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The role of irak-1 transcripts in sepsis

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ABSTRACT

THE ROLE OF IRAK-1 TRANSCRIPTS IN SEPSIS

by
Adithya Subramanian Sahasranamam

Sepsis is a complex, life-threatening syndrome that can lead to systemic organ failure and dysfunction. Its high morbidity and mortality rates makes it a critical global health issue. The primary factors at play during sepsis are abnormal inflammation and a lack of oxygen supply to the tissues and muscles. The toll-like receptors play a crucial role in eliciting innate immune in response to infection, primarily through the interleukin-1 receptor-associated kinase (IRAK) pathways. Disturbances in the homeostasis of IRAK signaling cascades can lead to immune dysfunction. n this paper, we review the molecular mechanisms of IRAK-1, an important mediator of TLR-induced inflammation and compare the effects of its splice variants and polymorphisms in the context of inflammation and sepsis.

THE ROLE OF IRAK-1 TRANSCRIPTS IN SEPSIS

by
Adithya Subramanian Sahasranamam

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APPROVAL PAGE

The ROLE OF IRAK-1 TRANSCRIPTS 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.

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TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

Chapter **Page**

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES
(CONTINUED)

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's 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 [1-6]. 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. 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's 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-1, and tumor necrosis factor (TNF) [14, 16] occurred, together with a substantial rise in the recruitment and release of immature myeloid leukocytes associated with chronic inflammation [17]. These studies have supported the design of a more fluidic model of sepsis with simultaneous inflammatory and immunosuppressive processes. This

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 such as immune and metabolic failure, increased T-cell suppression, and inhibition of proinflammatory signaling are now thought to be a part of PICS [1, 22, 23, 28–33]. The definitions and diagnostic criteria for sepsis and PICS are depicted 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].

Table 1.1 The Terminologies and Definitions of Sepsis

Term	Definition	
Infection	The invasion of an organism by a pathogen that elicits a pro-inflammatory response.	
Sepsis	An extremely dysregulated immune response from the host in response to infection.	
Onset of Sepsis	Observation of dysfunction in a new organ, away from the original site of infection.	
Sequential Organ Failure Assessment score (SOFA score)	The SOFA score is used to track a patient's status by determining the extent and severity of a patient's organ function over time. It uses data from the Cardiovascular, respiratory, hepatic, renal, and neurological organ systems for calculation.	
Rapid bedside organ dysfunction score – qSOFA	A quick test that suggests potential Organ dysfunction if at least two of the following is present: Altered Mental Status – Glasgow Coma Scale ≤ 14 Systolic Blood Pressure ≤ 100 mmH Respiratory Rate 22 breaths per min	
Organ Dysfunction	A significant change in the SOFA score by at least two points at a site remote from infection.	
Septic Shock	A state of sepsis that elicits a cascade of profound changes in the vasculature, metabolism, cellular, and organ functions.	
PICS	Critically ill patient	Admission to the ICU > 14 days
	Persistent inflammation	CRP > 50 μg/dL
		Retinol binding protein < 1 mg/dL
	Immunosuppression	Total lymphocyte count < 0.80 $\times 10^9$/L
	Catabolic state	Serum albumin < 3.0 g/dL
		Creatinine height index < 80%
		Weight loss > 10%
		BMI < 18 during hospitalization

Source: Mira, J. C., Gentile, L. F., Mathias, B. J., Efron, P. A., Brakenridge, S. C., Mohr, A. M., ... & Moldawer, L. L. (2017). Sepsis pathophysiology, chronic critical illness, and PICS. Critical care medicine, 45(2).

CHAPTER 2

THE MOLECULAR PATHWAYS OF SEPSIS

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 [34]. Upon recognition, the PRRs activate various receptors such as the nucleotide-binding oligomerization domain (NOD)-like receptors and Toll-like receptors (TLRs). The activation of these receptors causes 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 [38–41].

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-1β and IL-18. They are also involved in the formation of caspases,

which are implicated in apoptosis [42]. 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 [43, 44].

The name interleukin was suggested in 1979, which means "communication between leukocytes" [45, 46]. 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 [47-49].

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.

2.1 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 receptors was initially found in drosophila and were confirmed to possess an extracellular leucine-rich repeat domain and a highly conserved intracellular Toll/IL-1 receptor (TIR) domain across plants and animals. This TIR domain enables interactions between proteins and has been shown to play a vital part in the evolution of immunity.

In mammals, scientists have uncovered ten 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 as compared to antibodies. This low specificity of these receptors allows them to recognize a plethora of microbial products. Due to their low ligand specificity, TLRs have become a topic of great scientific interest [53–57].

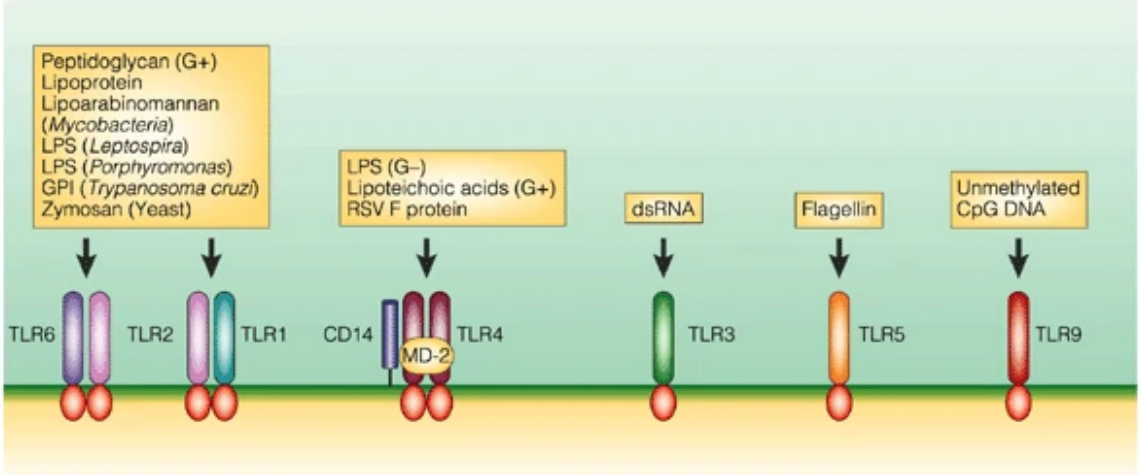

Figure 2.1 Toll-like Receptors possess an affinity for a diverse range of microbial ligands. This feature enables them to recognize a wide variety of PAMPs/DAMPs. Upon recognition of these ligands, TLRs initiate downstream pro-inflammatory responses that lead to the production and recruitment of pro-inflammatory cytokines.

Source: Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews Immunology*, 1(2), 135-145.

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 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 TLRs are activated, they recruit adapter molecules from the cell's cytoplasm that initiate signaling cascades like MyD88 [58–60] and Toll-interacting protein (TOLLIP), a protein kinase, IL-1 receptor (IL-1R)–associated kinase (IRAK) [58, 59, 61]; and another adaptor, TNF receptor-associated factor 6 (TRAF6) [58, 59, 62]. Recent studies have confirmed the crucial roles of these adaptor molecules in regulating innate immunity through targeted deletions of genes of the adaptor molecules [63–65]. Other studies have reported that MyD88-deficient mice fail to produce pro-inflammatory cytokines like IL-1, TNF, IL-6, and IL-12 when stimulated with ligands of the TLR systems, respectively [60, 65–68]. However, later detailed analyses have revealed the existence of a MyD88-independent pathway called the TRIF-dependent pathway that promotes the transcription of pro-inflammatory cytokines as well as trans-inflammation through the production of interferons (IFNs). All TLR families except TLR3 use MyD88-dependent pathways. Further studies of MyD88 pathways have revealed an important regulatory adaptor protein family, the IRAK family of kinases.

2.2 Interleukin-1 Receptor-Associated Kinases

Among the many genes involved in the TLR-signaling cascade, Interleukin-1 receptor-associated kinase 1 (IRAK-1) has been portrayed as an essential modulator of inflammation. IRAK-1 belongs to the interleukin-1 receptor (IL-1R) associated kinase (IRAK) family, [69] which orchestrates the pro-inflammatory responses to pathogenic invasions through signal transduction and mediation of the toll-like receptor (TLR) and

interleukin-1 (IL-1) signaling pathways [25]. These IRAKs serine-threonine kinases are expressed on the membranes of various immune cells such as dendritic cells, neutrophils, macrophages, and other non-immune cells like the epithelial and endothelial cells and fibroblasts) [70]. Together with the Interleukin-1 receptors (IL-1Rs), these TLRs form a receptor superfamily, called the "interleukin-1 receptor / toll-like receptor superfamily". All TLR family members exhibit the TIR (toll-IL-1 receptor) domain, a highly conserved domain homology that promotes acute inflammation and other additional adaptive immune responses.

When ligands bind to the TLRs, it recruits the adaptor protein MyD88. MyD88 possesses a TIR domain (Toll and IL-1-receptor homology domain to the intracellular TIR domain of TLRs. The TIR domain is connected with the death domain (DD). Upon TLR activation, the DD of MyD88 interacts with the DDs of the IRAK family of kinases and recruits IRAK-1, IRAK-2, and IRAK-4 onto the receptor complex's intracellular domain [58, 59].

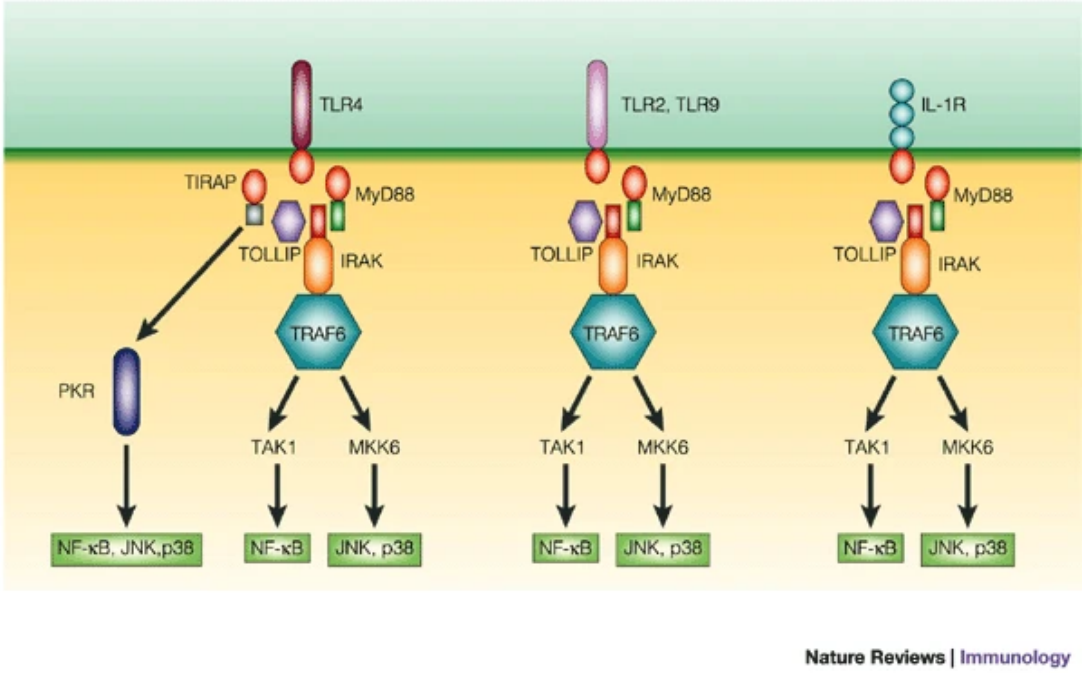

Figure 2.2 The TLR-induced NF-κB signaling is primarily mediated by IRAKs.

Source: Medzhitov, R. (2001). Toll-like receptors and innate immunity. Nature Reviews Immunology, 1(2), 135-145.

This TLR–nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) transduction plays a vital role in governing the initiation of innate pro-inflammatory immune responses by increasing the expression of pro-inflammatory mediators such as IL-6, TNF, HMGB1, adhesion molecules, and chemokines that are implicated in sepsis-induced organ dysfunction [71–74]. Studies on mice have shown that macrophages deficient in IRAK-1 express an inadequate pro-inflammatory response to TNF in response to LPS-induced sepsis and are thereby resistant to LPS lethality [75]. However, this makes the mice also very vulnerable to infections by Gram-negative bacteria [76].

TLR-mediated cell signaling in immunity is negatively regulated by another vital member of the IRAK family, known as IRAK-M (or IRAK-3) [77–79]. Unlike IRAK-1, IRAK-M does not possess kinase activity but instead regulates signaling through MyD88-

dependent TLRs [79]. Initially, the IRAK-M expression was thought to be present only in monocytes; however, recent studies have shown that it is present in both hematopoietic and nonhematopoietic cells. IRAK-M is primarily involved in inflammatory and anti-microbial responses. IRAK-M determines the levels of endotoxin tolerance, thereby dictating the magnitude of pro-inflammatory sepsis-induced changes [79–83]. Recent studies indicate that IRAK-M mediates macrophages' tolerance to LPS and the tolerance level in nonhematopoietic cells such as biliary epithelial cells [79, 83].

Patients suffering from sepsis also appear to express higher IRAK-M levels in their blood monocytes, with some studies indicating that IRAK-M levels were highest during the most profound blunting of LPS-induced cytokine responses. The relatively high expression of IRAK-M is associated with adverse clinical outcomes in sepsis patients [82, 84]. While the mechanisms accounting for IRAK-M–based endotoxin tolerance and the negative regulation of inflammation are not entirely known, they are believed to be associated with impaired TLR-mediated activation of both NF-κB and mitogen-activated protein kinase (MAPK)-dependent genes [85–87]. It is thought that IRAK-1M represses IRAK-4 activation, leading to a subsequent blockage of IRAK-1 phosphorylation [79]. Meanwhile, IRAK-1 hyperphosphorylation results in decreased protein stability, providing a potential mechanism by which to regulate IRAK-1 activity [88].

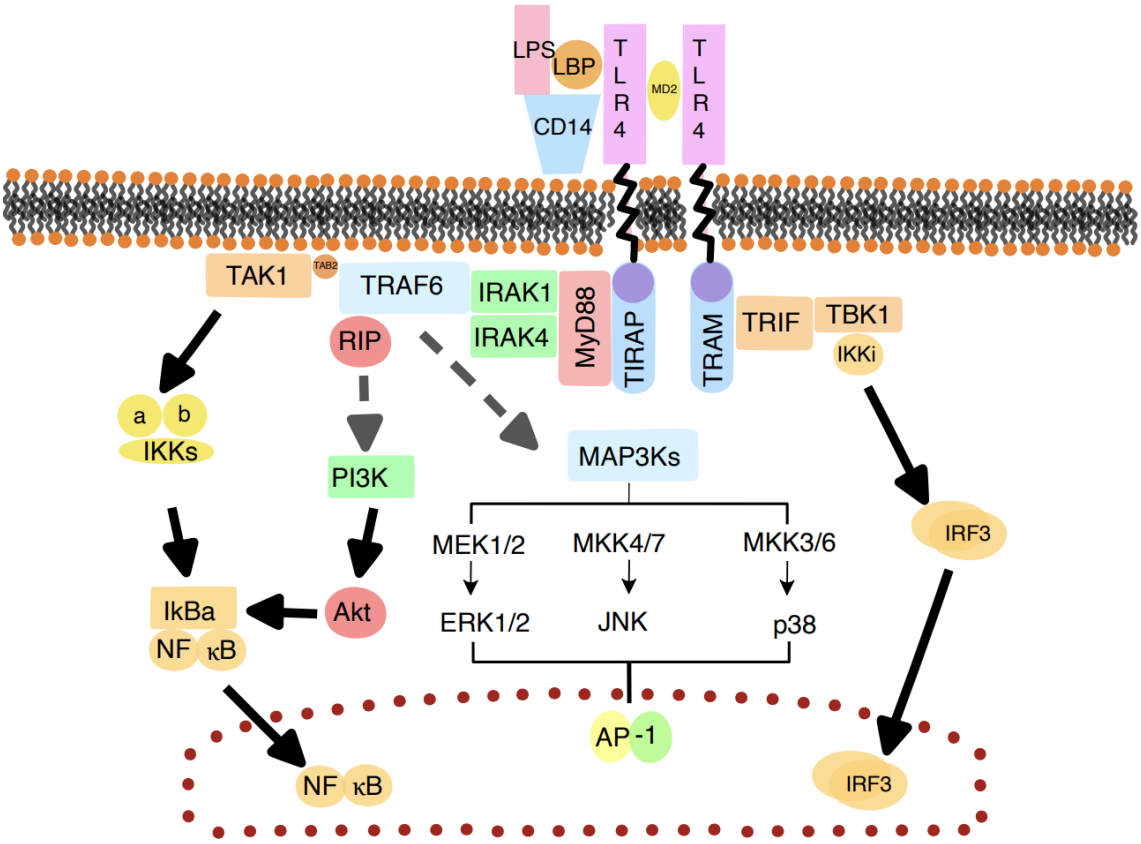

Figure 2.3 TLR4 mediates the recognition of the antigen LPS. The TLR4 pathway is responsible for initiating an anti-microbial inflammatory response via the NF-κB, Map3k, or the IRF3 pathways.

Source: File: Toll-like receptor pathway.svg. (2020, September 29). Wikimedia Commons, the free media repository. Retrieved 16:12, March 8, 2,021 From https://commons.wikimedia.org/w/index.php?title=File:Tolllike_receptor_pathways.svg&oldid=475789885

2.3 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-Cells (NF-κB)

NF-κB is a protein complex that is highly conserved and expressed in almost all animal cells. This protein complex has been labeled as a "rapid-acting primary transcription factor" as it is found in an inactive state but does not require protein synthesis for its activation [89]. This allows NF-κB to act rapidly and function as the first responder to harmful stimuli responsible for both the innate and adaptive immune responses [90].

NF-κB can be activated by a wide variety of stimuli, such as TNF, IL-1β, and reactive oxygen species (ROS), and through the stimulation of a wide variety of cell-surface receptors such as TLRs [89]. All TLRs other than TLR3 use MyD88 and are dependent upon IRAK-1 for activation. Following its activation, NF-κB translocates into the cell nucleus, promoting the transcription of a wide variety of genes involved in inflammation, cell survival, and cell proliferation. Any disturbance of the normal expression and function of NF-κB can lead to cancer and inflammatory and autoimmune diseases, together with greater vulnerability toward microbial infection and sepsis.

2.4 The Complement System

The complement system consists of small proteins synthesized by the liver that circulates in the blood as zymogens or inactive precursors. When the system is activated, proteases in the system begin to cleave proteins, releasing cytokines that initiate a cascade of zymogen cleavages that are highly amplified by each successive enzymatic reaction, resulting in the rapid and exponential generation of a large complement inflammatory response [91].

These responses result in the recruitment of phagocytes to the site of the inflammation, the stimulation of said phagocytes to eradicate foreign material, and the activation of the cell-killing membrane attack complex. The complement system can be activated by any of the following three pathways: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP) [92]. These three pathways all converge at the complement protein C3 convertase and share a common cascade from C5 to C9, which leads to the formation of the cell-killing membrane attack complex and its subsequent pursuit of target cells. This is complemented by the release of chemo-attractants (C3a and C5a) to aid in the recruitment of inflammatory cells [93].

The classical pathway uses the complement component 1q (C1q) as the PRR; C1q is a part of the C1 complex together with the proteases C1r and C1s. When C1q binds to the antigen-antibody complexes together with immunoglobulins G and M or apoptotic cells or structures on microbial cells, the proteases activate, cleaving C4 into C4a and C4b. C2 reacts with C4, creating the C4bC2a enzyme (C3 convertase) [94].

The PRR used in the lectin pathway is the mannose-binding lectin (MBL) or ficolins, which binds to apoptotic host cells and can also recognize and bind with carbohydrates. Such PRRs are similar to the CP pathway but are bound with MBL-associated serine proteases, which cleave C4 and C2 to yield C3 convertase [95].

The AP uses C3 as its central molecule. C3 is cleaved by CP/LP convertases or C3 (H_2O) formed from B (fB) binding. This cleavage of C3 exposes a thioester moiety in C3b, which allows for binding with surfaces [96]. fB then undergoes cleavage by the serum protease factor D (fD), producing AP C3 convertase C3bBb [or C3(H_2O) Bb]. The AP C3

convertase is stabilized by properdin, which allows for the recognition of several PAMPs and DAMPs, initiating a complement response [97].

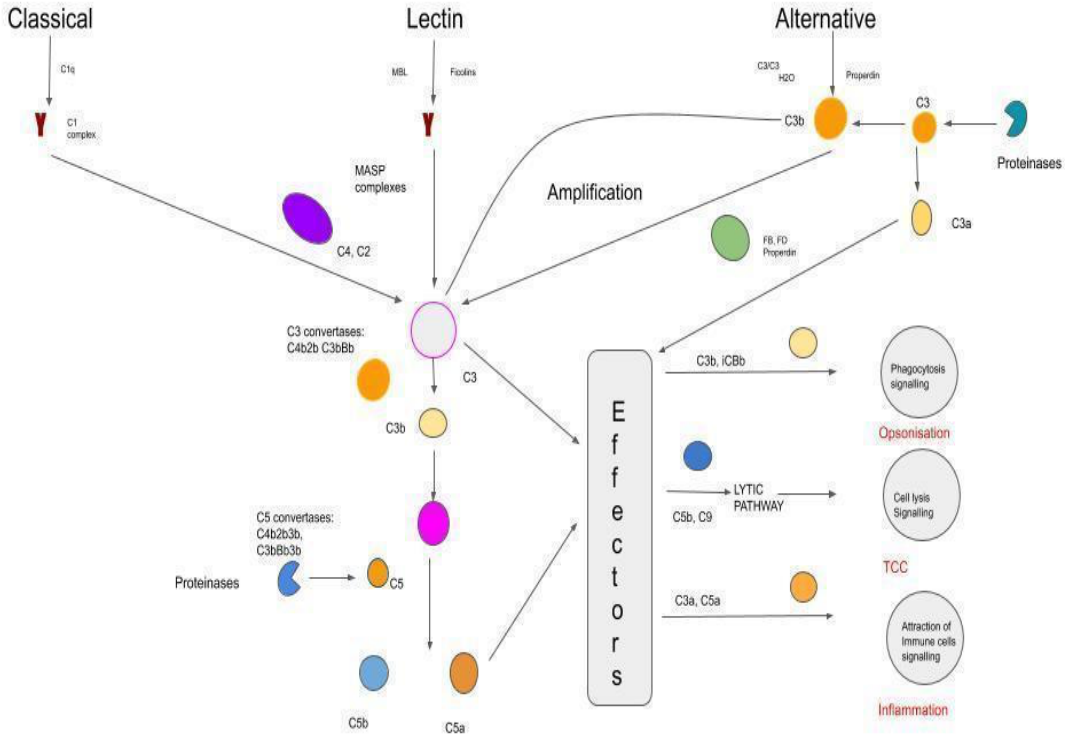

Figure 2.4 The Complement system mediates the activation of phagocytes in response to inflammation.

Source: Sarma, J. V., & Ward, P. A. (2011). The complement system. *Cell and tissue research*, *343*(1), 227-235.

CHAPTER 3

MULTI-ORGAN FAILURE IN SEPSIS

3.1 Organ and Tissue Dysfunction

The primary pathophysiology of sepsis occurs through the combined effect of hyperinflammatory humoral and/or cellular responses to the presence of PAMP/DAMP by humoral or innate APCs. This causes the APCs to trigger a one-to-many pro-inflammatory signaling cascade that leads to leucocyte recruitment. Although the pro-inflammatory response is controlled under normal conditions, sepsis induces a hyperinflammatory response that leads to a barrage of pro-inflammatory activity, which can culminate into multiple organ failure.

3.2 Cardiovascular Dysfunction

The primary cause of sepsis-induced organ failure and tissue dysfunction is a reduced supply of blood flow and oxygen to organs and tissues. Sepsis correlates with a broad spectrum of cardiovascular abnormalities that can lead to hypoperfusion, which is the initial hemodynamic profile of severe sepsis brought on by cardiogenic, hypovolemic, and distributive shock. In the early stages of sepsis, multiple changes occur in sepsis patients' vascular beds that are primarily elicited by the action of inflammatory cytokines such as TNF and IL-1 on the endothelial layers [98–100].

During sepsis, there is an overwhelming and dysregulated pro-inflammatory response that causes a massive buildup and release of pro-inflammatory cytokines like IL-1α, IL-1β, TNF, and IL-6. These cytokines interact with endothelial cells at the site of the injury to dilate the capillaries at the vascular bed and increase their permeability, enabling

the accumulation and flow of platelets and WBCs to handle repairs and infection at the site in question. IL-1 is composed of two genes: IL-1α and IL-1β. Upon its release, both IL-1α and IL-1β bind to the IL-1 receptors (IL-1R) on the walls of the vascular endothelium and promote the breakdown of the endothelium and its junctions by triggering the MyD88 signaling cascade [101, 102]. While these processes are regulated during normal conditions, the lack of immune homeostasis in sepsis leads to enhanced endothelial dysfunction. Endothelial dysfunction is marked by increased vasodilation and vascular capillary permeability. This results in the leakage of cytokine-rich fluid into the peripheries, causing edema and compromising vascular volume and blood pressure, resulting in hypotension and hypovolemia. Furthermore, the lack of a functional endothelial layer allows for microbial invasion deeper into the host, enabling the pathogens to potentially disturb regulatory mechanisms and ultimately cause dysfunction of remote organs [103].

In response to increased cytokine activity, neutrophils, fibroblasts, APCs, and macrophages are recruited to the site of the injury in concert with the production of secondary pro-inflammatory mediators by both macrophages and mesenchymal cells. TNF stimulates macrophages to produce IL-6, an interleukin with both pro-and anti-inflammatory properties [104]. IL-6 is released following a spike in TNF levels and acts as a signal for tissue damage; it is primarily involved with the mediation of the acute-phase response [105, 106], which triggers a systemic cascade that promotes leukocytosis, combined with symptoms of fever along with the release of Hepatic acute-phase proteins (APPs) such as CRP, complement components, fibrinogen, and ferritin [107]. These hepatic proteins are vital markers of inflammation and tissue damage and are primarily

associated with the recruitment of peripheral leukocytes, circulating neutrophils, and their precursors. These proteins also play a crucial role involved in coagulation in response to endothelial damage [100-107].

Mesenchymal cells such as those of the subendothelial layers and fibroblasts often have a repressed expression of Tissue Factor (TF). Either TNF stimulation of monocytes or the exposure of subendothelial cells in response to tissue injury can lead to the expression of TF, which binds with factor VII, causing the activation of the intrinsic coagulation cascade. During this coagulation cascade, prothrombin, a zymogen of thrombin, gets cleaved into thrombin, a powerful activator of platelet cells (thrombocytes) [108]. Thrombocytes are primarily involved in the homeostasis of injury and use oxygen radicals called ROS for signaling, which possesses powerful anti-microbial and antiviral properties to damage the DNA, RNA, and proteins of bacteria and viruses [109–112].

Thrombocytes release ROS into the bloodstream to signal for more thrombocytes. However, in the case of sepsis, dysregulation of the immune response leads to an accumulation of thrombocytes at the site of the injury, triggering an autoimmune response that damages the host's genetic and protein materials [113]. This can lead eventually to the formation of cancerous lesions [114].

ROS induces chronic inflammation by the induction of cyclooxygenase-2, inflammatory cytokines such as TNF, IL-1, IL-6, and IFNs and promotes the transcription. This production of IL-1, TNF and IFN promotes the recruitment of neutrophils to the site of inflammation [115, 116].

Neutrophils are phagocytes that are considered the first responders to injury and infections. Neutrophils are primarily produced in the bone marrow and migrate towards the

site of injury via chemokine chemotaxis. They promote phagocytosis through the release of ROS and the formation of extracellular traps called neutrophil extracellular traps (NETs) [117]. Activated thrombocytes promote the formation of NETs and have been implicated in the extravasation of neutrophils across the endothelium [118, 119]. This process has been associated with the release of TNF and nitric oxide (NO). NO is a potent vasodilator, among its many roles [120, 121]. Together with NO, TNF upregulates the expression of adhesion molecules like selectins and integrins on the endothelial surface, which promote the binding of leukocytes with the endothelium, allowing them to cross over into the tissue [120–122].

Excess levels of ROS can elicit a state of constant oxidative stress in erythrocytes and cause them to recruit leukocytes even after the pathogenic threat has been eradicated. This state of constant oxidative stress often results in a positive feedback loop that promotes pro-inflammatory and homeostatic repair. This constant state of oxidative stress elicits profound morphological changes in the vasculature and the vascular bed of a sepsis patient through persistent endothelial damage through the action of cytokines, ROS, leukocyte extravasation, and the formation of multiple thrombi along the vasculature [123].

Various studies have demonstrated the impact of the role of inflammation in coagulation and the role of coagulation in the pathophysiology of sepsis [124, 103]. Due to the lack of functional vascular blood–tissue barrier, there is a leakage of cytokine-rich blood into the periphery, which causes edema. This condition is actually made worse by the onset of rapid coagulation. The thrombi formations often restrict the flow of blood, promoting localized cell death and tissue damage due to hypoxia [103]. This formation of

multiple thrombi across the arteries and veins is referred to as disseminated intravascular coagulation (DIC) [125].

DIC is a serious effect of sepsis that can lead to rapid multiple thrombi formation along the arterial and venous pathways, causing severe obstruction of blood flow. This condition rapidly uses up the clotting proteins and factors in the blood, causing an inability to form clots; this state can increase one's susceptibility to bleeding and is known as hemorrhagic diathesis [125, 126].

Clinical studies have associated DIC with the development of multi-organ dysfunction, leading to mortality in patients with sepsis [127]. DIC occurs in approximately 83% of the patients with bacterial sepsis [128]. The prognosis of DIC and the associated mortality rates depend on the severity of sepsis and thrombosis. A recent study suggests a 47.7% mortality rate for DIC, with some statistics purporting higher mortality rates among African American (52%) and Native American (57%) populations [129], suggesting possible genetic predispositions towards less favorable outcomes during DIC and sepsis in certain groups.

Various studies have implicated the roles of TLRs in heart disease and failure. TLR4 plays a central role in promoting myocardial inflammation, primarily mediated via the NF-κB pathway [130]. The activation of the NF-κB pathway in cardiomyocytes results in the expression of various inflammatory mediators such as TNF, IL-6, IL-1, and NO. The chronic release of these pro-inflammatory cytokines promotes interstitial fibrosis and collagen deposition in the non-infarcted zone leading to ventricular dysfunction [131, 132]. This affects the heart's ability to pump blood to the periphery.

Furthermore, as a result of clotting and blood loss, the volumetric blood return to the heart is severely compromised, which results in a state of cardiovascular dysfunction called myocardial depression (MD) categorized by a lower decreased stroke volume and ejection fraction. The heart responds to this state of hypoxia with a tachycardia response with increased ventricular distensibility. Despite the intense distensibility of the right ventricle, the reduced arterial resistance [24] leads to a decreased volumetric return of blood at the left auricle, which facilitates the depression of the auricular contractility. There is also leakage in and out of the myocardial sections, affecting the ventricular ability. Furthermore, NO has been implicated given its role as a cardiac dilator; NO induces the muscle cells to relax and hyperpolarize, preventing their response to vasoconstrictors and thus perpetuating hypotension.

This state of relaxation combined with the arterial and venous dilation caused by the pro-inflammatory cytokines results in a state marked by hypotension and distributive shock [10], the latter of which causes improper perfusion of blood and hypoxia all across the body. These initial stages of inflammation followed by a lack of proper circulation are the primary cause of systemic organ failure in patients with sepsis.

3.3 Hepatic Dysfunction

Ischemic hepatitis or shock liver is characterized by acute liver injury due to inadequate blood flow and hypoxia. This condition primarily occurs as a result of cardiac failure and septic shock.

The liver plays an important role in maintaining normal metabolic, hormonal, and host-defense activities; ensuring overall systemic homeostasis; and regulating coagulation.

It is responsible for clearing bacterial and toxic materials from the bloodstream [133]. Animal models show that more than 60% of bacteria injected intravenously are trapped in the liver by 10 minutes post-injection [134]. The liver is also responsible for the clearance of endotoxins like LPS from the bloodstream [135]. Various kinds of hepatocytes like Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and stellate cells are involved in microbial clearance. Given the crucial roles the liver plays in metabolism and homeostasis, the liver is perfused by 25% of the cardiac [136].

Studies have shown that ongoing inflammation and hypoperfusion can cause liver damage and failure. Clinical statistics suggest that hepatic dysfunction is an early sign of sepsis, with a mean incidence rate of 39.9%, and is a risk factor that has been associated with poor outcomes [137]. While the exact pathophysiologies of liver dysfunction and failure are yet to be fully understood, liver dysfunction is often associated with chronic inflammation, hypoxia, and initial tissue hypoperfusion, either through alterations to the hemodynamic profile or through a direct or indirect assault on the hepatocytes—or a combination of both.

KCs are the first line of defense in the liver. The liver harbors approximately 80% of all macrophages in the human body as resident KCs [138]. Upon their activation by TLR4, KCs produce high levels of pro-inflammatory cytokines like TNF, IL-1, IL-6, and IFN-γ along with secondary mediators of tissue injury, e.g., NO and ROS [48]. These cytokines then transduce intracellular signals, causing hepatocytes to lower bile transporter expression and function [139-141] and triggering a shift in hepatocyte metabolic pathways, promoting inflammation and repair responses, which are responsible for an increase in the

synthesis of APPs mediated predominantly by IL-6, which results in the formation of a procoagulant phase [142].

Another major cytokine responsible for hepatic dysfunction is IL-18, which is secreted by KCs in response to LPS stimulation. IL-18 triggers the release of , IFN-γ which promotes HC apoptosis, and also increases TNF levels. [143]. Furthermore, IFN-γ upregulates TLR4 expression [144], which can further promote inflammation and the adhesion of neutrophils to sinusoidal endothelial cells, promoting thrombi formation in the hepatic sinusoids and impairs liver microvascular perfusion [145] through TNF- and IL-1–induced migration of leukocytes into the liver, causing hepatic damage.

As damage to hepatocytes spreads, the liver's ability to transport bile acid and bilirubin is damaged. There is a profound alteration in bile acid bilirubin's transportation into the hepatic canaliculi, which causes cholestasis as the bile fluid leaks within hepatic tissue. Cholestasis has been linked to well-defined apoptosis in rat hepatocytes and is thought to be high in pro-inflammatory DAMP-like molecules. Cholestasis is thought to promote inflammation [146].

3.4 Pulmonary Dysfunction

Acute lung injury (ALI) can be defined as a hypoxic failure of the respiratory system due to inflammation. It is characterized by inflammatory injury to the alveolar-capillary barrier, along with neutrophil extravasation and protein-rich edema fluid leakage into the alveolar spaces, significantly affecting the lungs' ability to engage in gas exchange. This can be caused by direct damage to the alveolar tissue or through extrapulmonary causes like

sepsis. ALI, in its worst phase, is known as acute respiratory dysfunction syndrome (ARDS).

The primary cause of ALI/ARDS is endothelial dysfunction due to inflammation and neutrophil extravasation into the alveolar spaces. This onset of inflammation is primarily the result of resident macrophages called alveolar macrophages. These macrophages are the first line of defense in the lungs and play a central role in orchestrating inflammation upon activation via the TLR4/NF-κB pathway, which then promotes the release of pro-inflammatory cytokines like cytokines (e.g., TNF and IL-1β), chemokines (e.g., IL-8), and thrombin, together with the actions of a plethora of mediators like proteases, ROS, and NO that support the vasopermeability and disintegration of the endothelium. This results in the leakage of protein-rich fluid extravasation into the pulmonary interstitium and alveolar spaces. The resultant edema reduces the alveolar volume and gas capacity available for gaseous exchange, culminating in a reduced exchange of oxygen and CO_2 at the blood–alveolar barrier.

The Alveolar Macrophages (AMs) also interact with other cells like the epithelial cell lymphocytes and mesenchymal stem cells [147-149] using paracrine methods. AMs stimulate the recruitment of neutrophils and dendritic cells through the secretions of TNF and IFN. Neutrophils clear pathogens by phagocytosis and by releasing NETs. NETs are a complex system of anti-microbial components that trap and kill bacteria [149] due to the release of NET-associated components (NET-ACs), which are primarily made up of anti-microbial peptides [150, 151]. The majority of NET-ACs are considered to be DAMPs, and their excessive release results in inflammatory responses that can lead to cell death, inflammation, and organ failure [152–157]. NETs amplify the pathophysiology of sepsis

through multiple pathways: among others, they induce endothelial and epithelial cell damage, provide [155] a scaffold for platelet binding, and promote thrombosis in sepsis [156]. This inflammation/damage site serves as an entry point for macrophages and facilitates the activation of dendritic cells. NETs trigger macrophages to produce cytokines [157] such as IL-1β by releasing DNA and serine proteases (among NET-ACs). Furthermore, the combined activation of neutrophils and thrombocytes results in the promotion of endothelial and tissue damage. Preclinical studies have reported that blocking the formation of neutrophil–platelet aggregates slow down ARDS development [154, 157-160].

Several neutrophil-derived mediators, including proteases, cationic peptides, ROS, and matrix metalloproteinases, appear to be important in triggering an increase in epithelial permeability caused by neutrophils and may be important in alveolar epithelial injury [161]. The role of oxidants derived from neutrophils and other sources has been explored in several experimental studies [162].

Neutrophils and APCs also induce the apoptosis of distal alveolar type 2 epithelial cells, which maintain lung fluid homeostasis through ion transport and also produce the pulmonary surfactant (PS), a crucial substance that coats the surface area of the inner lungs to minimize the surface tension of the alveoli. Damage to these cells leads to the development of an acute exudative phase that results in significant impairment of lung mechanics and gas exchange, which leads to hypoxia and hypercapnia (excess CO_2) [163].

Some endotoxins like LPS are recognizable by both the complement system, the AP, and the TLR4 receptors. Exposure to PAMP/DAMP triggers the production of and releases pro-inflammatory peptides such as C3a and C5a. C5a elicits ROS production from

neutrophils. Through ROS-induced IL-1 and TNF release, the TLRs activate, resulting in alveolar damage and inflammation [164]. C5a can also promote NET formation [165].

The blood supply to the site of injury is usually restricted due to increased thrombosis formation caused by the primary injury. Moreover, at sites of inflammation, O_2 consumption is elevated due to increased demand from immune cells. Furthermore, a reduced supply of metabolic substrates by blood clots, compression of blood vessels, and atelectasis of the lung contribute to tissue hypoxia during inflammation. The leakage of protein-rich fluid extravasation into the pulmonary interstitium and alveolar spaces results in decreased gas capacity and interrupts the exchange of oxygen and CO_2 at the blood–endothelial barriers. All these issues collectively support the onset of hypoxia, hypoglycemia, and acidosis, which lead to increased production of free radicals and ROS [166, 167].

The lungs showcase one of the most severe pathophysiologies to arise from sepsis in the form of ARDS. Due to persistent and chronic expression of pro-inflammatory cytokines, there is a disruption of the fluid mechanics between air and blood at the alveolar–blood barrier as a result of extensive damage by both neutrophils and alveolar macrophages. Hypoxia results in hyperacidosis due to increased levels of lactate in the blood. This triggers the release of ROS [169], which further exacerbates the damage. The combination of inflammation, hypoxia, lactic acidosis, and improper perfusion facilitates pulmonary failure and often death.

3.5 Immunosuppression in Sepsis

During PICS, inflammation is marked through high levels of acute-phase proteins, neutrophilia, and the release of immature myeloid cells. While the mechanisms behind the immediate inflammatory response of PAMPs and DAMPs have been elucidated, the etiology behind the chronic and persistent inflammation in PICS remains unclear. It has been hypothesized that PICS follows the same pathways as an immediate inflammatory response. The alarmins "Danger" signals might arise from one's own injured tissue or organ structure. It might as well be the histones, nucleosides, or DNA [166]. The presence of these "Danger" molecules can trigger a variety of immune signaling pathways through various receptors and in various cell types, including immune, epithelial, and endothelial cells [170, 171]. As a result, the production of both pro-and anti-inflammatory cytokines and reactive oxygen and nitrogen species results in tissue wasting and apoptosis [12]. There have also been alternative theories as to why the pathophysiology of PICS accounts for opportunistic infections arising from viral reactivation [172], suggesting changes in gut microbiota, or by mechanically induced trauma caused by mechanical ventilation or administration of catheter. As sepsis progresses, one can almost definitely see a trend towards increasing cases of opportunistic infections, coupled with the compounding complexity of the interactions between the paradoxical states of inflammation and immunosuppression [173, 174].

Compared with control individuals without sepsis, patients with sepsis have increased reactivation rates of latent viruses. Among populations of critically ill patients without sepsis, only 5% of the population expressed viral-DNA in their blood. By contrast, 42% of critically ill sepsis patients expressed detectable viral DNA [172]. Autopsies of

human cadavers of patients with sepsis have shown the presence k persistent infection foci along with the presence of microabscesses in over 80% of the victims [175].

The downstream effects of persistent inflammation are numerous. of particular interest is a host immune environment similar to that of an elderly individual at baseline – i.e., inflammaging (constant low-grade inflammation in the aged) contributing to immunosenescence (the innate and adaptive dysfunction immune systems of the aged) [176, 177].

During sepsis, there are drastic changes in adaptive immunity. In response to cytokine and chemokine, production granulocytes often demarginate and follow the cytokine and chemokine trail. This denigration of granulocytes stimulates the HSCs in the Bone Marrow (BM), which are stimulated to produce Immature Myeloid Cells (IMC), which later differentiate and mature into lymphocytes, granulocytes, or dendritic cells. This emergency and rapid replenishment is termed “emergency myelopoiesis” [178-180]. At this stage, the production and recruitment of myeloid cells take precedence over the formation of lymphocytes and erythrocytes. This results in a deficiency of lymphocytes (lymphopenia) and the loss of thrombocytes, causing anemia [181, 182].

While normal conditions, these IMCs will mature into granulocytes, lymphocytes, or dendritic cells; septic shock elicits an intense storm pro-inflammatory cytokine-like, which prevents immature myeloid cells from differentiating and maturing. Instead, these IMCs form a heterogeneous population of immature myeloid cells called myeloid-derived suppressor cells (MDSCs) [183-186]. These MDSCs exhibits both inflammatory and immune suppressive properties. This is accompanied by the formation of an immature neutrophil (polymorphonuclear) phenotype [183, 186] accompanied by a loss of monocyte

inflammatory cytokine production and antigen presentation [185], following the ROS-induced neutrophil recruitment caused by C5a. As these neutrophils lack any sufficient levels of Adhesin proteins from extracellular traps made up of DNA, Chromatin, or granular proteins, this inhibits them from exerting anti-microbial actions. The formation of MSDCs and the immature neutrophils are classic biomarkers that indicate sepsis and immunosuppression, as illustrated in animal models [186, 171].

During PICS, there is an overall decrease in the number of helper and cytotoxic T-cells due to apoptosis and resistance towards pro-inflammatory cytokines [188]. Post-mortem studies of patients who died of sepsis in intensive care units show a marked deficiency of CD4+ and CD8+ T-Cells in the lymphoid system. Other studies have shown decreased TNF and IL-6 production levels in response to endotoxins [188-190]. This role has been prominently linked with MDSCs.

Poor clinical outcomes have been associated with the expansion of MDSCs, specifically after sepsis. Unlike other myeloid cell types, these MDSCs exhibit strong immunosuppressive properties. Even though the complete workings of MDSCs are not yet elucidated, MDSCs play a central role in propagating their anti-inflammatory effects even during times of sepsis and trauma. They are implicated in the inhibition of adaptive immunity for their role in downregulating the T-cells, B-cells, and APC populations. MDSCs are divided into two types: monocytic and granulocytic [191]. MDSCs carry out their immunosuppressive actions in a variety of ways based on their subtype [192]. Mathias et al. demonstrated that MDSCs are persistently increased in the circulation, predominantly granulocytic, transcriptomically unique, and immunosuppressive to T lymphocytes after severe sepsis or septic shock in the Surgical ICUs (SICU) [191]. Persistently increased

percentages of blood MDSCs in this study were associated with increased nosocomial infections, prolonged intensive care unit stays, increased mortality, low poor functional status at discharge [191].

While the Medical ICU (MICU) patients showed a higher population of monocytic and granulocytic MDSCs, granulocytic MDSCs (G-MDSCs) count was particularly prominent with sepsis patients. In addition, sepsis causes APCs to lose expression of the human leukocyte antigen-antigen D related (HLA-DR) and activating MHC type II. This molecule is associated with immune stimulation, and HLA-DR repression is associated with immunosuppression and is a predictor of unfavorable outcomes during sepsis [190]. This HLA-DR REPRESSION combined with the increased surface expression of inhibitory T-cell ligands by APCs, promote promotes the release of anti-inflammatory along with a directional skew towards immunosuppressive TH2 phenotypes

These immunosuppressive processes lead to the phagocytosis of apoptotic lymphocytes resulting in the release of anti-inflammatory cytokines such as IL-10 by APCs and the suppression of proinflammatory cytokine transcription [193]. These anti-inflammatory cytokines upgrade the macrophages into a type II phenotype to upregulate T-regs. This is followed by the upregulation of arginase 1 (Arg1), which depletes arginase one. The G-MDSCs of sepsis patients exhibited high ARG1 activity, along with an increased expression of degranulation markers [194]. Along with ARG1, iNOS expression further impairs intracellular signaling and further promotes the apoptosis of T-cells. Then, nitric oxide, which arises as a byproduct of iNOS, then interacts with ROS generated by MDSCs, produces proximities. These peroxynitrites are used to nitrosylate several cell-surface proteins on the lymphocyte and the cysteine residents. This makes the T-Cell

express decreased responsiveness and alters its IL-2 signaling. Furthermore, Nitric Oxide affects the stability of Il-2's mRNA, and ROS suppresses the functions of the natural killer (NK) cells. Finally, MDSCs induce T-regs and upregulate the programmed death ligand-1 (PD-L1) by initiating direct contact through the CD40 receptor, while other checkpoint inhibitors in MDSCs cause T-cell apoptosis [192].

Lymphopenia occurs due to both acute apoptosis of effector T and B lymphocytes during sepsis, as well as the HSC shift to myelopoiesis [192, 195]. Lymphocytes also undergo TH2 polarization as well as the expansion of Treg cells. Neutrophilia occurs, but these effector, immature myeloid cells are suboptimal because they have a decreased capacity for antigen presentation, expression of adhesion molecules, and formation of extracellular traps, as well as an altered pattern of expression of cytokines and chemokines [193], coupled with low lymphocyte counts in the early days of sepsis, elucidate early lymphopenia as a biomarker for immunosuppression during sepsis [194]. Animal models that inhibit the sepsis-induced apoptosis of lymphocytes have shown increased survival rates. This data suggests the potential for therapies that focus on the inhibition of lymphocyte apoptosis as a potential therapy for sepsis [195-203].

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CHAPTER 4

THE GENETICS OF SEPSIS

The role of genetics in sepsis was first elucidated about 30 years ago when a study found out that adopted children expressed a fivefold increase in mortality rates when their biological parents (but not their adopted parents) died from infectious disease by the age of 50 years [201]. Further studies on sepsis and mortality have revealed a genetic aspect of human resistance or susceptibility toward sepsis/trauma outcome [205-212]. Recent genome-wide association studies have implicated gene polymorphisms that affected sepsis outcomes in all chromosomes, including the X chromosome (ChrX) [213-216]. The presence of gene polymorphisms in ChrX suggests that there might be sex-related differences in sepsis-outcome sepsis outcomes between men and women. This hypothesis is referred to as the nonhormonal hypothesis and states that the difference in sepsis-related outcomes is based on the differential homeostasis of the sex chromosomes. It is well-known that adult women have more robust immune responses than adult men [205, 206-214, 217, 218]. These data are supported by lower mortality rates among female sepsis patients than their male counterparts by almost 70% (27% vs. 46%; $p = 0.048$) [219]. As compared with their male counterparts, women express lower serum concentrations of cytokines during periods of critical inflammation. Women also express lower TNF levels, a critical biomarker of sepsis, whereas, in men, TNF levels continuously increase [220]. Furthermore, women also express higher levels of TLR7, which promotes macrophage recruitment and phagocytic activity. Dendritic cells also have greater type 1 IFN activity

[221]. Women also recruit higher numbers of antibodies, B-cells, CD4+ T-cells, and express lower numbers of T-regs [208, 209].

ChrX is rich in immunomodulatory genes. In contrast, the Y chromosome carries genes that predominantly govern sexual attributes in men with limited immunomodulatory effects [224]. Men inherit one copy of ChrX, whereas women inherit two copies of ChrX. One of these copies of ChrX is randomly inactivated (suppressed by methylation) during early embryogenesis as a mechanism to prevent increased protein production due to having two copies of ChrX [225]. This results in genetic mosaicism, which is the co-existence of cellular populations with different genotypes. This condition, unique to women, is thought to result in sex-biased differences in cellular phenotypes, as shown in Figure 4.1 [205, 217, 224, 225]. Genetic polymorphisms of any of these ChrX immunomodulatory genes could potentially explain the sex-based immune responses seen in men and women.

Many of the genes that are involved in sepsis are also involved in cytokine production through NF-κB stimulation and production. Genes that are involved in TLR signaling and innate immunity are found on ChrX, including IRAK-1, TLR7, TLR8, Bruton's kinase, and inhibitor of kappa B kinase gamma (NEMO). Hence, it is thought that the polymorphisms in the ChrX-linked key gene IRAK-1 could impact the TLR–NF-κB pathway in men and women differently [226, 227].

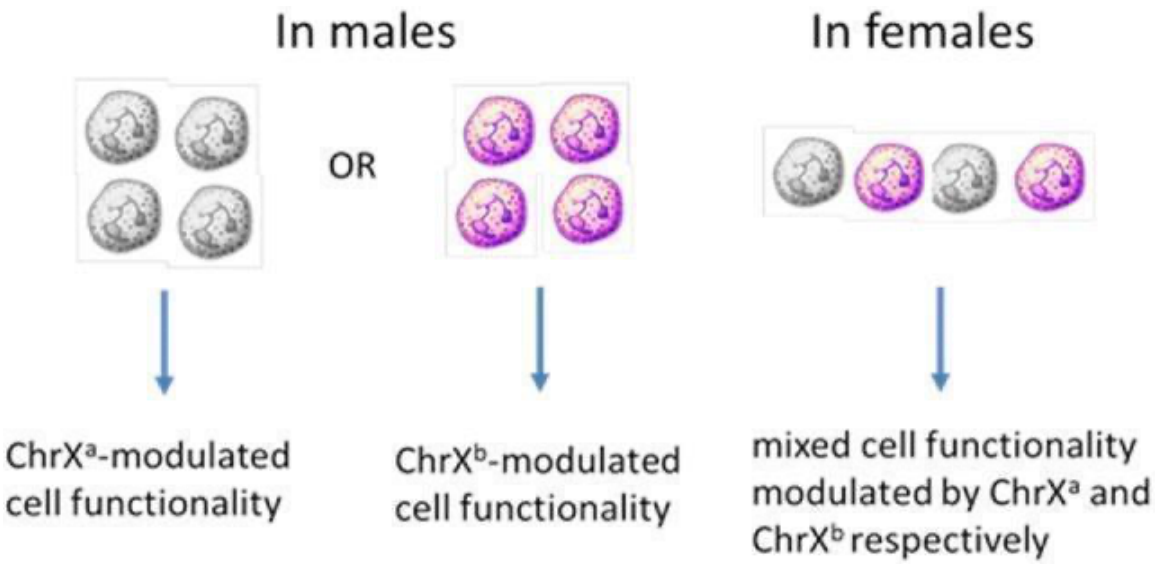

Figure 4.1 ChrX-induced cellular mosaicism as a result of protein variation induces phenotypic diversity in females and functional polarity in males.

Source: Spolarics, Z., Peña, G., Qin, Y., Donnelly, R. J., & Livingston, D. H. (2017). Inherent X-linked genetic variability and cellular mosaicism unique to females contribute to sex-related differences in the innate immune response. Frontiers in immunology, 8, 1455.

4.1 The Role of Gender in Sepsis

Genetic polymorphisms can play in the innate immune response may have far-reaching clinical effects that could be expressed in both haploid men and diploid women, as one copy of ChrX gets suppressed by methylation in early development leading to ChrX mosaicism in the latter. While women have equal levels of maternal and paternal ChrX inactivation (XCI ratio) in their blood, the expressions of ChrX mosaic cell subsets in the tissues and organ structures can sometimes be skewed [228, 229]. This skewed distribution of mosaic ChrX inactivated cell subsets can manifest itself at any age in healthy ChrX-heterozygotic women but often occurs more frequently in the elderly [229-231]. These skewed distributions are due to phenotypical and functional differences because of ChrX mosaicism. This mosaicism might manifest phenotypical dissimilarities between the subsets, which can lead to further ChrX skewing (conventionally, XCI ratio > 3) driven by

the unequivocal functional mechanisms of these mosaic subsets [231, 205]. For example, in heterozygotic women, differences in the functional pathologies between the mosaic stem cell subsets in the bone marrow [236] or mature WBCs at the periphery might further add to ChrX skewing [237].

During inflammation, acute ChrX cellular skewing toward a functional phenotype can occur as a product of differential cell trafficking and recruitment toward a site of injury or due to differential rates of necrosis, apoptosis, and cell proliferation driven by ChrX-linked allelic traits. This *de novo* ChrX-based cellular skewing in the blood is temporary and reversible, as the original cell ratio is reestablished following recovery from injury and after the inflammation dissipates [205].

However, sometimes an inflammatory response can result in irreversible and permanent skew in the bone marrow cells. This condition has been observed in mice-mosaic models of CYBB (gp91phox) and IRAK-1 under endotoxic stress and has shown permanent skewing in the blood and immune-competent organs. These studies have also revealed this skew often promotes increased survivability compared to homozygous wildtype animals [233–235]. This phenomenon is also observed frequently among healthy women or amongst women with severe phenotypically defective ChrX-linked proteins and is marked by increased populations of one mosaic subpopulations compared to the rest to the other subpopulations [60-62, 236-238]. Compared to men, this increased variability and flexibility often represents an advantageous condition especially under times of severe immune-pathophysiological conditions. Furthermore, ChrX skewing is often a confounder in sex-based or genetic association studies because it may mismatch the expected genotype-phenotype relationships. Therefore, studies investigating biological processes

associated with ChrX-linked genes need to test whether ChrX skewing occurs in heterozygous women and, if so, whether it is tilted toward or against the genetic variant of interest [205, 217, 239].

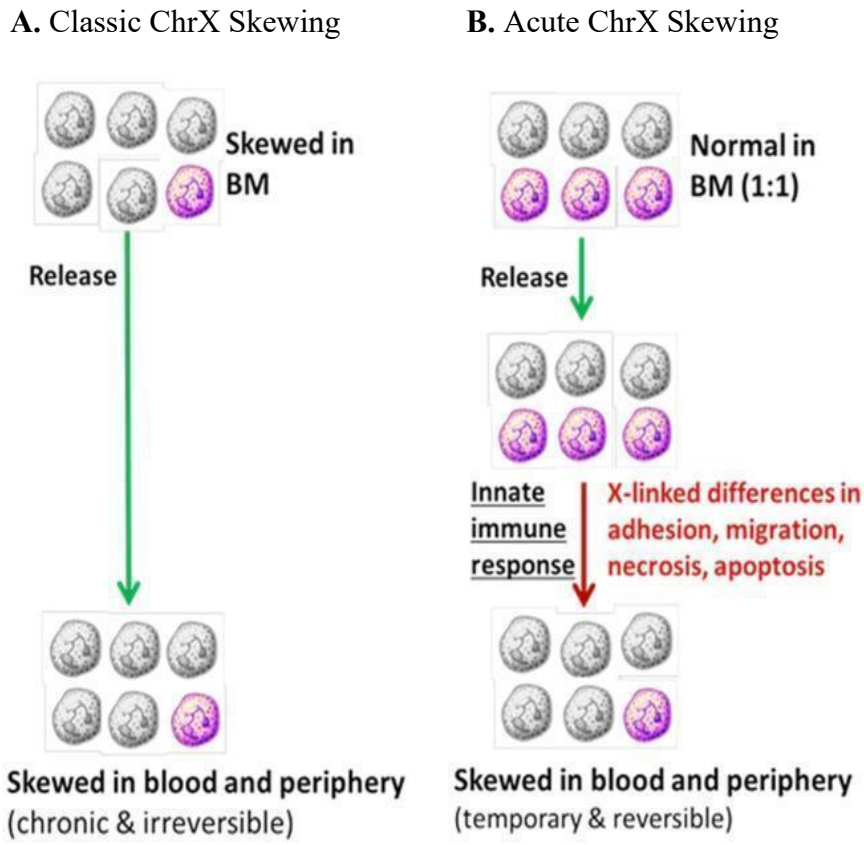

Figure 4.2 Cellular skewing can be temporary or permanent. When driven by primary changes in bone marrow progenitors can result in irreversible skewing (A) versus temporary mosaic skewing at the periphery driven by X-linked allelic variants (B).

Source: Spolarics, Z., Peña, G., Qin, Y., Donnelly, R. J., & Livingston, D. H. (2017). Inherent X-linked genetic variability and cellular mosaicism unique to females contribute to sex-related differences in the innate immune response. Frontiers in immunology, 8, 1455.

4.2 IRAK-1 Polymorphisms in Sepsis

Given the importance of IRAK-1 in immune regulation, the differential skewing of ChrX in heterozygous women can lead to significant phenotypic changes that may alter the course and the intensity of their immune responses. IRAK-1 exhibits differential messenger RNA splicing variants, IRAK-1B and IRAK-1C, which are formed due to improper splicing of exons 11 and 12. IRAK-1A is considered the correctly spliced version of IRAK-1. Meanwhile, the splice variant IRAK-1B expresses a shortened version of exon 12, whereas IRAK-1C completely lacks exon 11 (Fig. 4.3); overall, both these splice variants lack a functioning kinase domain. While the effects of both these splice variants are known, the full impact of a heterozygote with two different splice variants has not been elucidated yet [205, 217, 239].

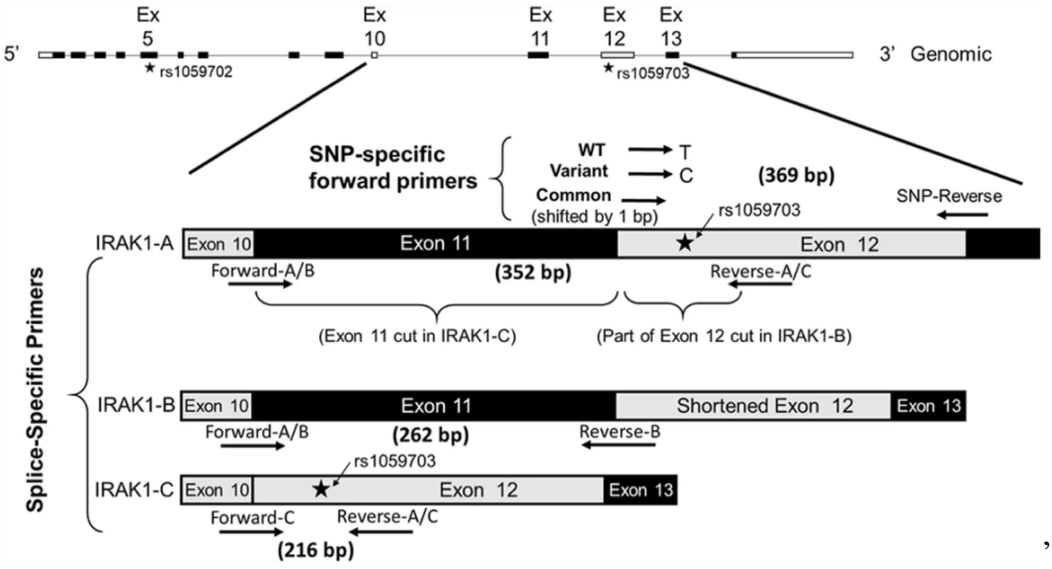

Figure 4.3 Graphical representation of IRAK-1 splice variants. The top boxes represent exons, and the lines represent introns of IRAK-1 of 5'-154.0200 to 3'-154.0105 Mb. The stars on Exon 12 represent the SNP rs1069703.

Source: Morcillo, P., Qin, Y., Peña, G., Mosenthal, A. C., Livingston, D. H., & Spolarics, Z. (2020). Directional X Chromosome Skewing of White Blood Cells from Subjects with Heterozygous Mosaicism for the Variant IRAK-1 Haplotype. Inflammation, 43(1), 370-381.

LPS stimulation and subsequent immunoblotting analysis of macrophages and dendritic cells revealed a decrease in IRAK-1A levels, suggesting protein degradation after activation, along with the induction of IRAK-1C expression. The data suggest that IRAK-1C might play a role as a pro-inflammatory TIR signaling regulator, like IRAK-M. These data further support a novel theory that cells could fine-tune downstream inflammatory cascades/responses. This is done by selectively depleting IRAK-1A and replacing it with a functionally defective kinase, IRAK-1C, which provides a novel insight into TIR signaling mechanisms [246].

While Rao et al. studied the role of the splice variant IRAK-1C, recent research conducted by Spolarics et al. suggested the existence of ChrX skewing at sites of the immune organs under inflammatory conditions. Furthermore, Morcillo et al. observed ChrX-based skewing in IRAK-1A/IRAK-1C heterozygous women toward leukocytes lacking any immunomodulatory capabilities. This condition promotes improved sepsis outcomes similar to as observed in relation to IRAK-1 deficiency [239, 246].

Given the importance of the IRAK-1 gene and the transcriptomic effects that IRAK-1C plays in reducing inflammation, IRAK-1C can be viewed as a potential alternative-splicing therapy. Furthermore, understanding the exact nature of IRAK-1C can lead to the production of highly precise inhibitors that can seek to inhibit IRAK-1A, preventing sepsis temporarily [246].

Apart from the splice variants, various single-nucleotide polymorphisms (SNPs) have been discovered in IRAK-1 that ensures significantly worse clinical outcomes during conditions of sepsis, trauma, cancer, and autoimmune diseases, like the SNP variant rs1059703 on exon 12, which is characterized by a thymine→ cytosine base pair change at

the 1674th base pair of exons 12, leading to the replacement of the amino acid leucine with serine at IRAK-1's kinase domain. This SNP is found only in the IRAK-1A and -1C splice variants, as IRAK-1B expresses a shortened form of exon 12 where the variant site is deleted, as shown in Fig. 4.3.

The rs1059703 SNP is widespread among various ethnic and racial groups in a 1:4 ratio, with 32% to 50% of heterozygotic women expressing WBC mosaicism for wildtype and variant alleles. Furthermore, this allele is found to exist in an almost perfect state of linkage-disequilibrium with five intronic SNPs ("rs3027898 T→G, rs731642 G→A, rs2239673 T→C, rs5945174 A→G, and rs7061789 A→G"), along with three more exon-based SNPs "rs1059701 C→T (synonymous), rs1059702 C→T (nonsynonymous), and rs1059703 T→C (nonsynonymous)" [247]. Even though these SNPs exist in near-perfect LDE with an R^2 value of almost 1.0, the importance of the rs1059703 haplotype, especially with the 532 L→S substitution, has been marked as a primary genotyping marker. Several studies have confirmed the marked negative impact of this haplotype, as it increases IRAK-1 phosphorylation rates, causing an increase in TLR-mediated NF-κB pathway activation, resulting in higher levels of pro-inflammatory cytokine transcription and release. Animal and human models of LPS-induced sepsis have shown the accumulation of leukocytes and APCs in organ systems, together with the increased expression of downstream pro-inflammatory cytokines and chemokines, owing to LPS-induced NF-κB translocation into the nucleus [248–252].

Further studies have identified and elucidated these LPS-induced NF-κB translocation patterns as critical biomarkers that enable the prediction of sepsis' clinical course. This prediction model has repeatedly proved vital, especially when assessing

patients who suffer from sepsis-induced ALI who express increased neutrophil levels. In these patients, LPS-induced ALI has also been associated with prolonged hospital stays, with extensive mechanical ventilatory support [253]. Patients with the variant IRAK-1 haplotype had higher 60-day mortality than wildtype patients (53% vs. 30%; $p = 0.05$) [253]. The rs1059703 SNP has also been implicated in causing a general increase in CRP levels, a marker of inflammation, and a predictor of cardiovascular risk [254, 255] in diabetic Caucasian women, along with high levels of hypertension.

While the SNP rs1059703 has been implicated as one with increased autoimmune effects, this SNP was also found to exert no effect on ALI, mortality rate, or ventilator-free days in the Han Chinese population. While authors have speculated that the increased prevalence of rs1059703 in the IRAK-1C allele in Han Chinese (87.5%) as compared with among Europeans (18.9%) might be the cause, this has not been confirmed yet [256]. This would be a crucial discovery that would confirm the role of IRAK-1C and the inflammation-induced skewing of ChrX cells among IRAK-1A/C heterozygotes as a crucial immunosuppression mechanism [257].

CHAPTER 5

METHODS OF DATA ANALYSIS

5.1 Source of Data

In order to study the transcriptional mechanisms of IRAK-1 and its splice variants and their combined effects with SNP rs1059703 mutant haplotype, we analyzed 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 [258].

Based on data from previous studies, the contributors Ferguson 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-1 mRNA levels [258].

5.2 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 Giga-base 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. These queries are oriented 5' to 3'. The four underlined bases at the 5' prime end represent Exon 12 in IRAK-1A and Exon 11 in IRAK-1C. The rs1059703 SNP at the 3' prime end is highlighted in bold. The combination of these two factors makes each oligonucleotide distinct.

Transcript	Query sequence
IRAK-1A WT	<u>CCCA</u>GGTGTACGAGAGGCTAGAGAAGCTGCAGGCAGTGT GGCGGGGGTGCCCGGGCATTT
IRAK-1A Var	<u>CCCA</u>GGTGTACGAGAGGCTAGAGAAGCTGCAGGCAGTGT GGCGGGGGTGCCCGGGCATT**C**
IRAK-1C WT	<u>ATCT</u>GGTGTACGAGAGGCTAGAGAAGCTGCAGGCAGTGT GGCGGGGGTGCCCGGGCATTT
IRAK-1C Var	<u>ATCT</u>GGTGTACGAGAGGCTAGAGAAGCTGCAGGCAGTGT GGCGGGGGTGCCCGGGCATT**C**

Source: Morcillo, P., Qin, Y., Peña, G., Mosenthal, A. C., Livingston, D. H., & Spolarics, Z. (2020). Directional X Chromosome Skewing of White Blood Cells from Subjects with Heterozygous Mosaicism for the Variant IRAK-1 Haplotype. Inflammation, 43(1), 370-381.

5.3 Statistical Processing and Data Analysis

All statistical analyses 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 [259]. To determine if there were significant differences between two groups of paired data, we performed a paired-T-test for parametric data [260] and a Wilkson-paired-T-test [261] for nonparametric data based on the Shapiro test results. Similarly, for unpaired and independent data, we conducted an independent t-test to compare two parametric samples and the Mann-Whitney U test for comparing two groups and the Kruskal-Wallis test while comparing more than

two groups with each other [261, 262]. When the Kruskal-Wallis test showed significance, we conducted a post hoc Mann-Whitney U test to visualize the differences between all the groups.

CHAPTER 6

RESULTS

6.1 Data Demographics

The dataset reported by Ferguson & Xue contains 30 samples from 15 patients reporting the SRA transcript sequences before and after endotoxin challenges. This dataset has data from 8 males and 7 females, with Caucasian (n = 9) and African American (n = 6) ethnic backgrounds. The authors had also categorized these patients into two groups of high and low inflammation, based upon the participant's previous sensitivity to the endotoxin challenge and the magnitude of their immune responses [255].

We used the search queries from table 5.1 with the SRA blast algorithm to determine the genotypes of the participants in this dataset and reported the results in Table 6.1. Our findings report the presence of 5 different genotypes expressed among the population. The genotypes reported are homozygotes for IRAK-1 (WT), IRAK-1 homozygotes expressing the rs1059703 SNP mutation, termed as "IRAK-1 (Var)", along with heterozygotes with IRAK-1 (WT) / IRAK-1 (Var) genotypes. The counts are reported in the table. Males are counted for this study as homozygotes despite the fact that they are (by definition) hemizygous.

Table 6.1 Demographics of the Sample Dataset

	Males (n)	**Females (n)**	**Total**
Total Participants	8	7	15
Ethnicity			
Caucasian	6	3	9
African American	2	4	6
Inflammation response:			
High	4	4	8
Low	4	3	7
By Genotypes:			
Homozygotes			11
IRAK-1 (WT) Homozygote	5	2	7
IRAK-1 (Var) Homozygote	3	1	4
Total Heterozygotes (WT/VAR)	-	4	4

6.2 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, we compared the total IRAK-1 expression levels before (Median = 2.7) and after the LPS (Median = 1.8) treatment. We tested for normalcy using the Shapiro-Wilk test, which showed that the data was not normally distributed. Hence, we conducted nonparametric tests. The Wilcoxon paired test indicated that there was a significant difference between the median of the two groups with p= 0.005 (Fig. 6.1A).

We performed a linear regression analysis with the IRAK-1 levels before LPS treatment against the expression levels after LPS administration to see if the decrease in IRAK-1 level is proportional to IRAK-1's baseline levels. The linear model had an R^2 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. As expected for lower levels after the LPS treatment, the slope was less than 1.

A.

B.

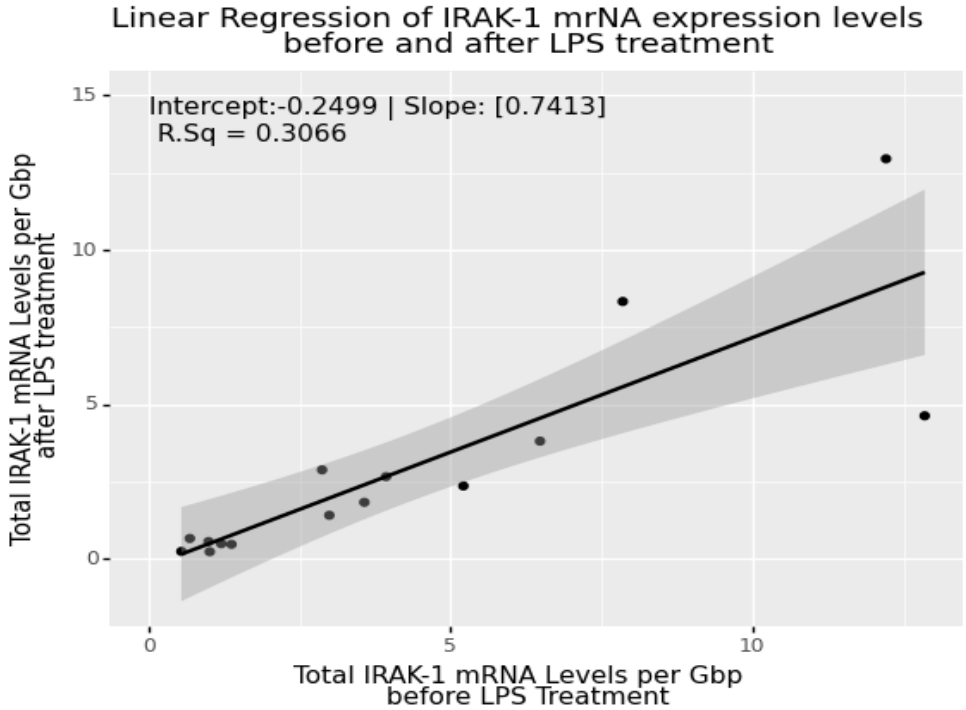

Figure 6.1 LPS administration reduces IRAK-1A expression levels. (A) Boxplot shows the overall IRAK-1A expression levels before and after LPS treatment. (B) Linear regression graph of IRAK-1 expression levels for baseline vs. control.

6.3 Exposure to LPS Does Not Elicit Any Significant Changes in IRAK-1C mRNA Levels

To investigate the effects of IRAK-1 splice variants on IRAK-1's expression levels, we compared IRAK-1A and IRAK-1C expression levels before and after LPS treatment. We used the Wilcoxon signed-rank test to assess the impact of LPS on IRAK-1A and IRAK-1C levels (Fig. 6.2 A & B). Our results indicated a significant decrease in IRAK-1A levels ($p=0.006$) following administration of LPS (Median = 1.74, SD = 3.52) compared to baseline (Median = 2.93, SD = 3.92) conditions. Furthermore, we did not find any significant differences in the IRAK-1C levels ($p = 0.7$) between baseline (Median = 0.06, SD = 0.13) and LPS conditions (Median = 0.04, SD = 0.08).

We conducted linear regression analyses on IRAK-1A and IRAK-1C levels to determine if IRAK-1A and IRAK-1C levels after LPS treatment are dependent on their initial levels. (Fig. 6.3). The IRAK-1A model had an R^2 value of 0.31 and a slope of 0.76, with an intercept of -0.34. The IRAK-1C model had an R^2 value of 0.0016, with an intercept of 0.73 and a slope of -0.13. This data suggests that only the splice variant IRAK-1A's expression is affected by endotoxin challenge, and IRAK-1C levels are not affected by endotoxins and/or are too low to measure and make accurate inferences.

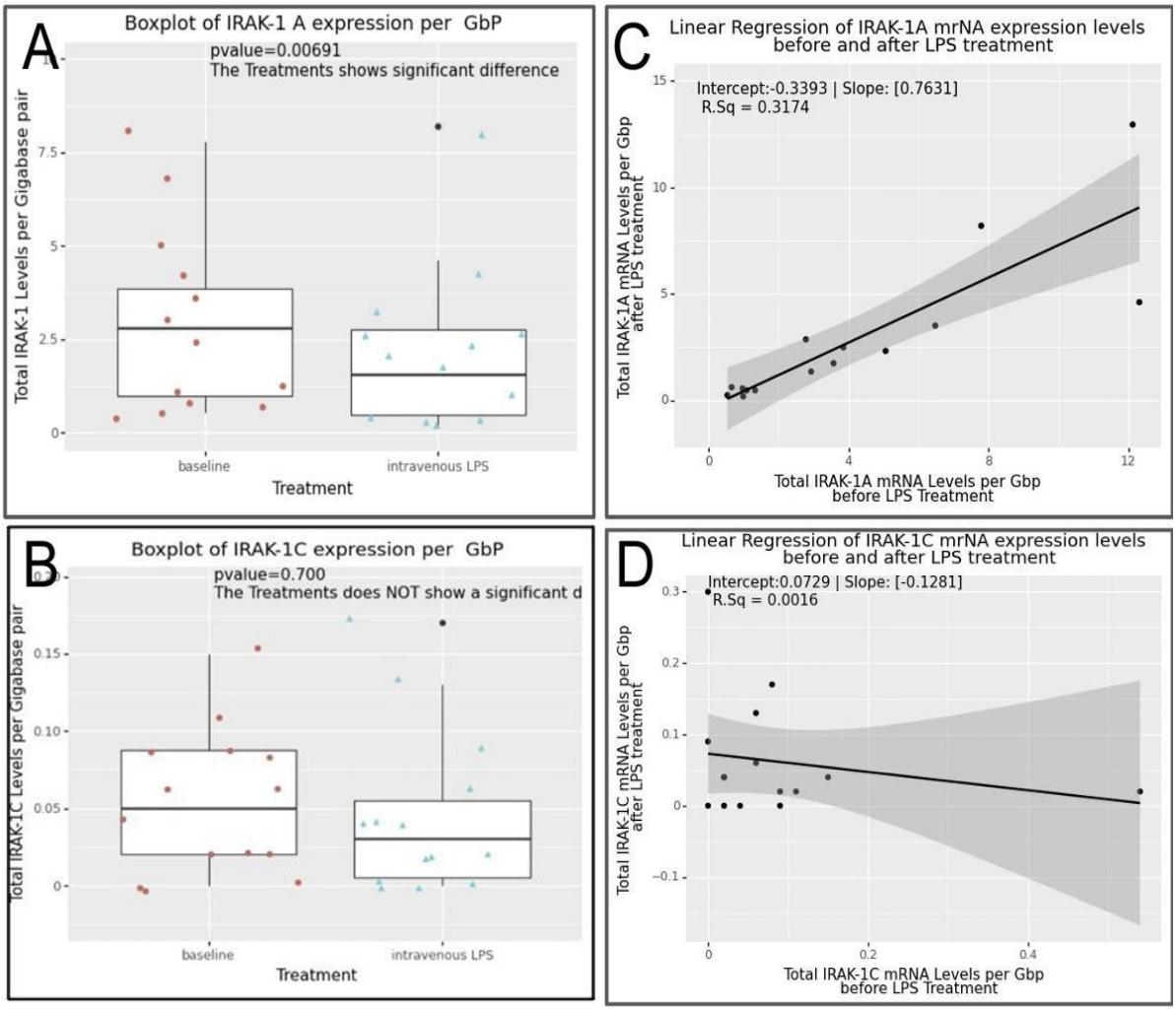

Figure 6.2 Changes in IRAK-1 levels are due to a decrease in IRAK-1A levels. While IRAK-1A levels decrease upon LPS treatment (A) whereas, IRAK-1C expression does not (B). Linear regression of IRAK-1A (C) and IRAK-1C levels (D) for control vs. LPS.

6.4 The Role of IRAK-1 in Hyperinflammation

A variety of studies have sought to understand the dynamics of the TLR-NF-κB 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 critical mediator in regulating the intensity of endotoxin challenges. High levels of IRAK-1 expression have been associated with increased pro-inflammatory activity, whereas studies on LPS-tolerance in human monocytes revealed unaltered TLR4 expression but suppressed MyD88-TLR4 and IRAK-1-MyD88 interactions, IRAK-1 activation. This might be due to a regulatory mechanism that is induced by miRNA-146A.

In order to compare the differences in IRAK-1 mRNA expression between groups with low and high inflammation levels, we used the paired t-test to check for significant differences among the means for IRAK-1A, IRAK-1C, and the total IRAK-1 expression levels. The Paired T-tests indicated a significant decrease in the IRAK-1 expression following LPS treatment in both the groups. The group with low inflammation had a $p = 0.03$ (Baseline: Mean = 2.23, SD = 1.81 vs LPS: Mean = 1.42, SD =1.66) and the group with high inflammation reported a $p = 0.0053$ (Baseline: Mean = 6.02, SD = 4.66 vs LPS: Mean = 4.21, SD = 4.22). As IRAK-1A levels are primarily responsible for changes in the total IRAK-1 levels, it also showed significant differences in both high ($p=0.01$, Baseline: Mean = 5.91, SD = 4.55 vs LPS: Mean = 4.12, SD = 4.42) and low inflammation groups ($p=0.01$, Baseline: Mean = 2.17, SD = 1.76). Similar to our previous results, IRAK-1C failed to show any significant differences for neither the high ($p =0.86$ Baseline: Mean =0.11, SD = 0.178 vs LPS: Mean = 0.088, SD = 0.097) or the low inflammation groups (p

= 0.85) Baseline: Mean = 0.57, SD = 0.052 vs LPS: Mean =0.038, SD = 0.060). (Refer Table 6.2 for detailed descriptive statistics)

6.4.1 Increased Baseline IRAK-1 Expression Levels Before Endotoxin-Induced Stress Are Associated with Hyperinflammation

To quantitatively analyze and understand the impact of IRAK-1 in endotoxin sensitivity, we compared IRAK-1 levels before and after the administration of LPS between two groups of low and high inflammation. Our results indicated the high inflammation group (Median = 6.02, SD = 4.66), compared to the low inflammation Group (Median = 2.23, SD = 1.81), had higher levels of IRAK-1, with p = 0.036. We observed a similar trend in IRAK-1A as well, where the high inflammation group (Median = 5.91, SD = 4.55) expressed increased IRAK-1A levels with a similar p-value of 0.036. However, when we performed the nonparametric Mann-Whitney test, we did not see any significant differences between the IRAK-1 levels of the low (Median = 0.66, SD = 1.16) and high (Median = 2.82, SD = 4.42) inflammation groups, with p = 0.136. IRAK-1A followed suit with p=0.138 for IRAK-1A levels between high (Median = 2.625, SD = 4.42) vs. low (Median = 0.620, SD = 1.13). Consistent with previous tests, IRAK-1C did not exhibit any significant changes (Fig. 6.3).

Table 6.2 Descriptive Statistics of IRAK-1 Expression Levels Before and After LPS Treatment for High and Low Inflammation Groups

	Treatment	Response Group	Mean	Median	Std Dev	Variance
IRAK-1A	Baseline	High	5.91	5.025	4.554	0.480
		Low	2.17	1.330	1.762	0.811
	Intravenous LPS	High	4.128	2.625	4.429	19.619
		Low	1.366	0.620	1.132	1.280
IRAK-1C	Baseline	High	0.110	0.06	0.178	0.032
		Low	0.057	0.04	0.053	0.004
	Intravenous LPS	High	0.083	0.05	0.098	0.009
		Low	0.039	0.02	0.061	0.003
IRAK-1 Total	Baseline	High	6.020	5.025	4.660	21.720
		Low	2.227	1.370	1.812	3.285
	Intravenous LPS	High	4.210	2.82	4.422	19.554
		Low	1.404	0.66	1.166	1.360

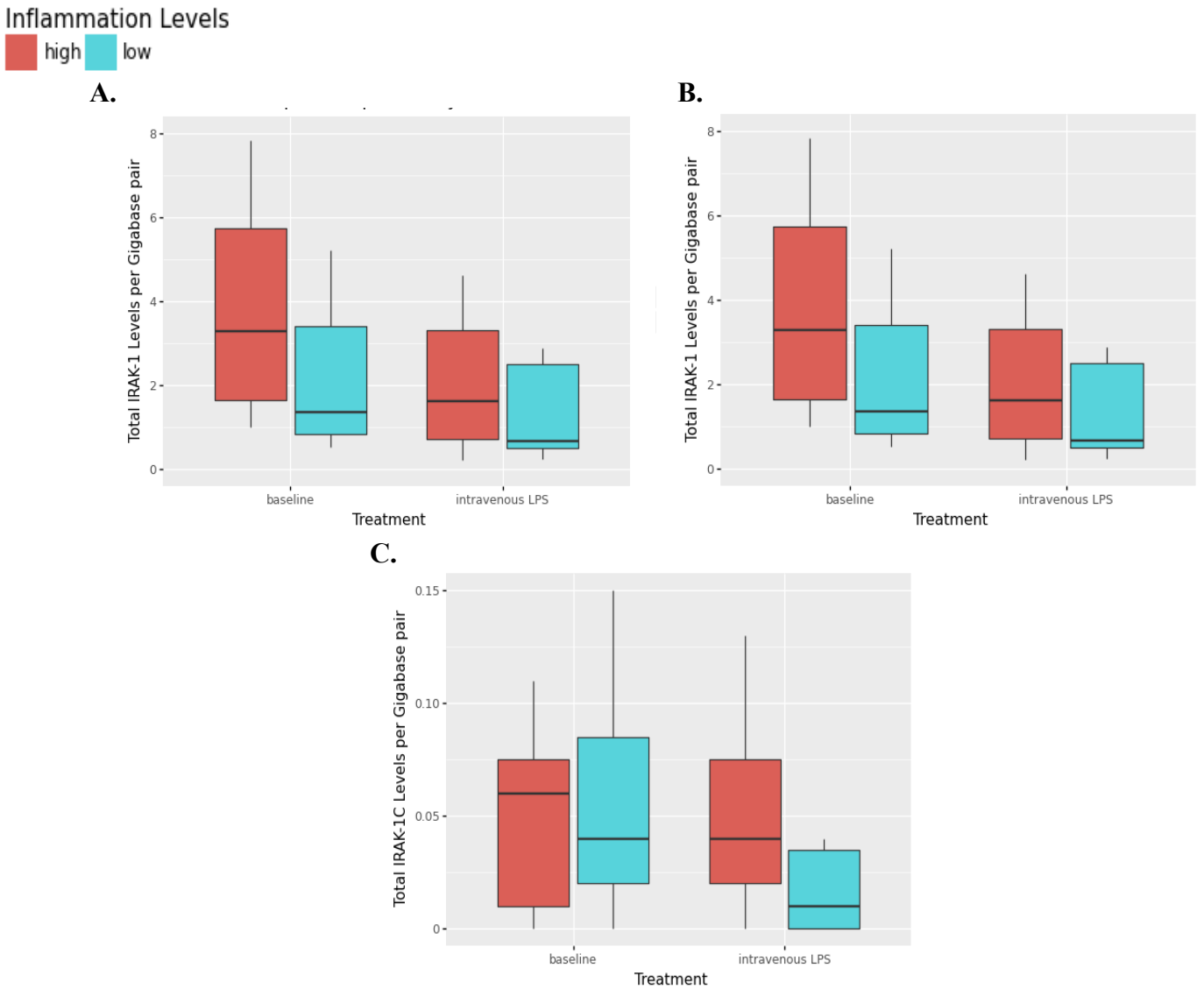

Figure 6.3 Patients with high inflammatory levels express higher IRAK-1 and IRAK-1A expression before LPS treatment. (A) There is a significant decrease in IRAK-1 (A) and IRAK-1A (B) following LPS treatment in expression levels. (C). There is no difference in IRAK-1C expression levels.

We hypothesized that this might be due to increased phosphorylation of IRAK-1A, promoting inflammation via cytokine release. Hence, we compared the changes elicited of LPS-induced toxicity between high and low inflammation groups by comparing the differences in IRAK-1A, IRAK-1C, and the total IRAK-1 levels (Table 6.3). Surprisingly, there were no significant differences in IRAK-1 levels p=0.36 in the changes elicited upon the IRAK-1 levels between the low (Median = 0.01, SD = 0.234) and High inflammation (Median = 0.1, SD = 0.06) groups.

Table 6.3 Descriptive Statistics of Changes in IRAK-1 Expression Following LPS Treatment

	Inflammatory response	**Mean**	**Median**	**Std Dev**	**Variance**
IRAK-1A	High	-1.810	-1.18	2.826	7.986
	Low	-0.823	-0.43	1.007	1.014
IRAK-1C	High	-1.783	-1.19	2.684	7.203
	Low	-0.804	-0.41	0.986	0.972
IRAK-1 (Total)	High	-0.028	0.01	0.234	0.055
	Low	-0.019	-0.02	0.065	0.004

6.5 The Role of IRAK-1C in Hyperinflammation

Past studies have shown that the rs1059703 SNP variant is linked to unfavorable outcomes in sepsis. In order to understand the combined effects of the splice variant along with the SNPs on inflammation, we compared the changes in the overall IRAK-1 expression levels for the WT or Var homozygotes and the WT/Var heterozygotes (Fig. 6.4).

As the rs1059703 SNP variant is associated with increased inflammation, and the IRAK-1C splice variant is a dominant-negative variant that suppresses inhibition, we hypothesized that there would be a significant difference in the total IRAK-1 levels between the homozygous IRAK-1 variants and both the homozygous IRAK-1 (WT) and the heterozygote groups. The results of the Kruskal-Wallis test ($F = 13.429$, $p = 0.001$) show that there was at least a significant difference in at least one of the groups. Consistent with our hypothesis, the post hoc Mann-Whitney test showed that there was a significant difference) between the homozygous variants (Median = 8.09, SD = 4.045) and the heterozygotes (median = 2.035, SD = 3.214) with $p = 0.003$. We also noticed a significant difference ($p = 0.005$) between the homozygous variants and the homozygous WTs (median = 1.180, SD = 2.025). However, we did not notice any differences between the homozygous WTs and heterozygotes.

We also noticed a significant difference between the three groups when we compared the IRAK-1 levels before ($F = 7.525$, $p = 0.023$) and after LPS treatment ($F = 6.225$, $p = 0.044$). The results from the post hoc Mann-Whitney test before the LPS treatment were similar to the results from the overall data and are shown in Figure 6.5. We noticed that there were significant differences between the homozygous variants and the heterozygotes (median = 2.035, SD = 3.215) and also between the homozygous variants (median = 8.090, SD = 4.045) and WTs (median = 1.370, SD = 2.109) before LPS treatment (Fig. 6.5A). This data suggests an increased IRAK-1 expression prior to LPS administration among the homozygous variants and is consistent with previous findings implicating SNP rs1059703 in inflammation.

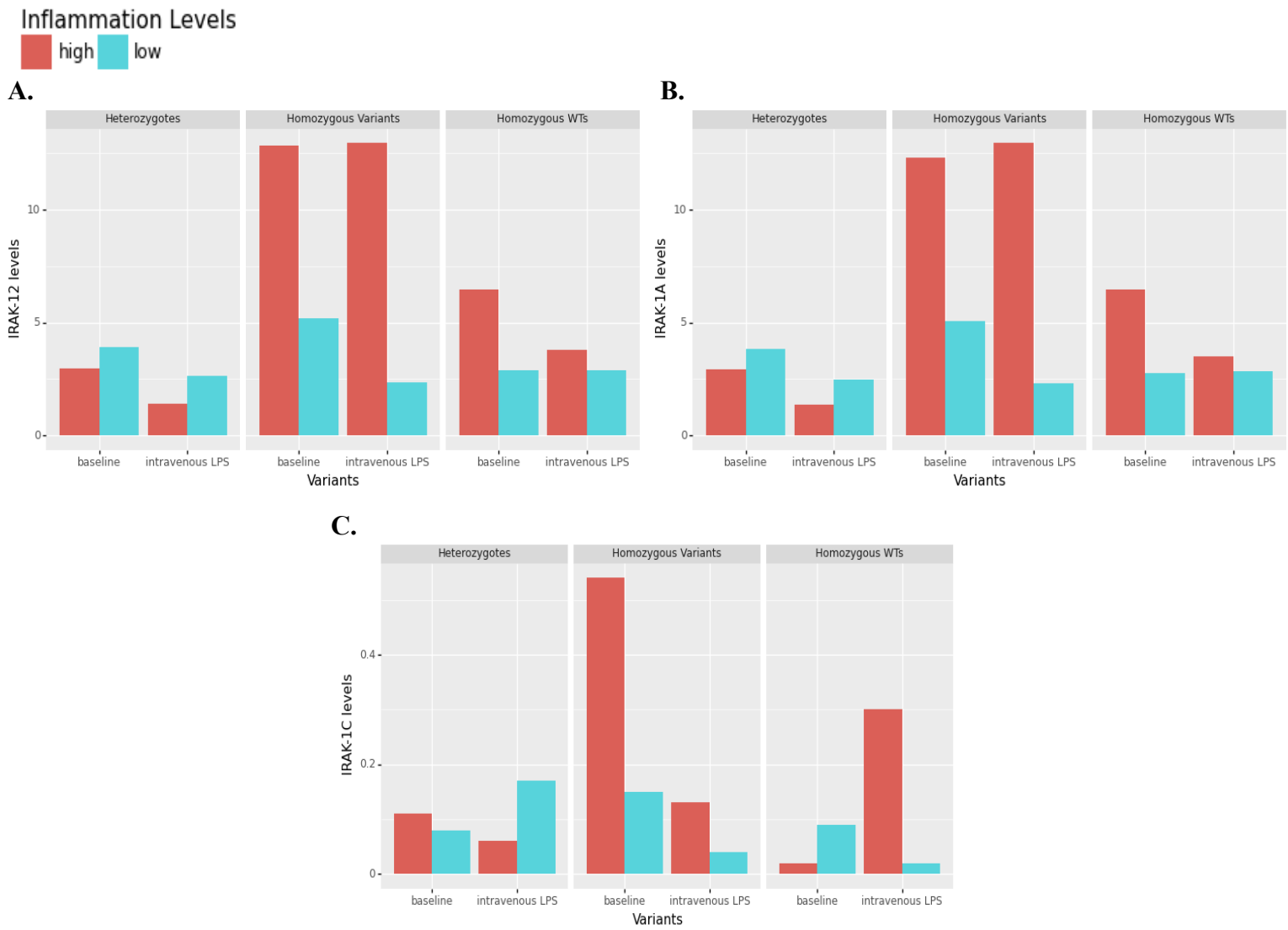

Figure 6.4 Comparative expression of IRAK-1 and its splice variants between Homozygous WTs, Homozygous Variants and Heterozygotes. (A) IRAK-1, (B) IRAK-1A and (C) IRAK-1C expression between Homozygous WTs, Homozygous Variants and Heterozygotes.

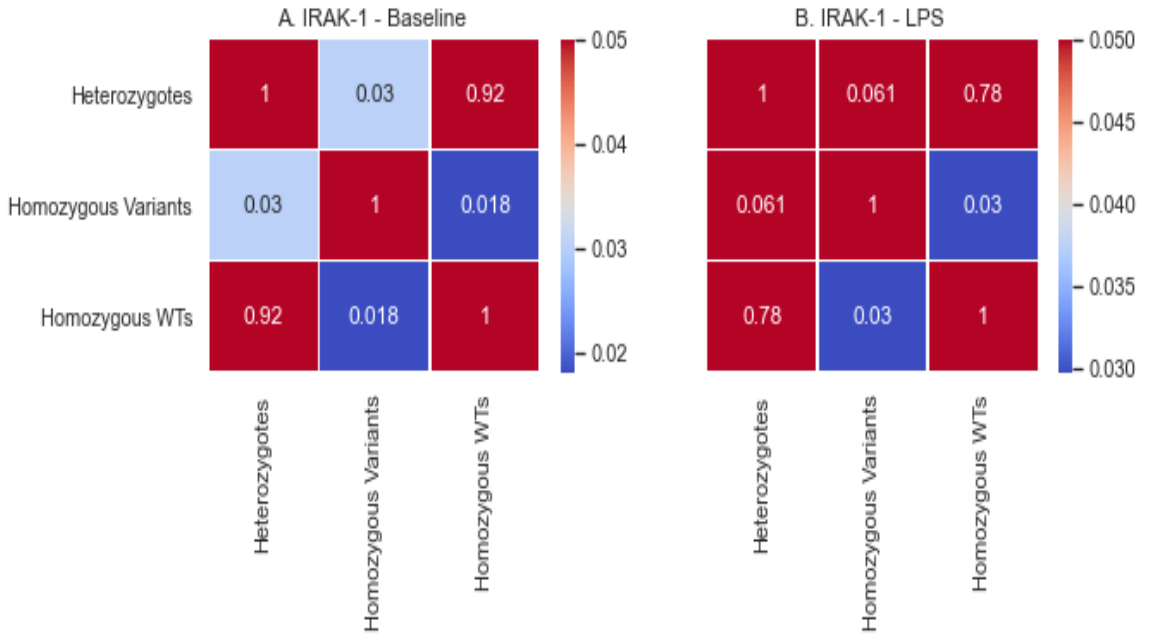

Figure 6.5 Heat map analysis of the IRAK-1 levels from the Kruskal-Wallis test followed by the post hoc Mann-Whitney ($p < 0.05$) between Homozygous WTs, variants, and the Heterozygotes (WT/Var) before (A) and after LPS treatment (B).

Furthermore, we also noticed a significant difference between the three groups when we compared the IRAK-1 levels in groups of low inflammation ($F = 10.250$, $p = 0.005$) but not amongst patients with high inflammation and after LPS treatment ($F = 6.225$, $p = 0.044$). The results from the post hoc Mann-Whitney test for patients with low inflammation showed a significant difference between the homozygous variants (median = 10.270, SD = 3.398) and heterozygotes (median = 1.305, SD = 1.055) ($p = 0.028$), between the homozygous variants and the homozygous WTs (median = 2.700, SD = 2.276) ($p = 0.024$). However, we did not find any significant differences between the heterozygotes and the homozygous WTs ($p = 0.45$).

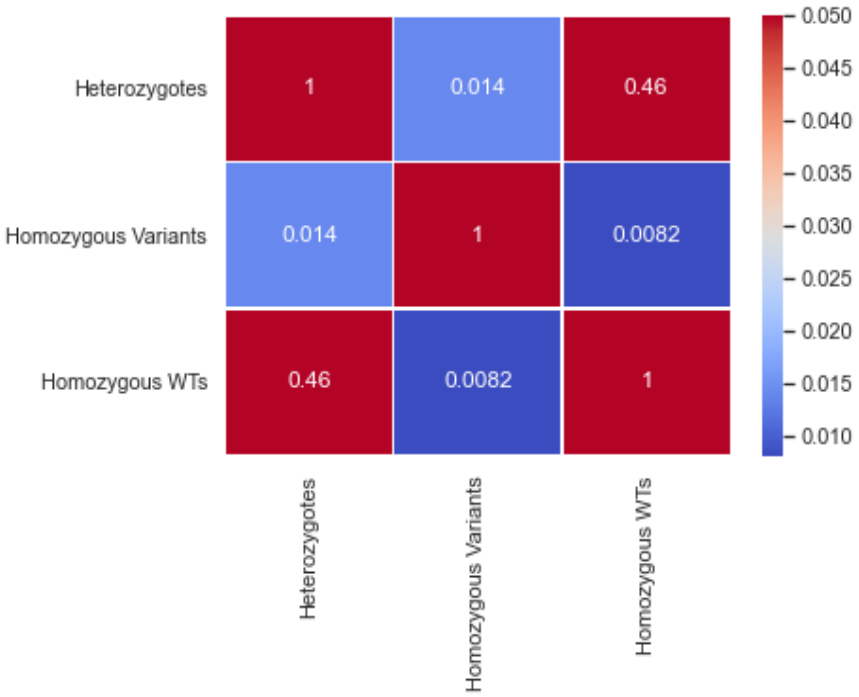

Figure 6.6 Heat map analysis of the IRAK-1A from the Kruskal-Wallis test followed by the post hoc Mann-Whitney ($p < 0.05$) between Homozygous WTs, variants, and the Heterozygotes (WT/Var) for patients with low inflammation.

As the SNP the rs1059703 is implicated with increased inflammation, we compared the IRAK-1A (Var) levels amongst the groups. The results from our Kruskal-Wallis analysis show that there were significant differences in the overall IRAK-1C levels between the heterozygotes and the homozygous variants groups before ($p = 0.015$) and after ($p = 0.015$) LPS treatment. We find an increased expression of IRAK-1A (Var) in homozygous variants before (median = 9.945, SD = 3.528) and after LPS treatment (median = 6.395, SD = 4.633) when compared to the heterozygotes (Baseline: median = 1.000, SD = 0.907; LPS: median = 0.655, SD = 0.615). Furthermore, we did not detect a significant difference in the IRAK-1A (Var) between the homozygous variants and the heterozygote groups in patients with low ($p = 0.468$) or high levels of inflammation ($p = 0.053$).

In order to investigate the effects of the rs1059703 SNP on the IRAK-1C splice variant, we compared the IRAK-1C, IRAK-1C (WT), and IRAK-1C (Var) levels between the three groups. Based on the results, we did not notice a significant difference in the overall IRAK-1C levels between the three groups ($F = 5.490$, $p = 0.064$). We were able to find a significant difference when we compared the IRAK-1C levels before and after LPS treatment, we found a significant difference in the IRAK-1C levels before LPS treatment ($F = 7.300$, $p = 0.026$) and we did not find any significant differences in the IRAK-1C levels post-LPS-treatment ($F = 1.167$, $p = 0.55$). Post hoc Mann-Whitney analysis of IRAK-1 levels before LPS treatment show that there was a significant difference between the homozygous WTs (median = 0.02, SD = 0.32) and the homozygous variants (median = 0.075, SD = 0.056) ($p = 0.024$). We did not find any difference between the heterozygotes (median = 0.05, SD = 0.136) and the homozygous WTs ($p = 0.101$) and between the homozygous variants and the heterozygotes ($p = 0.245$). We further investigated this by comparing the IRAK-1C (WT) and the IRAK-1C (Var) levels between the three groups.

When we compared the IRAK-1C (WT) levels before and after LPS treatment between the three groups, we did not find any significant differences in the IRAK-1C (WT) levels between the three groups before ($F = 3.248$, $p = 0.197$) or after LPS treatment ($F = 3.972$, $p = 0.137$) low. While we did not find any difference amongst the three groups in patients with high inflammation ($F = 1.498$, $p = 0.473$), we found significant differences amongst patients with low inflammation ($F = 7.395$, $p = 0.025$). The results from the post hoc Mann-Whitney analysis are shown in Figure 6.7.

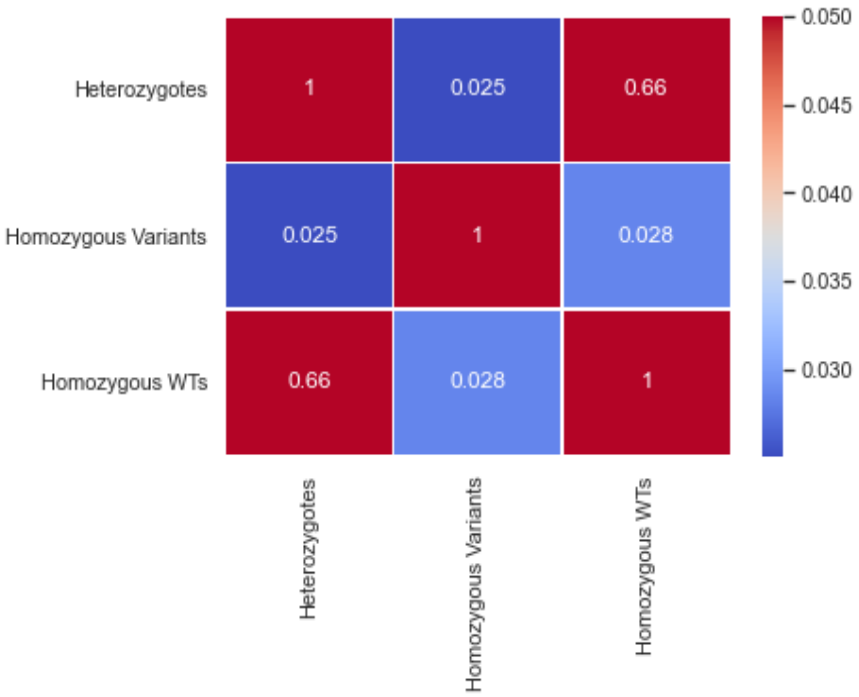

Figure 6.7 Heat map analysis of the IRAK-1C (WT) from the Kruskal-Wallis test followed by the post hoc Mann-Whitney ($p < 0.05$) between Homozygous WTs, variants, and the Heterozygotes (WT/Var) patients with low inflammation.

To understand the impact of the IRAK-1C splice variant with the rs1059703 SNP on inflammation, we compared the levels of IRAK-1C (Var) before and after LPS treatment for heterozygotes and homozygous variants. However, we did not find any significant differences between the heterozygotes and homozygous variants before ($p = 0.055$) and after ($p = 0.5$) LPS treatment. Furthermore, we also did not find any significant differences between the two groups amongst patients of low ($p = 0.226$) or high ($p = 0.308$) levels of inflammation. Thus, our data finds IRAK-1C with the rs1059703 mutation does not play a significant role in inflammation.

CHAPTER 7

DISCUSSION

7.1 Analysis of Results

Sepsis is a complex, life-threatening condition that arises from a severely dysregulated immune response to infection. This dysregulated immune response can lead to a state of simultaneous chronic inflammation, and immunosuppression termed PICS, which can lead to multiple organ failure resulting in death.

Innate immunity is primarily mediated by toll-like receptors. These receptors recognize pathogen-associated molecular patterns and initiate inflammatory responses primarily through the TLR-NF-κB pathway. This pathway is mediated mainly by the IRAK family of kinases, which are crucial mediators of inflammation. IRAKs play a multifunctional role in IL-1R signaling, TLR-based PAMP recognition, and the propagation of downstream-inflammatory signals via the NF-κB pathway. NF-κB has been identified as a critical propagator in inflammation due to its role in initiating the transcription of inflammatory proteins and cytokines [11-16]. There have been extensive studies linking the expression levels of both NF-κB and IRAK-1 to sepsis. Increased levels of IRAK-1 have been associated with increased inflammation due to an overproduction of inflammatory cytokines through the NF-κB pathway.

Past studies have implicated a correlation between IRAK-1 and NF-κB pathways in sepsis. Past studies indicate an increased activation of NF-κB in alveolar macrophages, PBMCs, and neutrophils in sepsis patients. Increased expression of NF-κB is also correlated with increased levels of downstream pro-inflammatory cytokines such as TNF,

IL-1, IL-6, IL-8, and IL-12. These pro-inflammatory cytokines are crucial mediators of immune responses and are responsible for the recruitment of leukocytes to the site of infection. Furthermore, they also promote vasodilation and increase the permeability of the endothelial layers at the site of the infection to enable the extravasation of leukocytes into the site of the injury.

While the expression of these pro-inflammatory cytokines is tightly regulated under normal conditions, sepsis causes an overwhelming and extremely dysregulated immune response that causes an overwhelming release of pro-inflammatory cytokines that lead to adverse effects such as endothelial dysfunction and damage to the peripheral tissues by promoting vasodilation and vasopermeability of the endothelial layers. This results in the leakage of cytokine-rich fluid into the periphery, causing edema and compromising the vascular volume and blood pressure. This comprises the overall blood circulation resulting in hypoxia. Furthermore, the increased cytokine activity from the increased cytokine activity promotes the recruitment of leukocytes and their extravasation into the peripheral tissues while stimulating them to produce secondary pro-inflammatory mediators. This leukocyte accumulation coupled with the lack of a functional endothelial layer allows pathogens to invade deeper into the host's tissues, resulting in the positive feedback loop that promotes hyper inflammation and tissue damage that can potentially disturb homeostasis mechanisms and ultimately cause dysfunction of remote organs, through the combined and reciprocal actions of inflammation and hypoxia [103].

A plethora of studies has shown that IRAK-1 and NF-κB levels correlate with crucial markers of sepsis, and sepsis-induced MODs like myocardial depression (CRP), ARDS (neutrophils), and Ischemic Hepatitis (CRP), confirming their roles as critical

moderators of inflammation and sepsis-based multi-organ failure. This makes IRAK-1 a crucial regulator of inflammation.

Upon its activation by TIRs, IRAK-1 undergoes phosphorylation and is degraded by the ubiquitin-proteasome pathway [12]. Current results suggest a significant decrease in IRAK-1 mRNA expression levels following administration of intravenous LPS (Fig. 6.1A). However, subsequent results show a linear correlation between total IRAK-1 levels before and after the administration of LPS (Fig. 6.2), suggesting that the initial levels of IRAK-1 before exposure to PAMPs/DAMPs determine the levels IRAK-1 after the LPS treatment. IRAK-1 levels are primarily dependent on the levels of inflammation. While this model has a poor fit, most of the variation in Figure 6.1 is explained by changes in the expression levels of IRAK-1A, the wildtype splice variant levels, as illustrated in Figure 6.2C.

On the other hand, there are hardly any changes in the levels of IRAK-1C. IRAK-1C fails to contribute towards changes in the overall IRAK-1 Levels. This splice Variant has been associated with significantly lower neutrophils extravasation in ARDS and minimal levels of NF-κB and TNF activation. This splice variant is a dominant-negative and has been proposed a role that is similar to IRAK-M. It is thought to be a novel regulator of inflammation that prevents the conduction of downstream inflammatory signals. Consistent with their findings, we also fail to report significant changes in IRAK-1C levels upon LPS administration [246] (Fig. 6.2 B, D).

To investigate the role of IRAK-1 and its splice variants in endotoxin-sensitivity and hyperinflammatory responses, we compared the levels of IRAK-1 and its splice variants before and after LPS treatment among groups of high and low inflammation.

While there were no significant changes in IRAK-1-C, IRAK-1A levels and the overall IRAK-1 levels did vary significantly. When we compared the levels of IRAK-1, IRAK-A, and IRAK-1C levels before and after LPS treatment, between groups of high and low inflammation, we noticed that while patients with high inflammation expressed higher levels of IRAK-1 and IRAK-1A mRNA, before LPS treatment, we failed to notice any significant differences in the IRAK-1 and the IRAK-1A after the administration of LPS between the groups of high and inflammation. These results suggest that the magnitude of inflammation depends on IRAK-1 levels before the endotoxin challenge and is consistent with previous studies on IRAK-1 and LPS tolerance.

Past studies have implicated a decrease in the IRAK-1 mRNA levels with increases in microRNA-146a (miRNA-146a) expression. This miRNA is produced as a result of NF-κB and hinders IRAK-1 mRNA expression through the RNA-interference pathway [263]. Past studies show that the miRNA-146a levels are continuously elevated for 24 hours in T-helper cells following LPS administration. However, the exact role and mechanisms of miRNA146 and its relationship with TLR signaling has not been elucidated yet [264, 265].

When compared to adult men, women generally express more robust and regulated immune responses [205, 207-209, 217, 218]. Past studies have shown that women patients with sepsis express significantly lower mortality rates by almost 70% compared to their male counterparts [220]. This is thought to be a result of ChrX skewing. ChrX is rich in immuno-modulatory genes such as IRAK-1, TLR-4, TLR-7, TLR-8, NF-κB, and TNF. As men only inherit one copy of ChrX, any mutations or alterations to the ChrX genes can have disastrous consequences. However, in females, one of the copies of ChrX is randomly repressed to prevent increased protein production, resulting in genetic mosaicism [205].

Recent research by Morcillo et al., [266] and Spolarics et al., [205] have shown a directional skewing among IRAK-1A/ IRAK-1C heterozygous that results in up-regulation of WBCs and immune cells expressing IRAK-1-C. This skew might explain why women express healthier and more balanced inflammatory responses than men under conditions of persistent inflammation. These studies are consistent with Rao et al.'s [246] findings on IRAK-1C that proposes an increase in IRAK-1C to IRAK-1A expression, thereby obstructing the activation of downstream inflammatory cascades.

In order to investigate the effects of IRAK-1 splice variants and the SNP rs1059703 on inflammation, we compared the levels of total IRAK-1 expression between the homozygous WTs, homozygous variants, and heterozygotes expressing both WT/Var. Our analysis indicated no significant differences in the overall IRAK-1and IRAK-1 expression levels between the homozygous WTs and heterozygous variants. However, the homozygous variants expressed significantly higher levels of IRAK-1 and IRAK-1A compared to both the homozygous WTs and variants. This data suggests the inhibitory role of IRAK-1C splice variants, despite the presence of rs1059703 amongst some of the heterozygotes.

When we compared the levels of IRAK-1A (Var) in sepsis, we noticed extremely high levels of IRAK-1A (Var) before and after LPS treatment. To our surprise, we did not find any significant differences in the IRAK-1A (Var) levels following LPS treatment. Previous studies show no significant differences in the levels of miRNA-146a between the IRAK-1 WTs and the IRAK-1 Variants. However, this SNP associated is an increase in the NF-κB levels [264-265]. In lieu of current findings, we think that there might the presence of hidden transcriptional regulatory mechanism that can control the levels of IRAK-1

mRNA expression. However, it does not seem to be affecting the IRAK-1A splice variant with the rs1059703 SNP. This might explain the constant state of inflammation expressed by IRAK-1A (Var) patients.

7.2 Limitations and Future Considerations

One of our primary concerns with this study is the small number of participants analyzed. Due to a lack of sufficient processing capability and time constraints, we could only analyze a small dataset with 15 participants and 30 data points. Due to the sample's small size, our data might not have strong resolving power and can result in type-II errors, where the null hypothesis is false and fails to get rejected [267]. We strongly feel that this issue might be particularly relevant regarding the IRAK-1C expression levels, given the variable's small effect on the total IRAK-1 levels. Therefore, the small sample size of this dataset might not possess the (statistical) power to expose such a small effect, possibly resulting in a type-II error. This limitation might also explain why we failed to notice significance while comparing the changes in IRAK-1 levels upon LPS administration between high and low inflammation groups. Furthermore, this sample set only includes patients of Caucasian and African American ethnicities and fails to consider the impact of age. Thus, this dataset would not be representative of the entire population.

In our future studies, we would like to implement an automated cloud-based system capable of blasting huge SRA datasets containing data from a wide variety of patients with diverse ethnic backgrounds and ages.

Furthermore, while there have been a plethora of studies investigating the role of IRAK-1A (with and without the rs1069703 SNPs) and their downstream effects in

inflammation, there is a scarcity of background data on the IRAK-1C mutant. Hence, we believe that it is prudent to measure the levels of downstream cytokines and relate the expression levels to the levels IRAK-1. This data will help elucidate the effects of the IRAK-1C splice variant and help correlate it with a wide range of markers.

7.3 Conclusion

Sepsis is a complicated condition that is characterized by highly dysregulated immune responses. These immune responses are generated through the activation of the NF-κB pathway, which is primarily governed by the adaptor molecule IRAK-1. A plethora of studies has elucidated the role of IRAK-1 and NF-κB in inflammation and sepsis-induced organ failure. Increased IRAK-1 expression has been associated with increased levels of crucial markers of sepsis such as C-Reactive proteins, decreased miRNA-146a levels, increased expression of hypoxia-induced genes, bilirubin, and blood lactic acid levels. Despite the plethora of evidence linking IRAK-1 in inflammation and hinting its role as a potential therapeutic target, there has not been much research conducted on the biochemical dynamics and mechanisms of its dominant-negative splice variant IRAK-1C.

In this paper, we reviewed the wide spectrum of downstream effects exerted by IRAK-1 and sought to understand the combined effects of the IRAK-1A and IRAK-1C splice variants along with the proinflammatory rs1059703 SNP mutant on inflammation. We characterized inflammation based on the total mRNA levels IRAK-1. Our results show a paradoxical decrease in the IRAK-1 mRNA levels following LPS administration. This phenomenon might be associated with the effects of miRNA-146a, a negative regulator of

IRAK-1 mRNA expression. We also report that patients with a high sensitivity to LPS express higher levels of IRAK-1A prior to endotoxin exposure.

Our results show a lack of significant differences in the overall IRAK-1 and the IRAK-1A levels amongst IRAK-1 (WT) homozygotes and the WT/Var expressing heterozygotes. We also noticed that there was a significant increase in IRAK-1 and IRAK-1A levels among the homozygous variants compared to the homozygous WTs and the heterozygotes. As past studies have associated increased levels of IRAK-1 and IRAK-1A with hyper inflammation, our results are consistent with previous findings that implicate the IRAK-1A (Var) with increased inflammation and the dominant-negative effects of IRAK-1C inhibiting inflammatory activities. However, we did not find any significant decrease in IRAK-1A (Var) levels of heterozygotes upon LPS administration. While many studies have associated this haplotype with increased NF-κB levels, recent research has shown that this haplotype does not exhibit a significant increase in miRNA-146a levels compared to the IRAK-1A (WT). These results suggest that miRNA-146a might not be able to inhibit or negatively interfere with IRAK-1A (Var) mRNA expression.

While our statistics also report any significant impact exerted of IRAK-1C (WT) and IRAK-1C (Var) between patients with low and high inflammation, we believe that the size of our dataset might have compromised the dataset's resolving power to successfully identify any significant differences in the levels of IRAK-1C.

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APPENDIX A

SUPPLEMENTAL DATA AND CODE

This appendix contains links to the complete dataset and the Python & Jupyter code used in the analysis.

A.1. Data Source Used in Analysis

The complete SRA Dataset used for analysis is available online on NCBI: https://www.ncbi.nlm.nih.gov/sra?term=SRP090337

A.2 Code for Statistical Analysis

The complete code used for our statistical analysis is available on my GitHub. Link to my code: https://github.com/ASSahasranamam/thesis

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