>LOS/TBSA(^a), median (IQR)</td>
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<td>Mortality, No. (%)</td>
<td>4 (24%)</td>
<td>4 (20%)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Numbers may not add to 100 due to rounding

\(^a\) Analysis restricted to patients alive until discharge

TBSA, total body surface area; LOS, length of stay.
**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 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 x FSC-H, SSC-W x SSC-H). The percentage leukocytes, monocytes/macrophages, T-cells, and IL-1β producing leukocytes in the SVF were identified using the following flurochrome-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-1β (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-1β. The gating strategy is outlined in Figure 4-2, where leukocytes were initially gated based on granularity and CD45 (side scatter x CD45), followed by size (forward scatter). These gated cells were then stained for cell surface markers for monocytes and macrophages (CD14$^{\text{hi}}$ CD16$^{\text{lo}}$). Finally, CD14$^{\text{hi}}$ cells were assessed for IL-1β 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-1RA 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 ± 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 two-tailed tests in order to explore the association between septic predictor index (SPI: percentage IL-1β 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.
4.1.4 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 Figure 4-2. The patient cohort that eventually developed sepsis recruited less macrophage (CD14+ cells) to the site of injury in contrast to non-septic patients (mean = 8.7 ± 1.4 vs. 29.3 ± 5.5; p<0.05; Figure 4-3A), within 96 hours post thermal injury. However, individual macrophages at the site of injury from patients that eventually become septic were secreting significantly more pro-inflammatory IL-1β cytokine than the patients that exhibited non-septic complications post burn (sepsis: 16.3 ± 3.9 vs. non-sepsis: 2.4 ± 0.74; p<0.01; Figure 4-3B). This relationship was also observed when septic patients were compared to controls for SVF macrophages and pro-inflammatory IL-1β (p<0.05 and p<0.001, respectively).
Figure 4-2. Flow cytometric analysis of the stromal vascular fraction. CD45+ leukocytes harvested from the stromal vascular fraction of burned patients were gated for monocytes/macrophages (CD14hi CD16lo) frequency and its IL-1β secretion levels. Top panel: non-septic cohort; bottom panel: septic cohort.
Figure 4-3. 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-1β from CD14$^{hi}$ cells at the site of injury. Control adipose tissues (normal) were obtained from donors that underwent plastic surgery for tissue reconstruction. Data expressed as mean ± SEM. *, ** & *** = p<0.05, p<0.01 & p<0.001, respectively.
**Systemic immunologic profile of burned patients**

We subsequently assessed systemic biomarkers in plasma samples from burn patients via multiplex. Amongst the myriad of soluble factors acutely released into the bloodstream within the first 96 hours post thermal injury, the anti-inflammatory cytokines IL-10 and IL-1RA exhibited significant differences between the septic and non-septic patient cohorts (Fig. 4A-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; p=0.017) and IL-1RA (183 ± 28 vs. 89 ± 18; p=0.014). These observations were also consistent when comparing septic patients and controls.
Figure 4-4. 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 ± SEM. *, ** & *** = p<0.05, p<0.01 & p<0.001, respectively.
**Septic Predictor Index**

To simplify this technique, we created a ratio of the aforementioned IL-1β from SVF-derived macrophages and macrophage proportion (proportion of CD14+ IL-1β+ cells / proportion of CD14hi CD16lo 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; Figure 4-5A). 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 (Figure 4-5B). 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 (Jeschke et al., 2015a; Stanojcic et al. 2016b), 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 (<65 years old) and elderly septic patients (Figure 4-6A). 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 (Figure 4-6B). These findings indicate that the SPI is 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; Figure 4-7).
Figure 4-5. Immune profiling burned patients to predict sepsis onset. (A) Frequency of IL-1β 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-1β+ 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 12^{th} 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 ± SEM. *, ** & *** = p<0.05, p<0.01 & p<0.001, respectively.
Figure 4-6. Analysis of the immunologic profiles of burned patients stratified by age and mortality. Frequency of IL-1β 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-1β+ cells / proportion of CD14hi CD16lo 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 ± SEM. *, ** & *** = p<0.05. p<0.01 & p<0.001, respectively.
Figure 4-7. **Comparison of the survival in septicemia patients.** Kaplan Meier survival curve of only sepsis patients that were divided into early (<12 days) and late (≥ 12 days) onset, with the later supporting approximately a 50% mortality rate.
4.1.5 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 these innate immune cells were producing more pro-inflammatory IL-1β. 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 predictor of sepsis. In contrast to conventional plasma biomarkers, by 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-1β secretion between the non-septic and septic cohorts. In the septic cohort, there was less macrophage recruited to the site of injury and heightened IL-1β production in contrast 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-1β into its mature form (Martinon et al., 2002) in the SVF after thermal injury (Stanojcic et al., 2014). Although increases in macrophages at the site of injury have been shown in the past (Saraf et al., 2016), 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-1β 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 recently conducted study that showed elderly burn patients
have delayed immune activity followed by a hyperactive immune response, which ultimately
results in consequential immune paralysis (Jeschke et al., 2015a; Stanojcic et al., 2016b).
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 (Varma et al., 2005).

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 (Cioffi et al., 1991; Heard et al., 1998;
Lorenz et al., 1994; Ono et al., 1993). 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 (Bohannon et al., 2009; Bohannon et al., 2008). However, despite its
promise the clinical benefit remains unknown. With this in mind, we are still unable to
rationalize both why and how the adipose tissue immune cells in burn patients produce high
amounts of IL-1β per macrophage post injury, and how further stimulation would improve the
immune system to prevent sepsis. Thus, our present findings accurately depict the patient
populations that are more susceptible to sepsis onset, however the greater paradigm of using these findings for therapeutic benefit remains elusive.

The intriguing finding of the smaller macrophage population observed at the site of injury from the septic cohort in contrast to the non-septic 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-1β, but they also dampen the immune response by preventing leukocyte trafficking (Fine et al., 2003; Olszyna et al., 2000; Saxena et al., 2013). 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, which leads to the prevention of macrophage recruitment to site of injury in septic patients, remains unknown. However, 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 (Pal et al., 2014; Pedersen et al., 2012a; Steensberg et al., 2000). IL-6 has been shown to be highly elevated post exercising and induces a systemic anti-inflammatory state (Pedersen et al., 2011; Pedersen et al., 2012b). Perhaps this state is mirrored in hypermetabolic burned patients. As the energy requirement is far more essential than maintaining immune competence, the immune system is shut down while prioritizing energy demand. 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) presenting with differential clinical course in burn patients (Patel et al., 2012; Shupp et al., 2010), 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 starts from a burn wound infection and develops into pneumonia and then systemic complications (Cohen et al., 2004; Ramzy et al., 1998). This was supported in our study by pneumonia occurring in 71% of septic patients, where as in non-sepsis burns it accounted for a 29% incidence (Table 4-1). The multi-organ dysfunction syndrome that is associated with burn trauma (Kraft et al., 2014; Jeschke et al., 2008a), was not significantly different between the groups indicating that our profiling is very specific to sepsis. The only difference that we found associated with sepsis was a higher incidence of renal failure in patients who underwent sepsis (46% vs. 6%). However, further elaborating our current profiling in terms of renal failure 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 of patients in order to accurately predict future sepsis onset. There are various approaches that have shown to alleviate the hypermetabolic response in burn patients (Gauglitz et al., 2011; Jeschke et al., 2007a; Kobayashi et al., 2011; Muthu et al., 2009), 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.
Originally described as an acute-phase pathogen sensor, the NLRP3 inflammasome has established itself as an integral inducer of pro-inflammation and pyroptotic cell death in various conditions and diseases. As such, it is a critical factor driving low-grade inflammation in diabetes, and it has been shown to have an important role in neural inflammation, autoimmunity, cancer, and as presented herein burns and sepsis. Despite its blockade resulting in numerous therapeutic benefits, as the present studies revealed, its implications to septic burn patients are unique and its activity is integrated among alternative pathways.

These studies were conducted based on the hypothesis that the NLRP3 inflammasome plays an important role in burns and sepsis. In most cell types, activity of the NLRP3 inflammasome is controlled by a two-step process: priming followed by activation. Priming involves increased transcription of the components of the inflammasome complex, such as the receptor, NLRP3, and its target, pro-IL-1β. The second step, activation, occurs when the NLRP3 receptor recognizes one of its many potential ligands and serves as a nucleator for the complex, attracting the adaptor protein ASC, and ultimately caspase-1 (Guo et al., 2015). The result is a protein complex capable of cleaving pro-IL-1β into mature, active IL-1β (Gross et al., 2011), which can then be secreted and have a myriad of local and systemic effects, namely impacting insulin sensitivity, both locally and by travelling to insulin-sensitive tissues, such as adipose tissue where IL-1 receptor signaling can directly interfere with insulin signaling (Wen et al., 2011; Jager et al., 2007). It is important to note, as well, that findings so far have not indicated robust expression of NLRP3 in adipocytes, themselves, indicating that it is most likely the inflammatory and/or stromal cells of the adipose tissue that are initiating and mediating these events (Koenen et al., 2011).
Overall, these studies successful established the NLRP3 inflammasome is activated in burn and septic burn patients and that its role becomes non-linear when it is genetically knocked out in rodents. In Chapter 2, we provide evidence that the NLRP3 inflammasome has elevated activation in white adipose tissue of burn patients relative to controls and furthermore, established that its expression increases as a function of greater injury severity. These observations occurred in the presence of increased ER stress and mitochondrial dysfunction in adipose and systemic inflammation. These results demonstrate the NLRP3 inflammasome is active during burn trauma. Furthermore, its synergistic relationship with injury severity suggests it may play a critical role in the acute phase after trauma.

Currently, we have not yet identified which DAMPs might be activating the inflammasome in the adipose tissue of burned patients, but there are several events that could be occurring, either alone or concomitantly, leading to the assembly and activation of this complex. For example, hyperuricemia (Ghaemi-Oskouie, 2011; Liu et al., 2010; Yamazaki et al., 2003), hyperglycemia (Shanmugam et al., 2003; Dasu et al., 2007; Vincent et al., 2007; Zhou et al., 2010), mitochondrial dysfunction leading to increased ROS (Zhou et al., 2011; Tschopp, 2011) and increased lipids (Wen et al., 2011; Vandanmagsar et al., 2011) (burn patients suffer extensive lipolysis, despite hyperinsulinemia) are all scenarios that have both been observed in burn patients and are known activators of the inflammasome. It is also likely that many of our burn patients experience a “highly primed” inflammasome state (Guo et al., 2012), as opportunistic infections (and hence bacterial-derived PAMPs, well-known inflammasome primers) are a common problem in burns. Current and future studies will focus on identifying the PAMPs and
DAMPs associated with burn and inflammasome activation in patients and animal models and whether genetic or pharmacological inhibition of inflammasomes improves post-burn outcomes.

In addition to inducing a canonical inflammatory response, the NLRP3 inflammasome was recently implicated in promoting obesity-induced inflammation and insulin resistance via detection of obesity-associated, endogenous DAMPs (Vandanmagsar et al., 2011; Schroder et al., 2010b). In obese mice, lack of NLRP3 expression prevents inflammasome activation in response to high fat diet-associated DAMPs and enhances insulin signaling in the adipose tissue and liver (Stienstra et al., 2011). Work by Ting and colleagues suggest that the mechanism of inflammasome activation in diabetes involves detection of saturated fatty acids, potentially resulting from lipotoxicity in obese mice (Wen et al., 2011). Others have suggested that mitochondrial dysfunction and subsequent increases in ROS may be responsible for priming and activating the inflammasome (Zhou et al., 2011; Sorbara et al., 2011) in metabolic disorders. Regardless of the method of activation, in terms of type II diabetes and insulin resistance, generation and release of IL-1β by the inflammasome interferes with insulin sensitivity via both direct (stimulation of the IL-1 receptor and downregulation of IRS1 (Jager et al., 2007) and indirect mechanisms (Wen et al., 2011; Vandanmagsar et al., 2011; Stienstra et al., 2012). When viewing all of this evidence together, it becomes clear that inflammation and insulin resistance are tightly linked via the NLRP3 inflammasome.

The results described in Chapter 3 suggest a complex role that the NLRP3 inflammasome plays during burn sepsis. Systemic knockout of NLRP3 resulted in improved bacterial clearance at the
site of infection and overall improved survival. However, unexpectedly this occurred in the presence of increased acute phase inflammation, cell death, and macrophage and neutrophil infiltration at the site of injury and adipose tissue. In the context of sepsis, perhaps the presence of an accelerated acute onset of pro-inflammation at the site of injury may be beneficial. Clinically, thermally injured septic patients exhibited prolonged increases in inflammasome gene expression in WAT well beyond the acute phase. In a recent clinical study we showed that patients unable to recruit sufficient macrophages to the site of injury acutely after burn have an overproduction of IL-1β, causing immune exhaustion and increasing the risk of sepsis (Chen et al., 2017). These observations suggest that having a sufficient immune response in the WAT during the acute phases post burn is imperative in determining outcome in the sepsis context. Perhaps an early and robust immune response in the WAT may hasten the energy production process and supply ample energy to the entire body acutely after thermal injury. This was shown in a recent study by Dror et al. where the presence of NLRP3 mediated IL-1β, was in fact required for glucose reuptake selectively into immune cells (namely macrophages) (Dror et al., 2017). Thus, this allows the body to allocate sufficient resources to strengthen the immune system in order to mount a defense against microbial infection, and to avoid sepsis. However, we currently cannot confirm whether the clinical observation is directly attributed to NLRP3-associated effects. Similar to the WAT clinical study (Chen et al., 2017), we are in the process of validating whether the differential immune response is attributed to patients’ NLRP3 activation status. If it holds true, then the induction of an accelerated pro-inflammatory state through NLRP3 inhibition during the acute phases post burn via inhibitor molecules (eg. Glibenclamide) may warrant consideration as a prophylactic regimen to severely burned patients to escape sepsis. Collectively, our findings propose possible mechanisms leading to sepsis in burn patients and support the interplay in cell death pathways during bacterial sepsis upregulated early after.
infection, and point to improved overall survival in the absence of NLRP3 inflammasome.

Chapter 3 also sheds new light on the role of IL-6 in burn sepsis and its function as an integral effector molecule in response to NLRP3 availability. A number of studies have shown that in order to balance and control inflammation, co-existent anti-inflammatory cytokines are produced in synchrony with pro-inflammatory ones during infectious states. One such cytokine is interleukin 6, which we have previously shown to be significantly up-regulated post burn injury as well as in septic burn patients (Stanojcic et al., 2017; Stanojcic et al., 2016b; Jeschke et al., 2008a). Other studies in non-burn patients have also shown that IL-6 levels in the serum are upregulated in both septic humans and mice, which can be used to predict outcome (Remick et al., 2000; Libert et al., 1992; Heremans et al., 1992). However, unlike other cytokines, IL-6 can function as both a pro-inflammatory and anti-inflammatory cytokine, depending on its source of release. In fact, we have recently shown that bone marrow derived IL-6 adversely alters adipose tissue metabolism via its effects on macrophage polarization, which exacerbates post-burn hypermetabolism (Abdullahi et al., 2017). In Chapter 3, we showed that IL-6 levels and macrophage infiltration at the site of injury and adipose tissue are significantly up-regulated in septic NLRP3−/− mice at 12-hours, suggesting that the lack of NLRP3 production facilitates a compensatory mechanism in which pro-inflammatory IL-6 is efficiently upregulated to fight off infection and improve survival. A similar IL-6 surge occurs in WT, however it occurs later, which may explain the differences in mortality. A previous study using cecal ligation induced sepsis in mice showed that blocking IL-6 early (0-12 hours after insult) attenuated pro-inflammation and improved survivability (Riedemann et al., 2003). Interestingly, the study also showed that high doses of IL-6 blockade (15 pg/ml in blood) had nearly the same survival as
CLP alone and suggests that complete acute phase absence of IL-6 is not beneficial. Thus, we believe that a deregulated IL-6 response (whether metabolic or inflammatory in origin) may be the key to understanding sepsis-induced mortality in certain populations.

In addition to shedding light on the role of the NLRP3 during the course of infection and sepsis, another important finding from Chapter 3 was the differential response within different tissues. When considering the site of injury, there was decreased bacterial presence in conjunction with increased macrophages and neutrophils early after infection suggesting that ablation of NLRP3 results in heightened immune infiltration and local responsiveness. This was further supported by neutrophil reduction at 72-hours after infection supporting a reduced demand for pathogenic clearance. In other words, NLRP3 are able to contain the pathogenic insult and by 72-hours the bone marrow increases its neutrophil supply to re-establish equilibrium and recuperate to normal state. Thus, the differential response within organs in response to burn sepsis sheds light on how the NLRP3 inflammasome is one component of a complex integrated network and ablation drives numerous compensatory pathways.

The unexpected onset of an acute pro-inflammatory state post-burn and infection due to the absence of the pro-inflammatory NLRP3 inflammasome is rather paradoxical. Therefore, instead of perceiving the inflammasome as an inducer of inflammation, we may need to redefine the inflammasome as a gauge moderating inflammation. In any case, the heightened pro-inflammatory response illustrated by both the cellular recruitment to the WAT and site of injury, and the elevated effector molecule expression underscores an important role of the NLRP3 inflammasome in determining sepsis outcomes for burned subjects under both a metabolic and immunologic context.
Combining the results of Chapters 2 and 3 in conjunction with the known components of the NLRP3 inflammasome, Chapter 4 used prospective immune appraisal in burn patients to assess patients at risk of immune exhaustion. Using WAT from the site of injury, we utilized the proportion of SVF macrophages (major producers of NLRP3 inflammasome) and IL-1β (by-product of inflammasome activation) to create the Septic Predictor Index ratio. Determined within 96-hours post injury, patients with ratios greater than 0.5 were determined to have sepsis occur subsequently. In contrast to the conventional plasma biomarkers, this immune response assessed at the site of injury embodies spatial precision, as it is a highly specific profile localized to the wound area. At the site of injury, we observed a reciprocal relationship of macrophage abundance and IL-1β secretion between the non-septic and septic cohorts. In the septic cohort, there were less macrophages recruited to the site of injury in contrast to the non-septic, whereas there was heightened IL-1β production by the macrophages from the septic cohort relative the non-septic. It appears that to compensate for the diminished macrophage “man-power” that the septic cohort is experiencing relative to the non-septic, individual macrophages must exert greater function in the acute phases of burn by producing more IL-1β. This high demand in IL-1β production from the small number of macrophages can quickly progress to immune exhaustion, which makes the patient unable to fend off future infection. This will enable invading microbes to proliferate and disseminate throughout the body, which ultimately leads to sepsis. This hypothesis is also supported by a recently conducted study that showed elderly burn patients have delayed immune activity followed by a hyperactive immune response, followed by consequential immune paralysis (Jeschke et al., 2015a; Stanojcic et al., 2016b). The implementation of the Septic Predictor Index ratio warrants validation in other burn cohorts and other populations with sepsis manifestations. Knowing that no current therapy exists, one could speculate that preventing the massive cytokine production of macrophages while concurrently
stimulating an early, consistent and controlled production would be a possible immunologically
oriented approach to prevent sepsis. Another approach would be to administer functional
macrophages to patients. One method of this administration may be possible via host patient
stem cells being differentiated into macrophages and then grafting them topically on the site of
injury to increase local immune “man-power”. Albeit interesting and innovative, these
experimental approaches would have to be investigated with caution seeing as there is no
evidence available that they will be effective. The subsequent sections will discuss a few
treatment approaches to combat sepsis and dampened immune function.

Treatment options for trauma patients with burn-induced injuries are regularly improved as
research reveals new methods of diagnosing and preventing infection or sepsis. It is already
known that a variety of factors may influence a patient’s outcome after a severe burn. These
include the size of the burn, age, sepsis and if there is a known inhalation injury (Herndon and
Tompkins, 2004). Despite providing the best critical care possible to patients, a grey zone still
exists of what additionally can be done to avoid sepsis. Unlike sepsis, currently there are various
approaches to alleviate the hypermetabolic response and support immune function, the most
well-established being beta-blocker treatment using propranolol, (Gauglitz et al., 2011; Jeschke
et al., 2007a; Kobayashi et al., 2011; Muthu et al., 2009). However, there is no globally accepted
therapeutic intervention to prevent or predict sepsis, thus making it very challenging to present
therapeutic ideas and vision to treat something that will inevitably occur. Outside of burn trauma,
sepsis therapy in critically ill patients focuses on supporting organ function and perfusion. The
ability to prevent sepsis would be a monumental medical advancement for burn patients as the
majority of the post-burn complications are sepsis-related. Unfortunately, previous studies and
trials involving neutralizing reagents to pro-inflammatory cytokines (eg. anti-TNF-α therapy)
were unsuccessful in reversing sepsis and the avalanche of sepsis-induced immune dysfunction
that ultimately leads to organ failure, and mortality. Using an opposite approach, studies have shown that administering G-CSF or GM-CSF can stimulate the immune system, however, these studies failed to show a therapeutic benefit (Cioffi et al., 1991; Heard et al., 1998; Lorenz et al., 1994; Ono et al., 1993). Combining the results of the present studies, other than traditional approaches to block the NLRP3 that proved complex, it introduces the role of immune “priming” and stimulation to counteract infections and sepsis.

Although the precise mechanism is not well understood, it has been proposed in models of aging that age “primes” the immune system and under conditions of chronic inflammation predisposes patients to negative outcome such as infections, sepsis and MOF (Nomellini et al., 2009). Other published data from our group suggests a dampened immune response in elderly. Using excised white adipose tissue taken during the first week after burn we found lower proportions of IL-1β+ and caspase-1+ macrophage compared to adults (Stanojcic et al., 2016b). Elderly patients represent a classic example of immune-senesce characterized by difficulty to mount a timely and adequate immune response. An elegant study conducted by Faist and colleagues showed that immune stimulation in elderly patients undergoing surgery results in increased lymphocyte proliferation and cellular immune response (Faist et al., 1988). Perioperative administration of immune activating thymopentin also reduced severity of infection in elderly (Braga et al., 1994). Anticipating a delayed immune hyperactivity does not reflect mortality predisposition and rather can be described as a “failure to launch” phenotype. However, the complete absence of cytokine production may predispose elderly along with burn patients to fatal outcomes. Ongoing investigations are being conducted to determine the culprits responsible for the prevention of early immune responsiveness. Currently, the exploration of immune activating agents in burn patients is lacking. Additionally, we need to conclusively determine whether "jump-starting" the immune system would promote homeostatic balance during the acute phase. As shown with
NLRP3 ablation in rodents, benefits to the site of injury were not upheld when comparing them to other organs such as liver. Thus, our data suggests that other tissues may prove to be better outcome predictors than those currently employed in order to overcome the temporal delays of cytokine detection in systemic blood. Also, the present observations directly relate the alterations in blood to adipose tissue-specific inflammatory mediators from the site of injury. With these alterations in tissue directly correlating with length of hospital stay it supports the feasibility of a prospective approach to predictive indicators of clinical course.

The immune-senescent state of trauma patients (as defined by the gradual deterioration of the immune system) entails their hampered ability to mount a robust immune response against external insults (Pritz et al., 2014). Thus, in attempt to produce a potent immune response, theoretically, each individual white blood cell of the immune system would have to function at a greater-than-normal capacity in order to achieve an immune output comparable to healthy individuals (due to the differential leukocyte count). This overload on leukocytes, despite the delayed production of effector molecules (as shown by the surge of cytokines in the later stages of the pathophysiology from our data) bankrupts the immune potential consequently making them susceptible to future insults, rendering them to an “immune exhausted” state. As immune activation post injury is required to ensure protection and survival of patients post thermal injury, we postulate the overall immune phenotype and the clinical outcomes observed may be attributed to the phenomenon of the aforementioned “immune exhaustion”. This notion would explain why rodents and burn patients with late onset sepsis have such a striking mortality rate, a consequence of depleting the necessary immune reserve required to mount an adequate response and ultimately succumb to their injuries. Other reports have also shown that early rapid decline is one of the best predictors of fatal outcome (Swanson et al., 2013).
Extending this notion of immune exhaustion, the following schematic was created to represent this phenomenon in septic burn patients. Figure 5-1 is a schematic of microbial clearance of a patient who experiences a burn-related infection. As shown in panel A (non-septic patient), in the pre-infected phase, the patient’s blood microenvironment is in a mild pro-inflammatory state due to the mounted immune response against DAMP molecules (generated in the process of trauma or burn). This low-grade pro-inflammatory state primes the patient’s immune system to sense external insults, thus facilitating efficient detection of microbial infiltration. During the early, or infected-phase the invading bacteria are detected by the circulating leukocytes that were recruited due to the original insult. This detection results in a heightened pro-inflammatory response, which leads to additional pro-inflammatory cytokine production and recruitment of leukocytes, ultimately leading to bacterial eradication. In the late or clearance phase, the invading microbes are eliminated, and the immune system is restored to a neutral state by a balance of pro-inflammatory and anti-inflammatory signals. On the other hand, panel B illustrates sepsis development. In the pre-infected phase, the net anti-inflammatory state of the blood microenvironment is defined by low-grade pro-inflammatory response mounted against the DAMP molecules, and an abundance of anti-inflammatory cytokines, produced possibly by the liver (the trigger for this response remains elusive). During the early/infected phase, an abundant pro-inflammatory response is mounted against the invading microbes by the recruited leukocytes. However, since the blood microenvironment prior to the infected-phase was anti-inflammatory, these recruited leukocytes must produce aberrantly higher levels of pro-inflammatory cytokines in order to achieve a net pro-inflammatory state to offset the effects of the pre-existing anti-inflammatory soluble factors. As a result, these leukocytes will likely over-exert their effector function and rapidly deplete their effector function potential, leading to
A Normal Clearance
Innate Immune Cells (Monocytes)

Pre-infected Phase
Net: Mildly Pro-inflammatory

Early (Infected) Phase
Net: Pro-inflammatory

B Sepsis
Innate Immune Cells (Monocytes)

Pre-infected Phase
Net: Anti-inflammatory

Early (Infected) Phase
Net: Highly Pro-inflammatory

Late (Clearance) Phase
Net: Neutral

Late (Septic) Phase
Net: Highly Anti-inflammatory
Figure 5-1. Systemic immunologic profile as a predictor of sepsis: a working model. Spheres with + symbols denote pro-inflammatory mediators; spheres with – symbols denote anti-inflammatory mediators; the immunological state of each phase is determined by the ratio of pro-inflammatory mediators relative to anti-inflammatory mediators. (Mildly pro-inflammatory, 2:1; pro-inflammatory, 3:1; neutral, 1:1; anti-inflammatory, 1:3; highly pro-inflammatory, 5:1; highly anti-inflammatory, 1:5) (Chen et al., 2014).
immune-exhaustion. This state is reminiscent of SIRS, or the classical cytokine storm that is observed in the acute phases of sepsis. In the late/sepsis phase, the immune system is exhausted and is therefore unable to produce additional pro-inflammatory mediators, despite having residual bacteria from the infected-phase. Furthermore, to counter the spike of pro-inflammatory signals in the infected phase, a massive surge in anti-inflammatory response is produced. This results in a net anti-inflammatory microenvironment, similar to the compensatory anti-inflammatory response syndrome, which is conducive to bacterial growth, dissemination, and ultimately, sepsis.

Although this study focused on macrophages and the NLRP3 inflammasome during sepsis, as evident by the data, there are multiple processes at work. The blockage of NLRP3 inflammasome may reduce inflammation and improve outcomes in septic patients, however it does not describe why it paradoxically induces increases in inflammation. The inflammatory process after burn trauma has long been thought to be negative and an initiator of the “slippery slope” to poor outcomes. The present findings challenge the paradigm that inflammation is negative. The author would like to propose that perhaps it is time that we rethink how inflammation is viewed after burn trauma: not all inflammation is bad, but rather it is a protective process that the body triggers to adjust to its new equilibrium. Thus, the elimination of this mechanism shifts the body’s homeostatic readjustment and as presently demonstrated, results in inflammation through other means none-the-less. In summary, inflammation is not the enemy. Nor should we consider treatments as liner processes. Rather, burn care providers should ensure that acute phase responses do not persist into the chronic phase. As new techniques and technology improve burn care, future studies need to be cognitive that predicting and mitigating sepsis is half the battle. The other half comes from mechanistic understanding of the inherent host response to mitigate sepsis and improve outcomes of burn patients.
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