ABSTRACT

THE ROLE OF IRAK-1 IN SEPSIS

by
Adithya Subramanian SAhasranamam

Adithya Subramanian Sahasranamam Sepsis is a complex, life-threatening syndrome that can lead to systemic organ failure and dysfunction. Due to its high morbidity and mortality rates, it has become a critical global health issue. Although many factors are at play during sepsis, the primary ones include abnormal inflammation and a lack of oxygen supply to the tissues and muscles. The interleukin-1 receptor–associated kinase (IRAK) family plays a crucial role in eliciting innate immune responses and switching to adaptive immune responses in the presence of pathogens. IRAKs are vital components in the interleukin-1 receptor signaling pathway and some Toll-like receptor signaling cascades that elicit inflammatory reactions in response to injury and infection. Disturbances in the homeostasis of IRAK signaling cascades can lead to a plethora of immunological problems. This paper seeks to understand the molecular mechanisms of IRAK-1 and compare its splice variants and polymorphisms while considering inflammation and sepsis.

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THE ROLE OF IRAK-1 IN SEPSIS

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To Dr. Grayson Davis,
A wonderful teacher of life sciences who also teaches about life.
I hope you like it, sir.

To Sahasranamam Subramania Iyer,
One of the smartest engineers I have met in this lifetime.
When my time comes we shall catch some films in heaven but,
until then I need your quirky advices and your blessings.

Thaazhndha puruvangal orunaal nimirum.
Kavizhndha imaigal orunaal uyarum.
Irugiya udhadugal orunaal thudithudikkum.
Karugiya parkkal orunaal narunarukkum.
Adhuvarai neengal engalai aaluga!
Adhuvarai ungal vallam onguga!

Shanmugam Sivalingam

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CHAPTER 1

INTRODUCTION

Systemic inflammatory response syndrome (SIRS) is a generalized immunological response against a vast range of pro-inflammatory pathologies, including infection, injury, trauma, and burns. SIRS is often characterized by significant changes in the body temperature and the onset of tachycardia, rapid breathing, and abnormalities in white blood cells (WBCs) and red blood cell (RBCs) counts and can give rise to systematic multi-organ dysfunction. When the onset of infection causes SIRS, the phenomenon is known as sepsis. Sepsis is a life-threatening inflammatory response that can give rise to systematic multi-organ dysfunction and failure caused by either trauma or infection.

Despite modern advances in elucidating the pathophysiology of sepsis, the condition remains one of the primary causes of mortality and morbidity in intensive care units (ICUs) worldwide. Current estimates suggest that sepsis affects more than 30 million people and accounts for more than six million deaths per annum worldwide. Based on the Surviving Sepsis Campaign data from 2012, 41% and 28.3% of reported deaths from sepsis occurred in Europe and the United States, respectively [1]. The study also found sepsis to be the most expensive health care condition in the United States annually, setting back American hospitals by USD 20 billion in 2011 alone [3]. These financial and mortality costs make the investigation of sepsis' molecular mechanisms a top priority to elucidate possible immune modulation therapies to more effectively treat patients afflicted by sepsis.

Severe sepsis is when the host's reaction to infection causes a systemic cascade of organ failures in a manner referred to as septic shock [1, 5, 6]. Sepsis is believed to cause organ failure through the uncontrolled upregulation of systemic

immune responses. However, in light of medical and scientific advancements, ICU survival rates have improved, which led to) the detection of the immunosuppression phase in the later stages of sepsis pathophysiology, ultimately explaining the high mortality rates. This syndrome was termed "compensatory anti-inflammatory response syndrome" (CARS) by Bone in his 1996 paper [4]. Similar to SIRS, CARS is a complex immune system response to severe infection; however, CARS is believed instead to be a condition marked by systematic inhibition of the immune system that restores homeostasis after the period of extreme inflammation. This led scientists and medical professionals to use the terms SIRS and CARS to differentiate the host's pro- and anti-inflammatory responses to a broad range of infectious and noninfectious stimuli [6–9].

While initial studies categorized CARS as the phase that appears at the end of or even after SIRS, researchers have since found evidence of pathways that support the idea that CARS is not a part of SIRS. Instead, CARS may exist entirely separately from SIRS and encompass an additional set of cellular and molecular interactions and pathogenesis pathways different from those of SIRS. However, CARS may also significantly influence sepsis and lead to adverse outcomes: while earlier studies of the pro-inflammatory phase of sepsis have helped to improve survival rates in the ICU, the emergence of an immunosuppression phase in the later stages of sepsis pathophysiology often left the patient vulnerable to secondary infections, which could explain the high mortality rates [10]. Indeed, later studies revealed that the anti-inflammatory responses elicited by CARS induce a severe immunosuppressed state wherein the immune system cannot recover despite eradicating pathogens from the body, which, as a phenomenon, has been termed "immune paralysis" [5].

Modern advances have reduced the rates of deaths occurring during sepsis' initial stages as homeostasis is reestablished early on in the disease's pathophysiology. However, those patients who fail to achieve homeostasis during the early phases of

SIRS/CARS enter a state marked by high mortality and morbidity rates, typically exhibiting severe weakness, malnutrition, chronic infections, and cognitive decline, which has come to be known as chronic critical illness [10–13].

Data from 2009 indicate that the annual health care costs for patients with chronic critical illness exceeded $20 billion. The majority of these patients (> 60%) were admitted with a sepsis diagnosis [12] and only 20% were ultimately discharged home; more than 40% were discharged to long-term acute care and skilled nursing facilities [11, 12], while 30% died in the hospital [12].

Due to its associated high mortality rates, CARS soon became a target for immune-modulating therapies [14]. However, despite extensive preclinical research into possible immunomodulatory therapies for CARS, not many treatment solutions to date have been implemented [15]. Later studies found that, during CARS' immunosuppressive phase, an increase in the levels of pro-inflammatory cytokines such as C-reactive protein (CRP), interleukin (IL)-6, IL-1Ra, and tumor necrosis factor (TNF) receptor [14, 16] occurred, together with a substantial rise in the recruitment and release of immature myeloid leukocytes associated with chronic inflammation [46]. These studies have supported the design of a more fluidic model of sepsis with simultaneous inflammatory and immunosuppressive processes. This evidence eventually led to the replacement of the traditional SIRS/CARS model with the concept of persistent inflammation–immunosuppression catabolism (PICS) [6].

PICS is characterized by a low but constant, chronic state of inflammation that paralyzes the host's immune system while exerting drastic catabolic effects on the body mass' nutritional intervention [7, 13] The key adaptive immune features that once typified CARS are now understood to fall under the larger umbrella of PICS. ("(PDF) A rapidly progressing lymphocyte exhaustion after...") These processes include immune cell metabolic failure, decreased T-cell numbers, lymphocyte dysfunction, increased apoptosis, increased T-cell suppressor function,

reduced T-cell repertoire, significant shifts in cytokine polarization toward humoral and TH2 cytokines, diminished membrane-associated human leukocyte antigen receptors, and epigenetic modifications secondary to the cell microenvironment [1, 22, 23, 28–33]. The definitions and diagnostic criteria for sepsis and PICS are defined in Table 1.1.

While the etiology or pathophysiology of PICS has not been completely elucidated, extensive studies on pro- and anti-inflammatory cytokines and chemokines have revealed the sheer breadth of sepsis and its many modes of action. Recently developed controversial theories suggest that the role of endotoxins and immunosuppressive SIRS medication might be secondary to the role of endogenous molecules like catecholamines [18, 36], corticosteroids [19, 20, 21], and IL-10 [22-27].

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$| **Term** | **Definition** |
| ---- | ---------- |
| Infection | The invasion of an organism by a pathogen that elicits a pro-inflamm
| Sepsis | An extremely dysregulated immune response from the host in response to
| Onset of Sepsis | Observation of dysfunction in a new organ, away from the origi
| Sequential Organ Failure Assessment score (SOFA score) | The SOFA score is used
| Rapid bedside organ dysfunction score - qSOFA | A quick test that suggests poten
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CHAPTER 2

CHAPTER NAME

THE MOLECULAR PATHWAYS OF SEPSIS.

2.1 The Innate Immune System.

The host's body initially promotes an innate pro-inflammatory response as a response to pathogens, which is arbitrated by antigen-presenting cells (APCs). These cells express pattern-recognition receptors (PRRs) on their surface, which can detect pathogen-associated molecular patterns (PAMPs) expressed on a pathogen's surface or through the release of damage-associated molecular patterns (DAMP) as a result of tissue damage [39]. Upon recognition, the PRRs activate various receptors such as the nucleotide-binding oligomerization domain (NOD)-like receptors and Toll-like receptors (TLRs). These cause a cascade of reactions across multiple pathways that promote the manufacture of pro-inflammatory cytokines and chemokines, which trigger second messenger cascades, resulting in amplified immune responses [35].

Cytokines and chemokines are crucial mediators of immune responses as they enable the recruitment of leukocytes to the site of infection/injury and increase the permeability of the endothelial vasculature, allowing for the localization of leucocytes [36, 37]. Cytokines and chemokines also facilitate communication between immune cells and their mediators and among adipocytes, fibroblasts, and endothelial cells. Additionally, cytokines and chemokines allow for interactions between the various cascade systems responsible for eliciting immune responses to occur. Given the complexity of the immune system, this particular intricacy has made it extremely difficult for researchers to understand the molecular mechanisms of the immune system [38–41]

The name interleukin was suggested in 1979, which means "communication between leucocyte" "[42, 43]. Many of these proteins are produced by and act on leukocytes, but cells from other tissues can also secrete them. They exert complex immune-modulatory functions, including cell proliferation, maturation, migration, and adhesion [44, 45]. The NOD-like receptor group aggregates to form larger protein complexes called inflammasomes [7]. These protein complexes play a vital role in the production and release of critical cytokines IL-1

$$\beta$$

and IL-18. They are also involved in the formation of caspases, which are implicated in apoptosis [48]. These pro-inflammatory cytokines elicit leukocyte proliferation, upregulate chemokine expression and express tissue factor production, and induces the production of hepatic acute phase reactants, which are important mediators produced in the liver during times of acute and chronic inflammation [46, 49]. During sepsis, these immune responses are amplified, leading to damage and death of tissues and cells. Recent studies that have analyzed the association between IL-18 levels and mortality [50–52] suggest the role of inflammasomes and autophagy as potential targets in the treatment of sepsis.

2.2 Toll-Like Receptors

TLRs are a type of PRR expressed on APCs. These TLRs are thought to play a very crucial role in the induction of innate immunity. This family of type I transmembrane receptorsSystemic inflammatory response syndrome (SIRS) is a generalized immunological response against a vast range of pro-inflammatory pathologies, including infection, injury, trauma, and burns. SIRS is often characterized by significant changes in the body temperature and the onset of tachycardia, rapid breathing, and abnormalities in white blood cells (WBCs) and red blood cell (RBCs) counts and

can give rise to systematic multi-organ dysfunction. When the onset of infection causes SIRS, the phenomenon is known as sepsis. Sepsis is a life-threatening inflammatory response that can give rise to systematic multi-organ dysfunction and failure caused by either trauma or infection. Despite modern advances in elucidating the pathophysiology of sepsis, the condition remains one of the primary causes of mortality and morbidity in intensive care units (ICUs) worldwide. Current estimates suggest that sepsis affects more than 30 million people and accounts for more than six million deaths per annum worldwide. Based on the Surviving Sepsis Campaign data from 2012, 41% and 28.3Severe sepsis is when the host's reaction to infection causes a systemic cascade of organ failures in a manner referred to as septic shock [1-6]. Sepsis is believed to cause organ failure through the uncontrolled upregulation of systemic immune responses. However, in light of medical and scientific advancements, ICU survival rates have improved, which led to the detection of the immunosuppression phase in the later stages of sepsis pathophysiology, ultimately explaining the high mortality rates. This syndrome was termed "compensatory anti-inflammatory response syndrome" (CARS) by Bone in his 1996 paper [4]. Similar to SIRS, CARS is a complex immune system response to severe infection; however, CARS is believed instead to be a condition marked by systematic inhibition of the immune system that restores homeostasis after the period of extreme inflammation. This led scientists and medical professionals to use the terms SIRS and CARS to differentiate the host's pro- and anti-inflammatory responses to a broad range of infectious and noninfectious stimuli [6–9]. While initial studies categorized CARS as the phase that appears at the end of or even after SIRS, researchers have since found evidence of pathways that support the idea that CARS is not a part of SIRS. Instead, CARS may exist entirely separately from SIRS and encompass an additional set of cellular and molecular interactions and pathogenesis pathways different from those of SIRS. However, CARS may also significantly influence sepsis and lead to adverse outcomes: while earlier

studies of the pro-inflammatory phase of sepsis have helped to improve survival rates in the ICU, the emergence of an immunosuppression phase in the later stages of sepsis pathophysiology often left the patient vulnerable to secondary infections, which could explain the high mortality rates [10]. Indeed, later studies revealed that the anti-inflammatory responses elicited by CARS induce a severe immunosuppressed state wherein the immune system cannot recover despite eradicating pathogens from the body, which, as a phenomenon, has been termed "immune paralysis" [5]. Modern advances have reduced the rates of deaths occurring during sepsis' initial stages as homeostasis is reestablished early on in the disease's pathophysiology. However, those patients who fail to achieve homeostasis during the early phases of SIRS/CARS enter a state marked by high mortality and morbidity rates, typically exhibiting severe weakness, malnutrition, chronic infections, and cognitive decline, which has come to be known as chronic critical illness [10–13]. Data from 2009 indicate that the annual health care costs for patients with chronic critical illness exceeded $20 billion. The majority of these patients ($> 60\%$) were admitted with a sepsis diagnosis [12], and only 20Due to its associated high mortality rates, CARS soon became a target for immune-modulating therapies [14]. However, despite extensive preclinical research into possible immunomodulatory therapies for CARS, not many treatment solutions to date have been implemented [15]. Later studies found that, during CARS' immunosuppressive phase, an increase in the levels of pro-inflammatory cytokines such as C-reactive protein (CRP), interleukin (IL)-6, IL-1Ra, and tumor necrosis factor (TNF) receptor [14, 16] occurred, together with a substantial rise in the recruitment and release of immature myeloid leukocytes associated with chronic inflammation [46]. These studies have supported the design of a more fluidic model of sepsis with simultaneous inflammatory and immunosuppressive processes. This evidence eventually led to the replacement of the traditional SIRS/CARS model with the concept of persistent inflammation–immunosuppression catabolism (PICS) [6].

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In mammals, scientists have uncovered 10 different kinds of TLRs, with each one playing a tailored role in innate immunity. These TLRs recognize highly conserved PAMPs as ligands and have exceptionally low specificity. In an attempt to understand their intricate workings to bind with a PAMP/DAMP, their ligand structures have become a topic of great scientific interest [53–57]

Although TLRs' complete workings have not yet been elucidated, recent data suggest that they often work as dimers. While most form identical homodimers,

some form heterodimers, with each dimer maintaining a unique affinity for ligands. These PRRs depend upon accessory proteins to aid in their binding with PAMPs. In particular, TLR4—the most studied mammalian TLR—plays a crucial role in recognizing the PAMP lipopolysaccharide (LPS), which is only produced by prokaryotes like Gram-negative bacteria. TLR4's recognition of LPS requires MD-2, a small protein with a currently unknown function that lacks a transmembrane domain, and CD-14, a high-affinity LPS receptor often expressed on macrophage surfaces. CD-14 and LPS-binding protein (LBP) present LPS to MD-2. When these PRRs are activated, they recruit adapter molecules from the cell's cytoplasm that initiate signaling cascades like MyD88 [58–60] and Toll-interacting protein TOLLIP.

CHAPTER 3

DATA ANALYIS

3.1 Source of the Data

To study the relationship between the SNP rs1059703 mutant haplotype and its combined effects along with IRAK-1A/ IRAK-1C heterozygous splice variants, data from NCBI's SRA database containing RNA-seq data of human peripheral blood mononuclear cells from 15 patients were taken. This study was conducted by Columbia University in 2016, with the Bio project ID PRJNA343985 and the Geodata set accession number GSE87290 [261].[**?**]

Based on data from previous studies, the contributors Fergueson and Xue, [] selected only the individuals in the top (high-responders) and bottom (low-responders) extremes for inflammatory responses from previous studies. These patients were subjected to inpatient endotoxin challenge (1ng/kg LPS) in healthy humans. RNA-Seq was conducted for peripheral blood mononuclear cells (PBMC, n=15) before and after LPS administration. As previously stated, human monocyte cells predominantly expressed IRAK-1A, with minimum levels of IRAK-1C expression. This allowed the sensitive recording of changes in IRAK-1C mRNA levels [261] (Refer Appendix A.).

3.2 Data processing.

The experimental SRA runs from NCBI were aligned with the identifier sequences in table 5.2 using SRA Blast. The total number of perfect matches was counted. These values were later normalized by dividing them by the number of Gigabase pairs (Gbp) of each SRA read's data file to give a normalized value. This data was processed using Python, R, and Jupyter Notebooks for statistical calculations.

Table 5.1 SRA Blast Query Sequences. Key sequences bolded and underlined.

3.3 Statistical processing & analysis

All statistical analysis were performed in Jupyter notebooks using python. The Shapiro test for normalcy to determine the normalcy of the variables under study and secondly subjectively by observing the histograms and box plot outputs [262]. To determine if there were significant differences between two groups of paired data, a paired-T test was performed for parametric data [266] and a Wilkson-paired T-test [264] for non-parametric data based on the Shapiro-test results. Independent and non-parametric groups were compared using the Whitney-Mann U test and the Kruskal-Wallis test for significant differences[265, 266].

1.

CHAPTER 4

RESULTS

4.1 Data Demographics

4.2 Exposure to LPS causes a reduction of IRAK-1 mRNA expression

While the roles of cytokines in inflammation have been extensively studied, the genetics of their regulatory molecules have not from the transcriptomic point of view. Recent studies on inflammatory responses have shown that differential gene expression can lead to changes in the inflammatory response, primarily through variances in mRNA levels. To detect changes in the mRNA levels, the total IRAK-1 mRNA expressions per Gbp before (median=2.7) and after the LPS (median=1.8) treatment were compared. We tested for normalcy using the Shapiro-Wilk test, which showed that the data was not normally distributed. Hence, we conducted non-parametric tests were conducted. The Wilcoxon paired test indicated that there was a significant difference between the median of the two groups. Fig. 6.1B a drop of 0.78 mRNA counts per Gbp (p-value:0. 0.005) (Fig. 6.1A).

To understandthe rate of change in IRAK-1 levels, linear regression analysis was performed with the IRAK-1 levels before LPS treatment against the expression levels after LPS administration. The linear model had an R2 value of 0.31 and a slope of 0.74, with an intercept of -0.249. This data suggests that the changes in IRAK-1 levels following LPS treatment are significant but are primarily dependent on IRAK-1's initial levels.

Figure 6.1 TLR4 mediates the recognition of the antigen LP and is responsible for the initiating-anti-microbial inflammatory response

4.3 IRAK-1C levels remain constant as they fail to disengage from the adaptor proteins

IRAK-1 has tw

4.4 The role of IRAK-1 in Hyperinflammation

There have been a variety of studies that have sought to understand the dynamics of the TLR-NF-kB pathway in inflammation, as an attempt to develop possible therapies aimed at restoring cytokine regulation in sepsis. These studies often implicate IRAK-1 as a key mediator in regulating the intensity of endotoxin challenges. High levels of IRAK-1 expression has been associated with increased proinflammatory activity, whereas Studies on LPS-tolerance in human monocytes revealed unaltered TLR4 expression but suppressed MyD88-TLR4 and IRAK1-MyD88 interactions, IRAK1 activation. As we had shown in section 6.1,IRAK-1 activation is which is marked by a sharp decrease in IRAK-1 expression. Based on previous studies, and our results from section 6.3, this drop is primarily due to the phosphorylation of IRAK-1A splice variant.

In order to understand the impact of splice variants on the inflammatory responses, it is essential to study the dynamics of IRAK-1 expression levels amongst patients with high and low endotoxin sensitivities. Figure 6.3 shows the levels of IRAK-1 and its splice variants amongst patients with high and low sensitivity to endotoxin challenges before and after LPS administration. Based on the results of the Shapiro-Wilk test, we conducted both parametric and non-parametric tests to compare IRAK-1A, IRAK-1C and the total IRAK-1 Levels before and after the LPS treatments.

Table 6.2 summarizes the descriptive statistics of the groups under study. We report a significant change in total IRAK-1 levels between patients with low ($p=0.03$), and the high levels of inflammation ($p=0.0053$). Similar to our results in 6.3, we

obtained s significant change in IRAK-1A levels as well low (p=0.013), and the high levels of inflammation (p=0.010). Similar to our previous results, IRAK-1C failed show exert any significant difference with. A p-value of 0.86 for patients with high IRAK-1

APPENDIX A

CELL SIGNALING SITES & PROTEIN DATABASES