Establishing a New Model of Endotoxemia-Associated Acute Kidney Injury in Zebrafish

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Sepsis is a leading cause of in-hospital patient deaths and is characterized through a systemic inflammatory response and subsequent organ failures. The kidney is known to be particularly susceptible to injury in this setting, with sepsis-associated acute kidney injury (S-AKI) being the most common organ dysfunction during sepsis. Notwithstanding this prevalence, the general mechanisms which cause S-AKI are largely unknown. Hypotheses regarding the roles of pro-inflammatory molecular pathways and related cellular responses have been of interest to explain such pathological effects. While recent studies have made strides to identify biomarkers of S-AKI from human patient data, and altered molecular pathways from animal and *in vitro* models, one source difficulty in making further progress are the model systems themselves. Current model systems are rife with their own complications; many fail to mirror known human S-AKI pathologies, and many are either difficult to work with, or do not give consistent results from one assay to the next. Here, we strove to develop a new *in vivo* model of S-AKI through microinjections of the endotoxin lipopolysaccharide from *Pseudomonas aeruginosa* into zebrafish larvae. While much is to be learned from this model, early data suggests this system is efficient, easy to work with, consistent, and shows hallmarks of kidney dysfunction and proinflammatory responses post-injection. As such, it may act as a valuable tool to understand the biopathology of S-AKI, as well as serve as a vital high throughput drug discovery model to identify novel therapeutics.

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Preface

With my deepest feelings of gratitude and appreciation: thank you to the University of Pittsburgh, the Hukriede lab, all the mentors, friends, and family who have shaped, inspired, and filled me and my life with meaning and significance. For Dad. For Mom.

The struggle itself toward the heights is enough to fill a man's heart. One must imagine Sisyphus happy.

-Albert Camus

1.0 Background and Significance

1.1 Sepsis as a Global Health Issue

> *Then again the messenger Argeiphontes spake to him: "Old sire, not yet have dogs and birds devoured him, but still he lieth there beside the ship of Achilles amid the huts as he was at the first; and this is now the twelfth day that he lieth there, yet his flesh decayeth not at all.*
>
> -Homer, *The Iliad*, Book 24 v.410-414

For over 2,700 years, sepsis has been described and studied; the term's first known usage—found in the above verses—can be traced to Homer's *The Iliad*, derived from the Greek verb sepo (σηπω), meaning "I rot"[1-3]. From Hippocrates to Semmelweis, from Koch to the current day, the general connotation of sepsis has been relatively unchanged, and though great strides have been made in the past few decades to research and treat this pernicious condition, there still remains a significant journey ahead to fully understand its pathology and to develop meaningful treatments and therapeutics for affected patients.

In the United States, sepsis is the leading cause of in-hospital deaths and has an associated annual cost of over $24 billion[4, 5]. As the understanding of sepsis has evolved throughout the centuries, so have the medical definitions and classifications. Most recently in 2016, the Third International Consensus Definitions (Sepsis-3) refined the general definition of sepsis as a life-threatening condition of organ dysfunctions caused by an individual's dysregulated infection response[6]. This definition arose through the study of large hospital patient cohorts and their associated clinical outcomes, as well as the current understandings of associated molecular and cellular mechanisms during disease progression. Having empirical benchmarks for organ

dysfunctions following an infection-related inflammatory response improved upon previous definitions (i.e. systemic inflammatory response syndrome, SIRS)[7, 8].

To estimate the global occurrence of sepsis, a team of researchers published an article in 2016 which analyzed a large collection of data from 27 clinical studies over the preceding 36 years. This study estimated the annual global incidence of sepsis to be a staggering 31.5 million cases with around 5.3 million deaths[9]. Of note, these conclusions were based on data originating from seven high-income countries; to further gain insight into the occurrence of sepsis, Rudd *et al.* (2020) analyzed multiple cause-of-death databanks from low, middle, and high-income countries. With this data set and metanalyses, this study remarkably concluded that the reality of sepsis appears much more grim, estimating 48.9 million cases of sepsis/year and 11 million sepsis-related deaths/year[10]. In context, this astonishingly would account for nearly 20% of all deaths worldwide being related to sepsis. The highest risk areas identified were low-income countries of sub-Saharan Africa and Southern/South-Eastern Asia.

These studies paint a sobering reality of a world beset by this devastating condition, and though research has been laudatory over the past decades, remarkably there is still a void of targeted therapies to adequately address sepsis-associated organ dysfunctions. As such, it is of importance to examine the current aggregate of knowledge on the initiation and progression sepsis, how this pathology is currently experimentally studied, and what data experimental model systems indicate as potential mechanisms for both causes of—and solutions to—organ dysfunctions.

1.2 Sepsis-Associated Acute Kidney Injury (S-AKI) Epidemiology and Pathobiology

During sepsis, though acute kidney injury is the most frequent organ failure, exact mechanisms causing organ dysfunction are relatively unknown, nor have effective treatments/therapeutics been developed[11]. Such understandings and treatments are greatly needed as sepsis is the leading cause of AKI, and more specifically, patients who develop S-AKI have upwards of 5 times the mortality rate as sepsis patients without S-AKI[12, 13]. Recent studies have indicated that ~60-70% of patients with sepsis have AKI[12, 14], and importantly, patients who survive S-AKI face increased long-term health risks of chronic kidney disease (CKD) and end-stage renal disease (ESRD)[15-17].

Through the studies of animal models, human patients, and septic human postmortem tissues, an initial picture of the pathobiology of S-AKI can be interpreted. Surprisingly, neither animal models nor postmortem human kidney tissues have shown significant cellular necrosis—a form of cell lysis death due to external sources of injury—during S-AKI, indicating that acute tubular necrosis is not a major factor contributing to loss of kidney functions in human patients[18-21]. Similarly, apoptosis—a form of programmed cell death—of tubular cells does not seem to be a consistent nor attributable phenomena of S-AKI pathobiology, though the apoptosis of infiltrating leukocytes during S-AKI is notable[18, 19, 22, 23].

Notwithstanding, patches of proximal tubule cells show a loss of brush border and apical vacuolization have been identified in human postmortem samples[19, 22]. Additionally, variable portions of proximal and distal tubules show dilation and cytoplasmic flattening[22]; all of which indicate significant functional changes to these cells. Together, these observations suggest that during sepsis, renal epithelial cells may undergo molecular reprogramming to favor cell survival over organ function.

During sepsis, alterations in microcirculation and subsequent heterogeneous blood flow is known to occur through mechanisms such as inflammatory vasodilation, autoregulation, endothelial dysfunction, and activation of the coagulation cascade[24-26]. Such phenomena are still intense areas of study as to determine to what extent these occur in the kidney during S-AKI, and how they contribute to progressive kidney injury and failure. Recent studies of renal blood flow (RBF) (both in animal models and human patient studies) interestingly have demonstrated that homeostatic microcirculation can even be altered in the presence of normal or increased global RBF[20, 27, 28]. These seemingly contradictory facts could be explained by mechanisms such as endothelial dysfunction leading to vascular shunting, microthrombi formation (though this is rarely seen in human S-AKI patients), and interstitial edema via damage of the glycocalyx and increased vascular permeability[29-31]. These factors could contribute to some nephrons becoming hypoperfused, even as RBF appears normal. Such alterations in microcirculation can be directly related to inflammatory cascades and the activities of the innate immune system during the infection response. Histological analyses of human postmortem tissues demonstrate a massive infiltration of leukocytes into renal capillaries, glomeruli, and—less frequently—into tubular lumens during S-AKI[22]. Proinflammatory effects and other pathological consequences of leukocyte infiltrations are discussed in future sections.

1.3 Molecular Mechanisms and Cellular Responses of S-AKI Pathology

1.3.1 Pattern Recognition Receptors and Their Agonists

To properly treat and prevent life-threatening organ dysfunctions during sepsis, scientists and clinicians need to have a firm understanding of the various molecular mechanisms which contribute to the pathology. Unfortunately, such mechanisms are not fully understood, and there are no proven therapeutics yet which can revive or regenerate organs in this state. A primary hypothesis to address the phenomena of sepsis-associated organ dysfunction focuses on the exacerbated, systemic inflammatory responses following infection. To identify and respond to pathogens, organisms throughout Eukarya have evolved a wide array of protein receptors to act as a first line of defense. In the innate immune response, Pattern Recognition Receptors (PRRs) evolved for this function and have remained significantly conserved throughout the Animal Kingdom[32-34]. The PRR family largely consists of subgroups of the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors, and the intracellular cytoplasmic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs)[35-39]. The variety of PRRs was an evolved array of mechanisms so that organisms could properly identify and respond to specific pathogenic stimuli appropriately.

Agonists of PRRs consist primarily of two classes of molecules: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), also known as danger-associated molecular patterns. PAMPs are a wide variety of various molecular motifs which, when detected by PRRs, initiate host non-self signaling to respond to the presence of foreign microbes[40]. Examples of PAMPs include bacterial peptidoglycan, lipopolysaccharide (commonly referred to as ‘endotoxin’), lipoteichoic acid, and viral dsRNA[41-43]. DAMPs refer

to a class of non-infectious endogenous molecules released by damaged or dying cells and tissues which interact with PRRs to initiate inflammatory responses[44]. As such, they act as an alarm that nearby cells/tissues have been damaged in some capacity and require attention. Examples of DAMPs include DNA (nuclear and mitochondrial), RNA, HMGB1, heat-shock proteins, and the extracellular matrix components hyaluronan and heparin sulfate[44-46].

The interplay between PRRs and PAMPs/DAMPs is of primary interest in the case of sepsis to understand the initial spark that ignites a systemic host response. Of importance in this setting are the TLRs, which can be found on the membranes of leukocytes, endothelial, epithelial, and fibroblast cells[47]. Within the PRR superfamily, there have been 10 identified TLRs in humans and 12 in mice, each with its own specialty for interacting with molecules particular to various pathogens[39, 48]. Many studies have examined the role of TLRs during infection and related sepsis, and while many can be active during these pathologies, TLR4 is of particular interest to address the molecular origins of systemic inflammation and subsequent organ dysfunctions[49-54].

1.3.2 TLR4 Signaling

TLR4 is a transmembrane receptor consisting of an extracellular leucine-rich repeat domain, and an intracellular toll/interleukin-1 receptor (TIR) domain[55]. A primary binding agonist of TLR4 is the gram-negative bacterial endotoxin lipopolysaccharide (LPS). LPS molecules are large multicomplex molecules which are embedded within the outer membrane of gram-negative bacteria. They consist of three major segments: the O-antigen, a glycan polymer and outer-most segment of LPS; the core oligosaccharide; and Lipid A, a phosphoglycolipid which anchors the whole complex into the membrane[56, 57]. During infection, hosts' LPS-binding

proteins (LBP) bind to LPS in serum and transfers it to the extracellular membrane-bound CD14 protein. This further stimulates the formation of the TLR4/MD-2 heterodimer complex as LBP transfers LPS to Myeloid Differentiation Factor 2 (MD-2). Of note, it is the lipophilic segment of LPS, Lipid A, that binds to MD-2[42, 58, 59]. Once this heterodimer complex is formed at the membrane, the signal is transferred intracellularly through two potential known pathways: the MyD88-dependent and -independent pathways. During the MyD88-dependent pathway, the signal leads to a series of protein interactions which culminate in the activation of TAK1 as well as NF-κB via the degradation of IκB. Together, these lead to the activation of several mitogen-activated protein kinases (MAPK), the production of key proteins in inflammatory processes (e.g. COX-2 and iNOS), and release of proinflammatory cytokines (notably TNF-α, IL-1β, and IL-6)[60-62]. Importantly, secreted proinflammatory cytokines—such as TNF-α and IL-6—have been well studied to further activate NF-κB in an autocrine and paracrine fashion, thus contributing to a positive feedback of inflammatory responses[63, 64]. During the MyD88-independent pathway, the TLR4/MD-2 complex become internalized as an endosome. Intracellularly, TLR4 binds to the adaptor proteins TRAM and TRIF, leading to the activation of IRF3/7. This culminates in the late-phase activation of NF-κB, as well as the expression of type-I interferons and cytokines, such as the chemoattractant CXCL10[50, 65]. Of note, TLR4 is also able to be activated via interactions with DAMPs, such as Hsp72[66] and HMGB1[67]. Figure 2 is a basic schematic of the TLR4 pathway activation via LPS.

1.3.3 Innate Immune Responses During Infection and Inflammation

As said cytokines and chemoattractants are secreted by stimulated cells, the innate immune system mounts an initial response to infection. Neutrophils are essential to combat pathogens as

they are the first immune cells to extravasate to sites of infection. Once localized, neutrophils act to combat infection through ROS production, NO production via iNOS, the formation of neutrophil extracellular traps (NETs), cytokine production, and phagocytosis[68-70]. These immune cells are short-lived, and have been observed to undergo apoptosis/pyroptosis within 24 hours[71]. This massive death of neutrophils, along with death of pathogens and various host cells, releases substantial DAMPs which can further exacerbate inflammatory responses and cell stress[70]. Moreover, NO is a potent vasodilator and contributes to lowering of blood pressure, and the cytokines produced by neutrophils adhere to endothelial cells to promote coagulation and thrombogenesis[31]. These effects are of significance as endothelial dysfunction and microcirculatory aberrations are well-established phenomena of sepsis[31, 72, 73].

Various cytokines also influence and attract macrophages which are present at sites of infection shortly after the initial infiltration of neutrophils[74]. During initial stages, proinflammatory cytokines induce macrophage polarization to the M1 phenotype. M1 macrophages act to combat pathogens and further secrete proinflammatory molecules, again notably via the NF-κB pathway[74-76]. Though they play essential roles in neutralizing the presence of pathogens, it is to note that they perpetuate proinflammatory responses which can further a positive feedback loop of inflammation in a paracrine fashion. The balance of their utility and detrimental effects during sepsis is not fully understood as more research is required. The actions and pathological alterations of the adaptive immune system during sepsis, though interesting and worth exploring, are outside the scope of this review.

1.3.4 Further Consequences of LPS and Proinflammatory Responses

Proinflammatory cytokines, such as TNF-α, have been documented to have further deleterious effects as they can increase the levels of reactive oxygen species (ROS) produced within cells[77, 78]. This rise in intracellular ROS induces mitochondrial dysfunction within cells, and can eventually lead to apoptosis[79-81]. The presence of ROS molecules, as well various PAMPs and DAMPs, have also been documented in the assembly and activation of the NLRP3 inflammasome[82, 83]. The NLRP3 inflammasome is a multimeric protein complex which, once activated, is known to facilitate the activation of caspase-1, a potent inducer of pyroptosis cell death. It also activates proinflammatory molecule IL-1β, and the pro-apoptosis molecule caspase-8[82, 84, 85]. Pyroptosis has recently been identified as a key contributor to the pathogenesis of sepsis in the kidney and other tissues in mouse and zebrafish models[86-90].

As LPS induces a variety of inflammatory responses, cells struggle to maintain homeostasis. By investigating particular downstream consequences post-TLR4 activation, several studies have identified significant increases in endoplasmic reticulum-stress (ER-stress), both *in vitro* and *in vivo*[91-93]. ER-stress is a product of the accumulation of mis-or-unfolded proteins within the ER, and this poses serious threats to cell function and survival. If the ER sentinel proteins IRE1α , PERK, and ATF detect mis/unfolded proteins, the unfolded protein response (UPR) occurs[94]. This is a safety mechanism which increases the production of chaperone proteins, decreases overall RNA and protein production, and triggers cell cycle arrest[95]. However, prolonged ER-stress—including stress induced via TLR4 activation—has been demonstrated to cause activation of the NLRP3 inflammasome, mitochondrial dysfunction, release of DAMPs, and apoptosis[91, 96, 97]. Interestingly, pharmacological inhibition of ER-stress via

4-phenylbutyrate significantly lowered inflammatory responses and tissue injury in an *in vivo* LPS-induced mouse model, as well as an *in vitro* model with human epithelial cells[92].

The deleterious effects of LPS on host cells and tissues extends further than known consequences of TLR4 activation. A growing body of research, first described in 2012[98], has been the discovery and elucidation of the unique form of cell death known as ferroptosis. Ferroptosis is a consequence of cellular distress stemming from iron-dependent lipid peroxidation. This process forms reactive lipid species (RLS)—notably malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE)—which have been found as mediators of multiple pathologies such as stroke[99], intracerebral hemorrhage[100], acute kidney injury[101], lung fibrosis[102], cardiac ischemia-reperfusion injury[103], and sepsis[104-107]. In an interesting 2016 study, researchers investigated the effects of LPS on red blood cells (RBC)[108]. Utilizing toxic and non-toxic variants of LPS (which conversely act as TLR4 antagonists), they found that both strains were able to directly interact with RBC membranes and cause hemolysis. This finding presents serious down-stream implications in pathogenesis. When RBCs lyse, free hemoglobin is released into circulation which is able to permeate endothelial barriers and enter lymph fluids[109]. To prevent free iron-induced damage, organisms have evolved specific heme binding proteins such as haptoglobin and hemopexin[110]. However, satiation of such binding proteins during overwhelming hemolysis can lead to free iron injury and subsequent ferroptosis of affected cells; such a condition—for example—has been studied to understand the progression and evolved defenses to malaria infection[111]. Significant increases in cell-free hemoglobin during sepsis has been documented, and how this and ferroptosis relate to associated kidney/organ dysfunction is a burgeoning area of study[112-117].

1.3.5 S-AKI Biomarkers: IGFBP7 and TIMP-2

As previously alluded to, it remains intriguing that initial kidney dysfunction can occur during sepsis with little-to-no histological aberrations[118]. As such, hypotheses regarding the prime causes of S-AKI began to focus on mechanistic changes within tubular cells to explain the initial loss of function. In 2013, Kashani et al. published a study which examined the urine of septic patients who eventually developed AKI to find novel biomarkers[119]. Indeed, they discovered two biomarkers—insulin-like growth factor binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases-2 (TIMP-2)—which together gave the best statistical power compared to other known biomarkers (e.g., NGAL, KIM-1, cystatin-C) to predict if a patient would develop S-AKI up to 48 hours before a loss of kidney function was clinically identified.

The exact roles of these biomarkers during S-AKI are yet to be fully elucidated, however, two main aspects are worth noting. Both IGFBP7 and TIMP-2 have several well documented functions, however they are both thought to enhance G_1 cell cycle arrest during cell injury[119-121]. There has been increasing evidence that cells (e.g. epithelial, renal tubular cells) go through transitions of cell cycle arrest and potential dedifferentiation during times of injury or stress. Data suggest these are protective and regenerative mechanisms to weather temporary insults, and have been studied in the kidney during scenarios of ischemia[122], nephrotoxic injury[123], and sepsis[124]. Moreover, evidence not only suggests that cell cycle arrest and dedifferentiation during kidney injury are essential to healthy adaptive repair, but require precise regulation and timing post-injury to be effective[123, 125]. Another recent study investigated the role of TIMP-2 during mouse models of S-AKI and cultured human kidney cells (HK-2) exposed to LPS. Findings indicated that TIMP-2 appears to associate with the NF-κB pathway, promoting the expression of proinflammatory cytokines. In culture, the increase in proinflammatory cytokines

promoted apoptosis, and when TIMP-2 was knocked down via a lentiviral vector during an *in vivo* mouse S-AKI model, proinflammatory cytokines significantly decreased, measured kidney function increased, and histopathological alterations were noticeably less compared to controls[126]. Together, these studies of TIMP-2 and IGFBP7 paint an intriguing picture of their roles during S-AKI, however, a more complete understanding of their potential positive and negative functions during this pathology still require more investigations.

1.3.6 Experimental Models of S-AKI

Of critical importance in the scientific and clinical progress to understand S-AKI are the experimental models which serve as the basis of knowledge which will stem mechanistic understandings and effective treatments for patients. While there have been several models of sepsis and S-AKI in a wide range of systems—such as porcine, ovine, and bovine—the three primary models which are in use and show unique promise are mouse, zebrafish, and human cell culture models. Within these models, S-AKI phenotypes can be triggered by three general mechanisms: exposure to an exogenous toxin (e.g. LPS), exposure to exogenous bacteria, or exposure to intrinsic microbes through surgical perforations of tissues[127].

Mouse models of S-AKI have utilized all three said modes throughout the past decades, though the cecal-ligation and puncture (CLP) has emerged as a leading system. The mouse CLP procedure consists of surgically perforating the cecum, causing endogenous bacterial flora to enter the body cavity[128]. This model system exposes the mouse to a wide range of microbes and can mimic known human S-AKI pathobiologies such as leukocyte infiltrations/apoptosis, systemic inflammatory responses, hemodynamic alterations, and various organ injuries/failures[129-131]. This model system has some drawbacks, however, as researchers are unable to fully control the

specific types and amounts of bacteria per subject, organ dysfunctions—or lack thereof—can be highly variable, and there is a high mortality rate in a short period of time (~70-80% mortality after 48 hours is common)[132].

Zebrafish models of endotoxemia, sepsis, and S-AKI have been emerging as well as unique and valuable model systems. By 48 hours post-fertilization, the zebrafish pronephros is functional; moreover, cell types and functions of the nephron are conserved compared to mammalian counterparts[133]. Additionally, the zebrafish adaptive immune system does not develop until 4 weeks post-fertilization, allowing researchers to utilize zebrafish larvae to investigate sole roles of the innate immune system within various models[134]. A key difference in zebrafish nephrons compared to those of mammalian organisms is their intrinsic ability to fully regenerate and even form new nephrons after injury[135, 136]. This presents a unique model to investigate mechanisms of healing and regeneration post-injury, as well as given the ability to use large numbers of animals which are ideal for drug-discovery experiments[123, 137, 138]. Philip et al. (2017)[139] detail a zebrafish sepsis model via exposure to LPS in solution. Compared to known human pathobiology, their model system demonstrated parallel sepsis phenotypes such as edema, organ injury, endothelial dysfunction, proinflammatory responses, increased ROS production, and immune cell activation. Wen et al. (2018)[140] developed a model system that more specifically analyzed S-AKI via microinjections of the known fish pathogen *Edwardsiella tarda*. This model system demonstrated edema, proinflammatory responses, expression of known kidney injury biomarkers, and increased expression of IGFBP7 and TIMP-2 within tubule cells. Additional specific examples of zebrafish and mouse models and related findings regarding S-AKI are detailed throughout previous sections. More thorough suggested reviews of S-AKI animal models include Doi et al. (2009)[127] and Dejager et al. (2011)[132].

The use of human cell cultures is another vital tool, allowing the ability to study human cell-specific responses in sepsis modeling. As such, utilizing cells of human origin may provide a greater potential to understand human-specific phenomena during sepsis as well as for applicable drug screenings to assay utility and toxicity. In 1994, Ryan et al. generated the immortalized human proximal tubule epithelial cell line (HK-2) which is still utilized in modeling of sepsis/endotoxemia[141]. Examples of S-AKI research using HK-2 cells include the study of proinflammatory signaling, apoptosis, roles of lncRNAs/miRNAs, metabolomics, and injury responses, among others[142-147]. In addition to HK-2 cells, the use of primary human cell cultures is also being utilized in some research. Donated human kidney tissues are dissociated to isolate particular cell types—such as glomerular, proximal tubule, and distal tubule—and are subsequently cultured and used for experiments. As mutations and genetic drift can occur in HK-2 cell lines after repeated passages, the use of primary cells presents a unique culture of cells directly from recently functioning human kidneys. Exemplarily, Emlet et al. (2017) dissociated human kidneys and cultured separately cells of proximal or distal tubule origin to identify differences in cell-specific expression of kidney injury biomarkers IGFBP7 and TIMP-2[143]. Figures 1-3 are schematics which combine knowledge gained from years of research which illustrate important molecular and cellular responses during sepsis.

1.3.7 Schematics of Known S-AKI Molecular and Cellular Phenomena

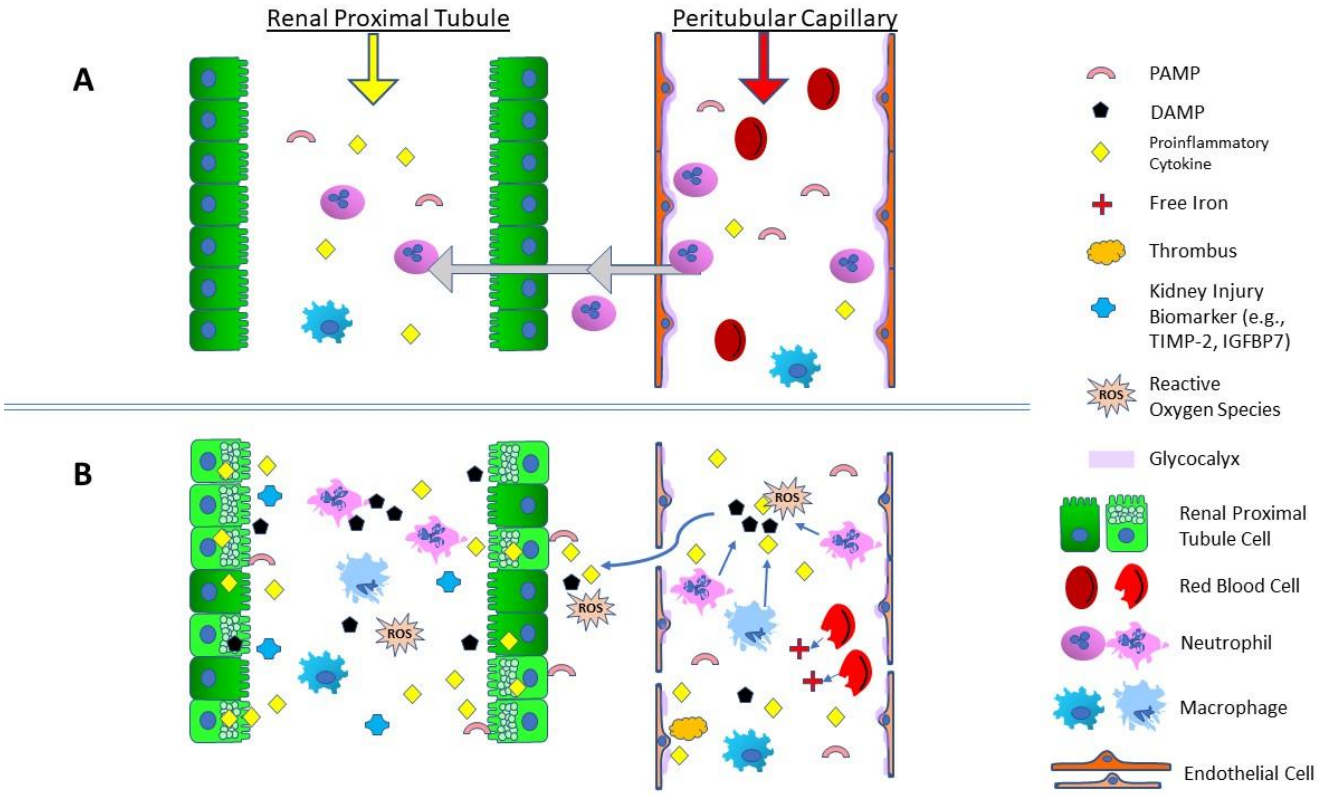

Figure 1: PAMP/DAMP renal cellular effects during endotoxemia. A) Initial phase of endotoxemia as circulating PAMPs first cause expression and release of proinflammatory cytokines from host cells. Neutrophils first localize to the kidney and extravasate from peritubular capillaries to enter the proximal tubule. Macrophages infiltrate typically after 24 hours. B) During S-AKI, endothelial cell dysfunction leads to increased permeability between cellular junctions, allowing circulating cytokines, DAMPS, ROS, and various circulating molecules to seep through into interstitial space. Leukocytes (primarily neutrophils and macrophages) undergo apoptosis, releasing more DAMPs and proinflammatory cytokines. LPS is also known to cause red blood cell lysis, releasing cell-free iron into circulation. Proximal tubule cells become vacuolized and their functions decrease, contributing to renal injury and failure.

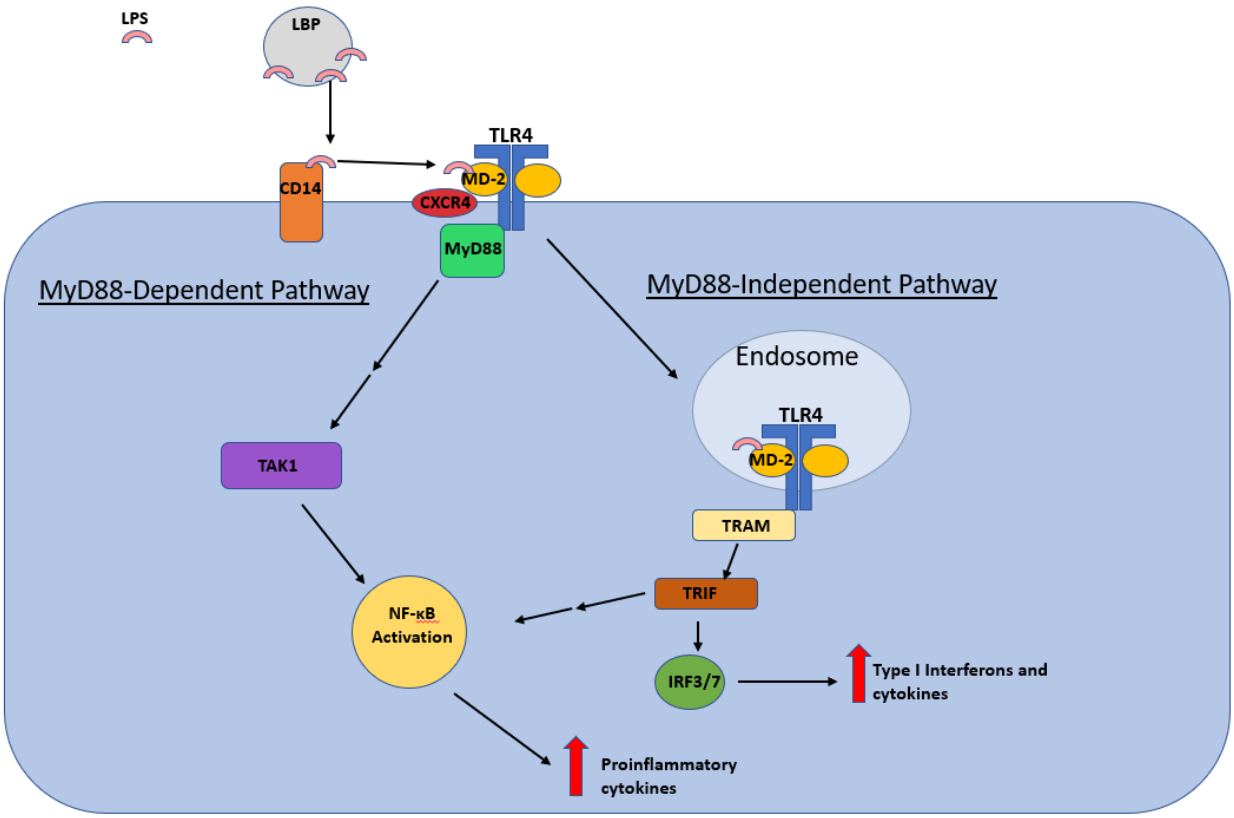

Figure 2: Schematic of TLR4 Activation via LPS. In serum, LPS is bound by lipopolysaccharide binding protein (LBP). LPS is transferred to CD14 at the cell surface, followed by its transfer to the TLR4/MD-2 complex. CXCR4 is known to cluster at this complex and may act to dampen downstream signaling. After LPS binding with the TLR/MD-2 complex, the signal can progress through either the MyD88-dependent or the MyD88-independent pathway.

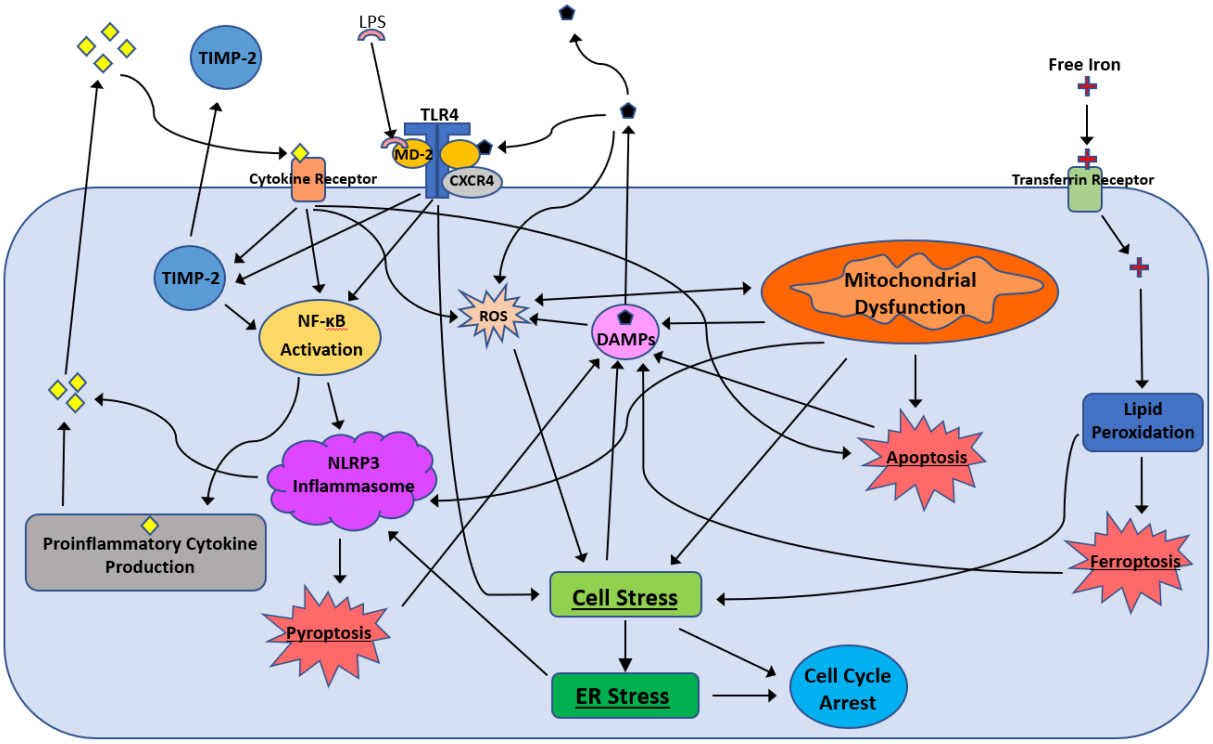

Figure 3: Intracellular signaling during endotoxemia. TLR4 is activated by LPS and DAMPs, causing proinflammatory pathways to become active. Various cell stresses caused by proinflammatory cytokines, PAMPs, and DAMPs contribute to ER stress, mitochondrial dysfunction, formation of the NLRP3 Inflammasome, and activation/release of TIMP-2. Consequentially, these factors can directly cause various cell cycle arrest, apoptosis, and pyroptosis. Cell-free iron, which is significantly increased in the serum during sepsis, causes lipid peroxidation and ferroptosis.

1.4 Creating a New Endotoxemia-Associated Acute Kidney Injury Model in Zebrafish

In 2018, Wen et al. [140] published a model of S-AKI in zebrafish via live microinjections of the fish pathogen *Edwardsiella tarda*. As previously mentioned, this model displayed mild edema, increased kidney injury makers, and expression of TIMP-2 and IGFBP7 within tubule cells

post-infection. While this model displays known pathogenic features of S-AKI, working *E. tarda* in our lab proved to have many difficulties. Results were highly variable from assay-to-assay, and zebrafish displayed little intermediate phenotypes; “sick” zebrafish progressed and died quickly, while the remainder displayed no signs of infection or sepsis. Additionally, *E. tarda* is a highly virulent fish pathogen, thus presenting a challenging situation while working closely with a large zebrafish facility. As such, we began to develop a new model of S-AKI without the use of live pathogens. Zebrafish were to be used as they have several desirable characteristics as a model system that would be of use in this setting. As they can be bred in large numbers, and their pronephros are functional within days, this model system allows for high throughput screening of both phenotypes, study of pathological molecular mechanisms, and eventually for drug discovery experiments.

2.0 Results and Discussion

2.1 Sterile *Edwardsiella tarda* Microinjections

Using the *E. tarda* model of S-AKI in zebrafish, attempts were made to kill the bacteria to assess the ability of a sterile injection to cause S-AKI phenotypes (e.g., edema) and mortality. An initial attempt utilized a heat-kill method, either with or without subsequent sonication to further shred bacterial cells. Various concentrations of bacteria were tested by measuring the optical densities (OD) prior to heat-killing. Tested OD levels ranged from 1-5; with an OD of 2.5 equating to ~300 colony forming units per nanoliter (the amount injected in the established *E. tarda* microinjection protocol). One nanoliter of each respective solution was microinjected into the sinus venosus of anesthetized zebrafish larvae on day 3 post-fertilization. No solution nor method attempted caused any edema nor death after microinjections compared to controls (Table 1).

Addressing the possibility that heating/sonicating caused significant denaturing of endotoxins, another set of experiments sought to create a sterile injection model without the use of excessive heat generation. Similarly, *E. tarda* cultures were grown and concentrated to various ODs (1-5) for freeze-fracturing. Samples were subjected to series of freeze/thaw cycles in liquid nitrogen, and one nanoliter of each respective solution was microinjected into the sinus venosus of anesthetized zebrafish larvae on day 3 post-fertilization. No solution resulted in the development of edema nor caused death after microinjection compared to controls (Table 1)

A further experiments utilized antibiotics to kill *E. tarda* bacteria prior to injection. Two common Gram-negative susceptible antibiotics were tested: streptomycin (a protein synthesis inhibitor) and meropenem (an inhibitor of cell wall synthesis). Minimum inhibitory concentrations

(MIC) of each antibiotic were identified previously (data not shown) and used in each experiment. *E. tarda* cultures were grown and concentrated to various ODs (2-5) and subsequentially treated with a respective antibiotic. One nanoliter of each respective solution was microinjected into the sinus venosus of anesthetized zebrafish larvae on day 3 post-fertilization. No solution resulted in the development of edema nor caused death after microinjection compared to controls (Table 1). For all sterile-based injection experiments, bacterial death was validated through plating of each solution on Tryptic Soy Agar plates with an overnight incubation at 28.5°C.

2.2 PAMP and DAMP Microinjections with Negative Results

Direct microinjections of multiple PAMP and DAMP molecules into day 3 post-fertilization zebrafish were attempted to elicit potential renal dysfunction. Post-injection, zebrafish were monitored for several days for signs of edema, death, or other overt phenotypes. Several compounds were tested—each at least twice—and all of which showed negative results. These included microinjections of: 20, 10, 5, and 1 ng of lipopolysaccharide from *Escherichia coli* (*E. coli*) dissolved in PBS; 20, 10, 5, and 1 ng of lipopolysaccharide from *Escherichia coli* (*E. coli*) dissolved in stearic acid; 1-2 nanoliters of 50, 10, and 1uM Lipid A; 1-2 nanoliters of 2,000, 200, 100, 10, and 1uM Lipoteichoic acid (LTA) from *Staphylococcus aureus*; 1 nanoliter of 500, 250, 100, 50 uM hemin; and 1-2 nanoliters of a 500uM hemin + 10ng/nl *E. coli* LPS solution (Table 1).

Table 1: Failed S-AKI/Endotoxemia Model Systems. A brief synopsis of the various chemicals/solutions which failed to produce evidence of S-AKI/Endotoxemia post-microinjection into zebrafish larvae. Tested concentrations for each is noted, and each experiment was attempted at least twice.

	Method	**Doses Tested**
Endotoxins:	LPS (*E. coli*) Injections	20, 10, 5, 1ng
	LPS (*E. coli*) Micells Injections	20, 10, 5, 1ng
	Lipid A Injection	50, 10, 1uM
	Lipoteichoic Acid Injection	2mM, 200, 100, 10, 1 uM
"Sterile" Bacterial-Based:	Antibiotic Killed *E. tarda* (streptomycin/meropenem)	4, 3, 2 OD
	Heat-Killed *E. tarda*	4, 3, 2, 1 OD
	Sonicated *E. tarda*	4, 3, 2 OD
	Heat-Killed+Sonicated *E. tarda*	5, 4, 3, 2, 1 OD
	Freeze Fractured *E. tarda*	5, 4, 3, 2, 1 OD
DAMP:	Hemin Injections	500, 250, 100, 50uM
DAMP + PAMP	Hemin+LPS (*E. coli*)	Hemin: 500, 250uM + LPS: 20, 10ng

2.3 Microinjections of Lipopolysaccharide Derived from *Pseudomonas aeruginosa*

An initial soak experiment was conducted to validate the toxicity of LPS derived from *Pseudomonas aeruginosa* with or without AMD3100, a chemical inhibitor of CXCR4. High concentrations of LPS (100ug/ml), with or without AMD3100 (10ug/ml), were fatal after 24 hours. A lower concentration of 10ug/ml LPS showed mortality only when combined with AMD3100 (Figure 3A).

In several subsequent experiments, day 3 post-fertilization zebrafish were microinjected with *Pseudomonas aeruginosa* LPS into the sinus venosus and monitored for several days (Figure 3B). An initial set of experiments investigated the survival and edema phenotypes of the fish after multiple days post-injection. Injections of 40 and 60ng LPS caused edema to varying extents, both with and without AMD3100 (Figure 3C, 3D). Additionally, LPS injections caused mortality in all groups when tracked for 5 days post-injection (Figure 3E). Control groups injected with PBS displayed no edema, nor mortality over this time frame. Statistically, a survival assay calculating the area under the curve via a Kaplan-Meier analysis yielded 500, 441.7, 458.3, 452.1, 461.7 arbitrary units (a.u.) for control, 40ngLPS, 40ngLPS + AMD3100, 60ngLPS, and 60ngLPS + AMD3100, respectively. Data did not reach statistical significance when comparing treatment groups (dosage and AMD3100 treatment). However, there was statistical significance comparing the control group to each treatment group: 40ngLPS, 40ngLPS + AMD3100, 60ngLPS, and 60ngLPS + AMD3100; $p = 0.001$, $p = 0.005$, $p = 0.0046$, $p = 0.0004$, respectively. As such, future experiments no longer included AMD3100 treatments.

To validate if the LPS injections were causing an innate immune response, Tg(TNF-α:GFP) zebrafish were injected. Within 24 hours, significant expression of TNF-α was increased

compared to control injected zebrafish (Figure 4A), demonstrating the activation of pro-inflammatory signaling pathways known to be linked to innate immune cells during endotoxemia.

To ascertain possible kidney dysfunction contributing to the pathology, a set of day 3 post-fertilization zebrafish were microinjected with 80ng LPS along with a control group injected with PBS. At 48 hours post-injection, zebrafish were fixed and subsequently stained for Na^+/K^+-ATPase. Under healthy conditions, this ion channel is primarily found concentrated on the basolateral membrane of renal tubule cells, however during renal dysfunction, it becomes diffuse through the cytoplasm, losing its polarity. Confocal analysis revealed that most LPS-injected zebrafish showed this loss in polarity in contrast to controls (Figure 4B).

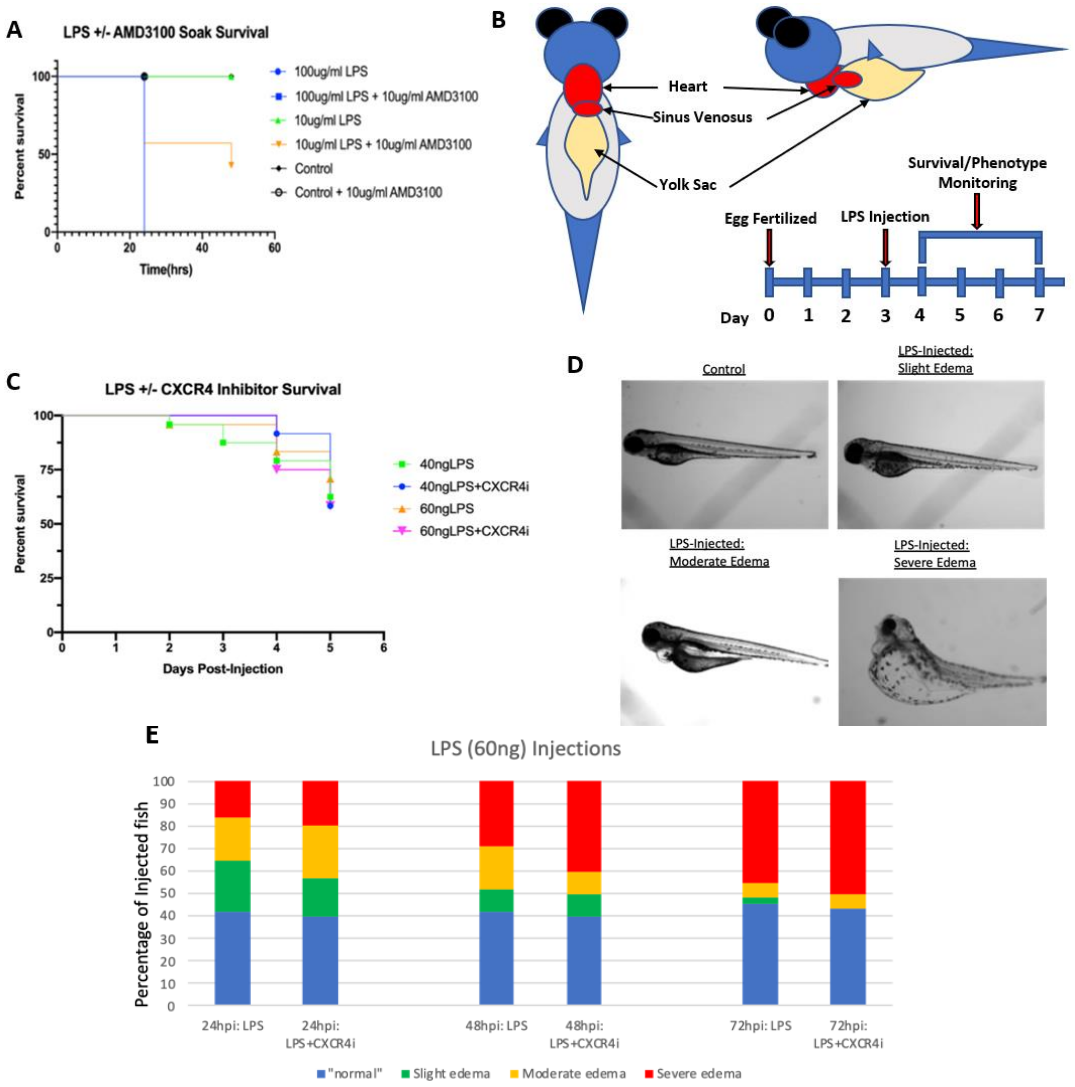

Figure 4: Pseudomonas aeruginosa LPS Experiments. A) Survival of zebrafish larvae during a soak experiment with LPS +/- AMD3100. 15 fish per condition were tested. B) Schematic of zebrafish anatomy in relation to microinjection experiments, along with respective timeline. C) Survival of zebrafish larvae after microinjections of LPS, +/- AMD3100 incubations. Data represents 24 zebrafish per condition; survival of PBS-injected controls was 100% over the timecourse. There was no statistaically significant difference in survival between the injected groups based on dose nor with AMD3100 treatment. Significance did exist between each group and control, however (40ngLPS, 40ngLPS + AMD3100, 60ngLPS, 60ngLPS + AMD3100; p=0.001, p=0.005, p=0.0046, p=0.0004, respectively) D) Images taken at 48hpi; array of edema phenotypes seen after LPS microinjections. E) Proportion of edema phenotypes from 24-72hpi of LPS. Data represents 24 zebrafish per condition tested.

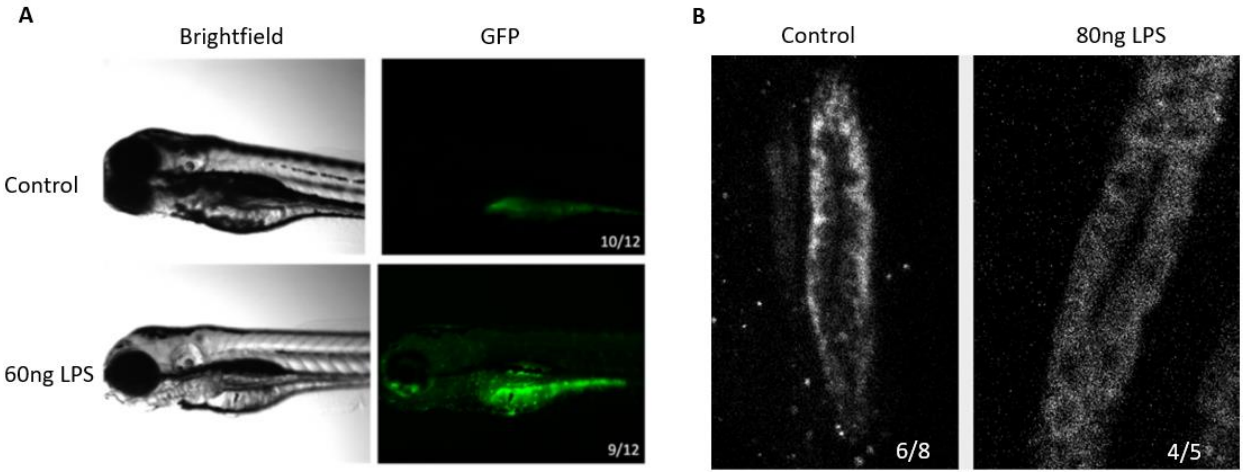

Figure 5: Inflammatory response and kidney injury post LPS microinjections. A) Brightfield/GFP representative images of Tg(TNF-a:GFP) zebrafish 24hpi with LPS. B) Na^+/K^+ ATPase stain of zebrafish pronephros, 48hpi with LPS.

2.4 Discussion

As previously detailed, zebrafish model systems have unique characteristics which make them of interest to study the progression of endotoxemia and sepsis-associated kidney injury. However, there have been few developed models of endotoxemia/S-AKI which are consistent and reliably parallel to known human pathologies. For example, while the zebrafish S-AKI model utilizing live *E. tarda* microinjections does show known hallmarks of the disease, it is highly variable, and overloading zebrafish with a virulent pathogen does not replicate what is seen in human patients. Moreover, it is difficult to conclude exact mechanisms of kidney injury in the background of overwhelming infection. With these factors in mind, we determined to establish a novel model system which overcame these critiques.

While multiple attempts failed to generate noteworthy phenotypes nor death, we eventually experimented with LPS extracted from *Pseudomonas aeruginosa*. Zebrafish are known to have evolved tolerances to many endotoxins, however, a study by Novoa et al. (2009) found that LPS from *Pseudomonas* was able to cause inflammatory responses and mortality in zebrafish[148]. These responses were amplified in the presence of AMD3100, a CXCR4 inhibitor, which is hypothesized to aggregate with TLR4 at the membrane and dampen downstream signaling. This compound was utilized at first in attempt to replicate data of Novia *et al.* (2009) (Figure 3A), however, further experiments of LPS microinjections did not find significant differences to mortality nor edema with AMD3100 treatment (Figures 3B and 3D). As such, AMD3100 use was not continued throughout further experiments. An initial experiment in which Tg(TNF-a:GFP) zebrafish were injected demonstrated the obvious activation of proinflammatory pathways due to LPS exposure. Further experiments should elucidate a more definite timeline of these proinflammatory pathways, and exactly which immune cells over this timeframe contribute to the inflammatory response.

Monitoring of the zebrafish for several days after LPS injections indicated the progression of edema and mortality. Edema can be caused by multiple sources of tissue injury; however, kidney dysfunction is a well-known cause of this phenotype. Depending on the concentration of LPS, this model system was found to cause around 25-50% of injected zebrafish to develop edema. This edema typically became more pronounced over several days following the experiment, typically causing death of the zebrafish.

To further provide insight into the possibility of kidney dysfunction within this model system, a Na^+/K^+ ATPase stain was performed on zebrafish 48 hours post-injection. Tubules of LPS-injected zebrafish notably lost polarization of this ion channel, with its expression being

primarily diffused throughout the cytosol. This observation contrasted with zebrafish control subjects which showed strong basolateral localization. Overall, this provides preliminary evidence that renal tubule cells are truly in a state of stress and dysfunction in response to LPS.

2.5 Future Directions and Conclusion

Future experiments should start by further characterizing this pathology through studying the time course of kidney dysfunction by viewing changes in kidney injury biomarkers, proinflammatory pathways, and cellular stress biomarkers. These studies should assess the pathology at the levels of RNA, protein, functionality, and histology.

Once a deeper understanding of this model system arises, it can serve as a platform to investigate the molecular pathobiology of S-AKI/Endotoxemia as there is still much unknown to explain the mechanisms of kidney injury in these settings. For example, the role of biomarkers TIMP-2 and IGFBP7 can be further explored, as can their role in cell cycle arrest, their interactions with inflammatory pathways, and their relationships with kidney dysfunction. Additionally, the exact cellular consequences of the proinflammatory response—initiated by TLR4—can be elucidated during kidney dysfunction. Along with this topic, the study of the innate immune system during S-AKI could be explored. The roles of neutrophils and macrophages—for better and for worse—could be investigated during a time course of injury and organ dysfunction. As these cells act to initiate and perpetuate the inflammatory response, their overall contribution may be deleterious to renal function. Discovering if/when the cluster around the pronephros, and how this affects kidney function are worthwhile areas to investigate.

If fortuitous, and the model does truly offer a window into this pathology, it may uniquely lend itself to the testing of novel therapeutic compounds to reverse injury and promote regeneration. This is a key strength of a zebrafish model as a high throughput system. Future experiments will help establish the molecular mechanisms of S-AKI, and how it may be possible for therapeutics to intervene during endotoxemia to prevent kidney dysfunction. As there is much to understand regarding S-AKI pathology, this model system could serve to address multiple pathways, interactions, and cellular responses. Indeed, this could even serve to study multiple organ dysfunctions during endotoxemia; the value of which is immeasurable given how grave a reality sepsis is, and how far we must go to develop meaningful and significant treatments. A first step in this process is to have the tools necessary to test these questions, of which a reliable model system will serve as the bedrock.

3.0 Methods

3.1 Zebrafish Husbandry and Microinjections

All studies were approved by the University of Pittsburgh IACUC, and zebrafish were maintained as per published guidelines[149]. Zebrafish were incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, buffered to pH 7.2 with 10 mM HEPES); eggs/larvae were incubated in E3 medium-0.01 mg/l methylene blue (Sigma-Aldrich) up to 72 hours post-fertilization. Throughout experiments, tricaine (Sigma-Aldrich, MS-222) in E3 medium was used for anesthesia (0.168 mg/ml), and for euthanasia (0.64 mg/ml). Day 3 post-fertilization larvae were microinjected with 1-2 nl of respective solutions in the sinus venosus as per previously published protocol[150]. PAMP/DAMP molecules injected included: Lipid A (Sigma-Aldrich, L5399), *E. Coli* LPS (Sigma-Aldrich, L2630), lipoteichoic acid (Sigma-Aldrich, L2515), *P. aeruginosa* LPS (Sigma-Aldrich, L9143), and Hemin (Sigma-Aldrich, H9039), all reconstituted as per manufacture guidelines. For relevant experiments, AMD3100 (Sigma-Aldrich, 239820) was used at 10µg/ml in E3.

3.2 Na^+/K^+-ATPase Staining

At 48 hours post-injection, zebrafish were euthanized, fixed, embedded in JB-4 resin (Polysciences, 18570-1), and stained with monoclonal Na^+/K^+ ATPase antibody (α-6F,

Developmental Studies Hybridoma Bank, Iowa) at 1:25 dilution, and secondary antibody goat anti-mouse cy3 (Jackson Laboratories, 155-165-003) diluted 1:500 as per published protocol[150]. Embedded zebrafish were sectioned on microtome (Shandon Finesse Thermo microtome) and examined by confocal microscopy (Zeiss LSM 700).

3.3 Bacterial Culture

Edwardsiella tarda (*Ewing* and *McWhorter* O1483:H1; ATCC 15947; American Type Culture Collection) was cultured in Tryptic Soy Broth (Sigma-Aldrich, 22092) at 28.5°C overnight while shaking while following published protocol[140]. Prior in injections, bacteria samples were resuspended in PBS to desired concentrations as determined by a spectrophotometer.

3.4 Bacteria Heat Kill, Sonication, Freeze-Fracture, and Antibiotic Incubations

To heat kill, bacterial cultures were placed in a dry bath incubator (Boekell Scientific) set to 65°C for 30 minutes. A sonicator (Branson Digital Sonifier) was set to 20% intensity, and bacterial samples were sonicated for 30 seconds with 5 second on/off pulses. Freeze-fractured bacterial samples were subjected to three freeze/thaw cycles by being submerged in liquid nitrogen for 30 seconds, followed by 5 minutes at room temperature. For antibiotic-kill bacteria

experiments, bacterial cultures were incubated in respective antibiotics (meropenem, Sigma-Aldrich, M2574; streptomycin, Sigma-Aldrich, P4333) for two hours prior to injections.

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