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Investigating the Sexual Dimorphism of Disease Tolerance in Sepsis

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Investigating the Sexual Dimorphism of Disease Tolerance in Sepsis

by

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Abstract

Sepsis is a dysregulated immune response to infection, with mortality rates as high as 30%, however, there are currently no disease-modifying treatments for this disease[1]. One of the reasons why preclinical sepsis discoveries have failed to translate into effective human therapies is that interindividual heterogeneity has historically been neglected in preclinical sepsis research, including the fundamental contributions of biological sex on disease pathogenesis and treatment response. Epidemiologic studies have observed that males have a higher incidence, severity and mortality rate than females in sepsis, however, the mechanisms underlying this bias have not yet been established[2–6]. This thesis aims to examine potential underlying mediators of the sexual dimorphism within sepsis illness severity. We investigated the impact of biological sex on host defence using a well-established mouse model of sepsis induced by fecal peritonitis[7,8]. We used this model to study three principal mediators of sex-based immune response differences: the gut microbiota, sex chromosomes and sex hormones. To uncover differences in these mediators we used a transgenic and germ-free mouse model. Further, we aimed to understand the sex-based influence on infection tolerance and resistance. Lastly, we completed preliminary studies on sex-based differences in infection tolerance using a tetracycline antibiotic as a potentiator of mitochondrial tolerance. This project addressed a critical gap within sepsis research and revealed biological sex differences in infection tolerance and potential therapeutic implications.

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Symbol	Definition
BHI	Brain Heat Infusion
CFU	Colony Forming Units
CLP	Cecal Ligation and Puncture
DPEP-1	Renal Dipeptidase
FIP	Fecal Induced Peritonitis
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GF	Germ Free
KO	Knock Out
LB	Lysogeny Broth
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
MSS	Murine Sepsis Score
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PLF	Peritoneal Lavage Fluid
qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SPF	Specific Pathogen Free
SRY	Sex Determining Region of Y
TNF	Tumor Necrosis Factor
WT	Wild Type
4CG	Four Core Genotypes

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Chapter 1: Introduction

1.1 Significance

Biological sex and its contribution as a critical variable within biomedical research has notoriously been neglected. In 1993, the NIH required that women be included in clinical trials, however, this did not expand to the use of female animals in pre-clinical studies[9]. Thus, male mice were historically used in preclinical studies to investigate various diseases, and these findings were translated into clinical trials and therapeutic design, based solely on the male model. It was not until recently, in 2016, that the NIH required biological sex to be accounted for as a variable in animal models[9]. While more studies have now included males and females in preclinical research, only a staggering 10% of immunology studies analyze their data by biological sex[10].

Sepsis is a prime example of a clear sex bias in the immune response, with little success in designing studies to understand and treat the disease accordingly. Previous pre-clinical discoveries of sepsis therapeutics, which primarily relied on the use of inbred male mice, have failed to translate to humans in clinical trials of diverse patient populations[11]. A key explanation for the failure of mouse-to human translation in sepsis has been the failure to consider the heterogeneity in humans with sepsis, including fundamental considerations like the impact of biological sex on disease pathogenesis and treatment response.

Therefore, the majority of our understanding of disease mechanisms, pathways and treatments in sepsis is based largely on the male response. With emerging evidence that sex bias plays an important modulatory role in immune responses including in sepsis, it is critical that we explore the mechanisms of crosstalk between biological sex and sepsis pathogenesis to fully

grasp the mechanisms of disease , and incorporate these learnings into sex-inclusive therapeutic innovation[10,11].

1.2 Sepsis

Definition

Sepsis is a complex and heterogenous disease that has undergone three refined definitions over the past few decades. Our understanding of the disease and its manifestations are quickly evolving, and the current definition of sepsis is "a life-threatening organ dysfunction caused by a dysregulated host response to infection"[12]. In its most severe form, septic shock, mortality rates are approximately 30%[13]. Because the underlying mechanisms causing this excessive and dysregulated immune response are incompletely understood, there are currently no disease-modifying treatments for sepsis, and treatment is based on antibiotics and supportive care.

Pathophysiology

The dysregulated host response to infection that characterizes sepsis can be triggered by a variety of infections, including bacteria, fungi and viruses. The response is initiated as an attempt by the host to eliminate the infecting pathogens. However, progression to an excessive and dysregulated response leads to collateral damage to the host and the multiple organ dysfunction characteristic of sepsis.

The first step in the host's attempt to combat the infection is recognition of the pathogen and initiation of an innate immune response. This response is initiated by engagement of pattern recognition receptors (PRR's) on host cells by pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs include a broad range of pathogen-derived molecules that typically have the characters of multiple repeating subunits, such as

bacterial endotoxins or fungal β-glucans, while DAMPs are molecules derived from damaged or dead cells from the host that reflects cell injury due to infection[14]. Signalling via PRRs (ex. TLRs, NOD-like receptors) on sentinel immune cells, such as macrophages or antigen presenting cell (APC) leads to the activation of various inflammatory signalling pathways (ex. NF- κB pathway)[15]. These pathways upregulate the expression of cytokines and chemokines (ex. tumor necrosis factor (TNF), Type 1 Interferons (INF)) to initiate a local inflammatory response, and activate the endothelium to recruit neutrophils to the site of infection[13]. Recruited neutrophils infiltrate infected tissues where they deploy their effector mechanisms to help eliminate pathogens, which include phagocytosis to engulf pathogens, release of reactive oxygen species (ROS) and proteolytic enzymes for microbial killing, as well as neutrophil extracellular traps (NETs) for immobilization and extracellular killing[16–18]. While these mechanisms are critical for appropriate and timely clearance of the infection, an excessive response can lead to damage beyond the target pathogen and collateral injury to host tissues [15,18]. Furthermore, when the inflammatory response extends beyond the site of infection and is upregulated systemically, this damage to tissues can occur in body sites further from the initial site of infection, causing multiorgan dysfunction or failure[19]. This dysfunctional response is what drives the progression of disease and damage within the host during sepsis. This largely includes indiscriminate inflammatory tissue damage, resulting in organ dysfunction and in many cases, death. Previous attempts to treat this disease have included blocking specific pro-inflammatory cytokines, such as TNF-α, to limit this pro-inflammatory response and its effects on endothelial activation, however this instead caused an increase in sepsis mortality likely through impaired host defense against infection[20]. To date, sepsis remains a global health priority as recognized by the World Health Organization, however we have not yet found disease-modifying treatments[21]. Altogether

this highlights the complexity of the disease and how treatment is challenging, as the damage to the host is not just from infection but driven by the immune response itself.

1.3 Biological Sex and Immune Response

Biological Sex and Gender in Disease Pathogenesis

Biological sex and gender are often used interchangeably; however, they hold distinct meanings and should be considered as separate variables within research. Biological sex is defined by chromosome and hormone makeup: females have XX chromosomes, ovaries, and estrogen and progesterone as primary sex steroid hormones. Males have XY chromosomes, develop testes, and produce androgens as primary sex hormones. Separate from biological sex, gender is more variable and includes socially constructed roles and behaviours[22]. Gender changes with time, culture and environment, and it is important to acknowledge how gender roles may influence human studies. Social expectations from different genders have been shown to impact the timely decision to seek medical care and increase risk factors based on behavioural patterns[22]. Although we appreciate the role that gender plays in health, our studies use mice which lack the social constructs required for gender, thus we will only be studying biological sex.

Biological sex has a long history of neglect within biomedical research, despite the prominent role it plays in various diseases[23]. Sex-based differences have been found to play a role in various physiological systems, including the immune system. Alongside factors such as age, biological sex has been found to play an important role in shaping in infectious disease response and outcome[24]. In general, females tend to exhibit more robust innate and adaptive immune reactions compared to males, this leads to a quicker elimination of pathogens when compared to males[10]. However, this heightened immune activity can also make females more

vulnerable to inflammatory and autoimmune diseases (80% of autoimmune diseases have a female predominance, such as rheumatoid arthritis and lupus)[25]. Alternatively, males have been found to less effectively tolerate severe infections, including sepsis[7,10]. Despite the longstanding appreciation of differences in immune responses and immune-mediated diseases between males and females, relatively little is known about the mechanisms mediating these differences. Hormonal differences have been hypothesized to differentially affect the immune response between males and females. In support of this, the incidence of autoimmune disease onset as well as disease flares are significantly increased during periods when hormonal differences are maximal, including during puberty and pregnancy. For example, it has been found in systemic sclerosis that women are often diagnosed during puberty (an increase in sex steroid hormones) and are at a much higher risk during pregnancy (significant hormonal increase)[26,27]. Other studies have hypothesized a role of differential X-linked gene dosage between males and females arising from incomplete X chromosome inactivation in females[28]. Additional details of the crosstalk between sex hormones and sex chromosomes and immunity will be discussed in detail below, but these examples demonstrate that much remains to be understood about the mechanisms mediating biological sex bias in infectious and immune disease.

Biological Sex in Sepsis

Within sepsis we see a significant male sex bias in incidence, severity and mortality compared to females[7,11]. While there are many complex variables alongside biological sex when studying humans, including age, gender, genetics and co-morbidities, epidemiological studies that have controlled for co-variables consistently demonstrate a sex bias in adverse outcomes in males[2,4,29,30]. Controlling for co-variables using inbred, age matched mice represents an opportunity to systematically isolate and determine the mechanisms by which biological sex

impacts sepsis pathogenesis. The few published studies of biological sex in rodent models of sepsis have replicated the sex-bias observed in humans, with females showing more favourable outcomes compared to males. A study using estrogen receptor agonists in male mice, and females that had been ovariectomized found that the excessive leukocyte-endothelial interaction were reduced by estrogen signaling, thus maintaining integrity of the microcirculation[31]. Conversely, testosterone depletion in male animals was previously shown to increase male survival in polymicrobial sepsis[32]. However, with the limited information available to date, we do not fully understand the mediators of sex biased outcomes in sepsis, nor the mechanisms driving differential responses. Below, I will review the putative mediators that may drive sex biased immune response that will be studied in this thesis project.

1.4: Biological Mediators of Sex Differences

Sex Chromosomes

At a basic genetic level, males and females differ on their 23rd set of chromosomes, males have XY composition and females have XX. This difference in chromosomes encodes the biological distinction between "male" and "female" sex. The *sry* gene (sex determining region of Y) encoded on the Y chromosome is a master regulator of sexual differentiation, the expression of which in males results in the development of testes[33]. The absence of this gene in XX females results in no formation of testes, and the development of female reproductive organs (ovaries). In an effort to balance X-linked gene expression between females with two X chromosomes and males with one, one X chromosome in females is randomly inactivated in each cell during development, this is called X chromosome inactivation (XCI)[34]. However, incomplete X-chromosome inactivation occurs commonly, resulting in differential X-linked gene dosages that can also contribute to phenotypic differences between males and females. In addition to sex

determining regions, each chromosome contains various genes that contribute to immunity. According to the National Human Genome Research Institute, the X chromosome is three times larger than Y, with approximately nine hundred genes and the Y gene contains only fifty-five[35]. The X chromosome also contains more genes for immune function than any other chromosome in the human genome[36]. Some critical genes include pattern recognition receptors (PRRs), such as toll-like receptors (TLR7, TLR8), which are important in recognizing and identifying pathogens to alert the immune system of infection. The standard inactivation of one of the X chromosomes (XCI) in females occasionally fails, resulting in two transcriptionally active X chromosomes and an increased expression of X-linked genes compared to a normal X-inactivated female or an XY male[37]. XCI failure resulting in an increased X gene dosage can either act as helpful or harmful depending on the disease in question and the X-linked genes being overexpressed. Increased expression of X-linked PRRs could aid in the strong female innate immune response, but persistence of these receptors could also drive chronic inflammation and the female predominance of autoimmunity[38]. Alternatively, a few studies have suggested that Y chromosome linked genes play a strong role in immunity bias, and the absence of this chromosome in females may drive immune differences[33,39]. Thus, chromosomal and genetic differences between males and females are one example of potential biological factors contributing to sexual dimorphism of immune responses.

Gonadal Hormones

Males and females have a differential production of sex steroid hormones that drive sexual development, secondary sex characteristics, and reproductive functions[40]. Hormones, including estrogen, progesterone, and testosterone, exert their effects by binding to specific receptors within target cells. These hormones can easily traverse cell membranes due to their

lipophilic nature, and binding to their respective intracellular receptors (ex. ERα, NR3C4) to form hormone-receptor complexes that traffic to the nucleus[41,42]. Within the nucleus, the hormone-receptor complexes modulate gene expression, either activating or repressing specific genes[42]. Hormone receptors are expressed by many cells throughout the body, including immune cells. For example, ER-α and ER-β estrogen receptors are expressed by natural killer cells and macrophages, and genetic deletion or blockade of these receptors has been shown to impair protective immune responses against viral infections and tumor immunity[37].

Estrogen has been proposed as a protective factor in females during acute illness , more specifically, estrogens have been found to play an important role in the modulation of inflammatory response[43,44]. Recent studies suggest that estrogens inhibit inflammatory signaling pathways such as NF-kB and the activity of the NLRP3 inflammasome, thus decreasing the production of pro-inflammatory cytokines such as IL-1β and IL-18[43,45]. Recently, it has been suggested that sexual dimorphism of immune responses in sepsis may be mediated by the protective effects of estrogen in females. In an article by Zhao *et al*., it was found that the administration of estrogen could reduce sepsis-induced muscle wasting in mice[44]. Septic mice administered 17β-estradiol displayed significant decreases in muscle wasting and hypothalamic inflammation compared to septic mice without treatment[44]. Alternatively, testosterone has been found to impart immunomodulatory effects, with recent studies reporting that testosterone exerts substantial immunosuppressive effects on both the innate and adaptive immune systems, diminishing the production of immunoglobulins and cytokines[46]. However, whether the differential sex hormone milieu in males and females mediates sexual dimorphism of acute inflammation and illness severity in sepsis remains unknown.

Gut Microbiota

The composition of commensal microorganisms within the gut microbiome between males and females has been shown to differ[7,47]. It is now well established that the gut microbiome is critical for mucosal and systemic immune development and homeostasis, and that dysbiosis contributes to a variety of immune and inflammatory diseases including Inflammatory Bowel Disease (IBD), Type 1 Diabetes (T1D) and autoimmune arthritis[48]. More recently, the gut microbiota has emerged as an important modulator of sepsis pathogenesis in mice and humans[7,49,50]. As the gut microbiome is a crucial regulator of mucosal and systemic immune development and function, sex-based differences in the microbiome may mediate sexual dimorphism of immune function. In numerous studies the microbial communities in the gut have been found to vary between males and females[51,47]. However, the specific differences at a species level have not been comprehensively determined for humans[52–54]. Different bacteria present in the gut have been known to either mount pro or anti-inflammatory responses, thus, the naturally occurring abundance of a species may play a role in the sexual dimorphism of immune response to disease[51]. Yurkovetskiy *et al*. and Markle *et al*. both found that in studying the gut microbiome between sexes, differences did not begin to appear until after puberty when hormone differences become apparent[55,56]. These studies examined sex differences and the influence of the gut microbiome using Germ-Free (GF) mice, which lack a gut microbiome in comparison with SPF mice containing a conventional laboratory microbiome. Yurkovetskiy *et al*. found that the incidence and early onset of Type 1 Diabetes (T1D) in SPF mice was greater in females compared to males, however this difference was abolished under GF conditions [55]. Further, GF mice that had been given the microbiota of male SPF mice, were protected from early onset T1D[55]. Similarly, in the Markle *et al.* study, transfer of adult male SPF microbiome to immature females, protected the females as adults from T1D[56]. Changes in the gut

microbiome can create a predisposition to sepsis by facilitating the growth of harmful pathobionts, fostering an imbalanced immune response, and reducing the production of beneficial short-chain fatty acids (SCFAs)[57]. Differences within the gut microbiota between males and females, and the known impact of microbiome dysbiosis on host immune response may play an important role in the differences between males and females in sepsis.

1.5 Infection Tolerance and Resistance

Definition

Successful host immune responses to infection require two fundamental components – resistance of the invading pathogens, and tolerance of resulting disease. The first, resistance, is the ability of the host to eliminate the infection and minimize pathogen burden[58]. Infection resistance has been well studied within the field of sepsis, including the various immune effector mechanisms that help capture and clear invading pathogens. In contrast, mechanisms of infection tolerance are less well understood. Infection tolerance refers to host mechanisms that are aimed at limiting the damage caused by infection, independent of pathogen burden[58, 59]. As such, differences in sepsis outcomes between males and females may be due to differential infection resistance and/or tolerance, which will be determined in this project.

Infection Tolerance and Resistance in Sepsis

Within sepsis, there are a number of resistance mechanisms that allow the host to eradicate the infection and limit pathogen replication and dissemination. The systemic response to infection as described in the section above is a key player in disease resistance to clear pathogens from the body. The activation of pro-inflammatory cytokines is critical in the initial steps to orchestrate immune cells to the site of infection[18]. Following an appropriate systemic

inflammatory response, innate immune cells recruited to tissues are critical in order for the host to resist the infection. Neutrophils are potent antimicrobial innate immune effector cells that are crucial for host defense against invading pathogens[60]. These cells carry a number of effector mechanisms to kill pathogens, including phagocytosis, neutrophil extracellular traps (NETs), proteases and reactive oxygen species release [61,62]. To defend against invading pathogens, neutrophils must be recruited from the blood into tissues at the site of infection. Neutrophil recruitment to infected peripheral tissues requires a carefully orchestrated molecular cascade of events between neutrophils and the vascular endothelium[63]. Neutrophils first begin by tethering and rolling along the endothelium, mediated by E and P selectins. This is followed by activation of integrins by chemokine receptor signaling in the neutrophil, resulting in adhesion and arrest. The neutrophil then uses integrins and chemokine gradients to crawl along the vascular endothelium. Finally, neutrophils exit the vasculature via transendothelial migration or paracellular migration with the assistance of adhesion molecules (ex. PECAM-1, JAMs and CD99)[18,61].

Some organs such as the lung and liver do not follow this classical paradigm. Instead, neutrophils are recruited to these organs via non-classical mechanisms; lacking a rolling step, recruitment occurring in the capillary vessels and direct adhesion using nonclassical adhesion molecules[64]. DPEP-1 was recently discovered as a novel nonclassical adhesion molecule that is critical for recruitment of neutrophils to the lung, liver and kidney during sepsis[65]. Until recently, the molecule DPEP-1, known as renal dipeptidase, had only been known for its function as a renal enzyme located on the kidney epithelium, responsible for hydrolyzing substrates[65]. A new function of this molecule as a mediator of neutrophil adhesion was recently discovered, independent of its enzyme activity. Choudhury *et al*. found that DPEP-1 was expressed on the

lung and liver endothelium, and mediated adhesion of neutrophils and recruitment from the blood into the lungs and liver[65]. In this study, blocking DPEP-1 in models of sepsis (endotoxemia and cecal ligation and puncture induced peritonitis) resulted in a significant decrease of neutrophil recruitment to inflamed lung and liver vasculature, translating into improved survival[65]. Due to the critical role of neutrophils in disease resistance in sepsis, DPEP-1 is a target for additional studies of acute inflammation of the lungs and liver.

While mechanisms of disease resistance focus on pathogen clearance, disease tolerance works to maintain health of the host during infection. During sepsis, homeostasis is heavily disrupted by both pathogen derived factors (toxins, enzymes, PAMPs) and host derived factors arising from pathological inflammation (oxidative stress, pH imbalance, metabolic alterations and a lack of energy substrates such as glucose and oxygen required for ATP production)[66]. Fortunately, the host engages a number of active mechanisms to mitigate cell injury and dysfunction caused by these harmful mechanisms, collectively referred to as infection tolerance. For example, during sepsis when the host is in oxidative stress and cellular hypoxia, cells upregulate hypoxia inducible factor (HIF) pathway, which shunts energy production towards anaerobic glucose metabolism to provide an alternate energy source in the absence of a reliable oxygen source[66]. The impact of biological sex on infection resistance and tolerance mechanisms has yet to be understood.

Mitochondrial Tolerance in Sepsis

The mechanism of infection tolerance that has been best studied is mitochondrial tolerance responses. Recent studies have found a mitochondrial stress response during infection that provides disease tolerance through adapting alternative metabolic pathways to provide adequate energy. It is well established that a breakdown of mitochondrial tolerance responses,

and mitochondrial dysfunction, represents a core mechanism of pathogenesis in sepsis. Interestingly, recent ground-breaking work from Colaço *et al.* revealed the ability to therapeutically potentiate mitochondrial tolerance mechanisms to improve outcomes in mouse models of sepsis through administration of tetracycline antibiotics (specifically doxycycline), independent of its antimicrobial effects[67]. Doxycycline was able to provide protection by selectively acting on mitochondrial ribosomes, resulting in a shifting of metabolism towards fatty acid oxidation and glucocorticoid sensitivity[67]. This drug potentiated tolerance pathways and upregulated alternative pathways to provide energy for the host during bacterial sepsis. This study offers insight to potential mechanisms that may be targeted therapeutically to improve disease tolerance within sepsis. However, like the majority of sepsis studies in the past, this landmark study only included male mice, and therefore the potential impact of biological sex on mitochondrial tolerance within sepsis remains unknown, as well as the generalizability of this therapeutic development to females with sepsis. When comparing mitochondrial differences, we see that in many tissue types, females have an increased antioxidant capacity and lower ROS production compared to males[68]. While this bias exists in mitochondrial function, we are still unsure of how this may translate to mitochondrial tolerance in sepsis.

1.6: Mouse Models of Sepsis

Together with collaborators from the National Preclinical Sepsis Platform (NPSP), members of our lab recently contributed to a systematic review of biological sex and sepsis in preclinical models that identified very few animal sepsis studies that investigate the effect of sex on treatment outcomes[11]. We have yet to understand the mechanisms underlying the male-sex bias in sepsis, and this is partially due to the lack of inclusion of biological sex as a variable in

preclinical studies. Table 1 below highlights the summary of the key animal models used to study sepsis. While endotoxemia and bacterial injection are other common sepsis models, they neglect the complex microbial interactions of polymicrobial infections. Additionally, CLP fails to provide consistent standardization of cecal content release, and subjects animals to additional tissue trauma during surgery. An alternative to CLP that yields a titratable, reproducible, and standardized sepsis model is fecal-induced peritonitis, wherein donor fecal (or cecal) contexts are weighed, homogenized, and injected as a slurry in the peritoneal cavity of recipient mice. In this thesis project, we utilized the FIP model based on foundational pilot studies carried out by the NPSP[69]. The NPSP has completed various pilot experiments using this model, thus we were able to collaborate with this national group, and adapt the model within our facility to standardize dosage and timepoints. By closely mirroring the human condition of abdominal sepsis, this model allowed for the study of illness severity, systemic inflammation, and immune pathophysiology in polymicrobial sepsis. Further, to verify our results, we additionally used a monomicrobial model of infection (*E. coli*) to further support our findings using a controlled model.

	Cecal Ligation and Puncture (CLP)	Endotoxemia	Feal Induced Peritonitis (FIP)	Bacterial injection
Method to induce sepsis:	Laparotomy is performed under anesthetic to ligate a section of the cecum.	i.p injection of lipopolysaccharide (a single component of the gram-negative bacterial membrane).	i.p injection of a standardized dosage of cecal slurry from a donor mouse.	i.p or i.v injection of a single bacterial strain to induce sepsis.
Advantages:	Theoretically similar to human bacterial peritonitis (polymicrobial insult followed by bacteremia).	Replicates sepsis physiologically without using a microbial source.	Minimally invasive model to induce polymicrobial sepsis.	Standardized dosage of infection.
Disadvantages:	Variability in size of ligation and cecal content released. Animal undergoes surgery.	Activates only one TLR pathway.	Variability in bacterial composition between donors.	Uses only one source of pathogen.

Table 1: Comparison of key animal models of sepsis
Description of each infection model (CLP, Endotoxemia, FIP, Bacterial Injection) in addition to the main advantages and disadvantages within sepsis studies[69,70].

1.6 Research Rationale

Sepsis is a global health concern that lacks disease-modifying treatments. Epidemiologic studies of human sepsis, as well as previous studies of rodent sepsis have reported a strong male bias towards adverse sepsis outcomes, but the mechanisms and treatment implications are unknown. Therefore, my thesis will aid in understanding the contribution of key biological sex mediators on the sexual dimorphism of sepsis illness severity. Given the prevalent sex bias in sepsis and lack of successful clinical studies and sex-based therapeutics, these findings will work to fill the knowledge gap within the literature and provide insight into translational studies.

1.7: Hypothesis and Aims

The overarching hypothesis of this project is that biological sex modulates the severity of sepsis through mechanisms driven by sex-based differences in the gut microbiota.

Specific Aims:

1. Define the impact of biological sex on host defense in a mouse model of polymicrobial sepsis.
2. Determine the relationship between biological sex and the gut microbiome composition on host defense and illness severity in polymicrobial sepsis.
3. Determine the impact of biological sex on neutrophil influx into the liver and lungs during sepsis.

Chapter 2: Materials and Methods

Animal Husbandry

All experimental mice used within this thesis were on a C57BL/6 genetic background and aged 8-12 weeks as indicated in figure legends. Specific pathogen-free (SPF) mice were housed and bred in the Mouse Barrier Unit (MBU) of Foothills Campus, University of Calgary. For experiments comparing Germ Free (GF) and SPF mice, SPF mice were purchased from Jackson Laboratories and acclimatized within the MBU for a minimum of 1 week prior to experimental use. GF mice were housed and bred in the Germ-Free Facility within the International Microbiome Centre located at the Foothills Campus, University of Calgary. These GF mice are born and bred under germ free conditions, containing no microorganisms. DPEP-1 knockout and wildtype SPF mice were provided by the Daniel Muruve lab and housed in the Clara Christie Centre for Mouse Genomics (CCCMG) facility at the Foothills Campus, University of Calgary. Transgenic Four Core Genotype mice embryos (XY^{-Sry} males) were imported from Jackson Laboratories and implanted in C57BL/6 SPF females to be born to the SPF background. Once these mice were adults, they were bred with wildtype SPF females within our MBU facility to produce all four genotypes of mice (Figure 19-21). Genotyping was completed by a laboratory technician using ear skin samples to track corresponding genotypes. Animal protocols performed were approved by the University of Calgary Animal Care Committee and followed in accordance with Canadian Guidelines for Animal Research (Protocol #: AC19-0139).

Infection Models

Fecal Induced Peritonitis

The Fecal Induced Peritonitis (FIP) model is a minimally invasive method used to induce sepsis in mice. The cecum contents from 8-12 week old donor female mice are collected,

weighed, and homogenized using a 30mL syringe. The homogenate is filtered through a 100µm strainer using 7mL/g of prepared phosphate buffer (50mM). The filtered slurry is centrifuged at 3000g for 25 minutes and the supernatant is poured off and the slurry content is weighed. The remaining slurry content is homogenized in a 5% dextrose 10% glycerol solution for a final concentration of 100mg/mL. This concentration is then diluted with PBS to a final dosage of 1.0mg/g based on the weight of each mouse. Inoculum is then injected into the peritoneal cavity of the mouse. Unless otherwise stated, the length of time for infection is 6 hours to encapsulate the acute inflammatory period induced by the model.

E.coli ST131

The monomicrobial model employed to induce bacterial sepsis within mice was an intraperitoneal injection of *Escherichia coli* ST131. 2µL of frozen glycerol stock was thawed and combined with 10mL of Lysogeny Broth (LB) in a shaking incubator at 37 °C overnight. 5mL of LB was then combined with 1mL of the overnight culture and placed back in the shaking incubator for 2 hours at 37 °C. 1mL of subculture was placed in the centrifuge at 8000rcf for 5 minutes and the supernatant was poured off. The remaining contents are vortexed with 1mL of PBS and diluted to the desired dosage ($5x10^7$ CFU) per injection volume (300µl). Unless otherwise stated, the length of time for infection is 6 hours to encapsulate the acute inflammatory period induced by the model.

Endotoxemia

Lipopolysaccharide (LPS) is a component of the gram-negative bacterial membrane and is used to induce endotoxemia within mice. Purified LPS glycerol stock is thawed from -80 °C and vortexed with PBS. Mice are given an intraperitoneal injection of 1mg/kg in a total volume of 300µl. Unless otherwise stated, the time of infection is 3 hours.

Murine Sepsis Score

The Murine Sepsis Score (MSS) is an observational behavioural scoring system for mice, used to quantify the degree of illness during an experiment. Mice are scored on a sale of 0-4 in 7 categories, 0 indicating no signs of illness, and 4 being extreme. The sections mice are scored in are: appearance, level of consciousness, activity, response to stimulus, eyes, respiration rate and respiration quality (Table 1). Mice exhibiting a total score of >21 or a score or >3 in the categories of respiration rate or quality, will have reached their humane endpoint and are euthanized.

Peritoneal Lavage

Peritoneal lavage is a technique used to collect fluid (PLF) from the area of infection. This is completed after the mouse has been euthanized. 3mL of cold PBS is injected into the peritoneal cavity of the mouse, and the abdomen is massaged for 10 seconds. After this, the fluid is withdrawn from the cavity and stored on ice until used for further analysis.

Flow Cytometry

After infection, mice were anesthetized with isoflurane until they became unconscious. Mice remained under anesthetic while cardiac perfusion was performed. 30mL of 30°C PBS was injected into the left ventricle after cutting the inferior vena cava. Consistent pressure was applied to push PBS through the syringe and perfuse blood throughout the vasculature. Liver, lung and spleen were removed and placed in cold PBS. Tissues were homogenized individually using gentleMACS tissue dissociator and homogenate was strained through a 100µm cell strainer. RBS lysis was completed using ACK lysis buffer and samples were centrifuged (2500rpm, 3min, 4°C). Live cells were counted using a hemocytometer and aliquoted into equivalent amounts for fixing and staining. Cells were fixed, labeled and stained with

fluorochrome-conjugated secondary anti-mouse antibodies. Cells were acquired using the BD FACS Canto and populations were identified using a consistent gating strategy (Figure 8).

Tissue Culture

Tissues samples of the liver (upper left quadrant), lung (entire left lung), kidney (entire left kidney), spleen (anterior half of spleen), PLF and whole blood were collected from mice after euthanasia. Tissue samples (liver, lung, kidney and spleen) were weighed in 1mL of PBS homogenized (30Hz, 3min). Serial dilutions in PBS were completed from 0 to 10^8 for each sample. 30µL of each dilution was plated on BHI (for FIP experiments) or LB (for *E. coli* experiments) for each sample. Plated samples were allowed to dry within the BioSafety Cabinet and then incubated at 37°C for 16-24 hours. After this point the lowest serial dilution of each sample that displayed a minimum of 6 colonies with clear isolated colonies, was counted and recorded for each sample.

MPO Activity Assay

This assay was completed to quantify the myeloperoxidase activity level within the homogenized lung tissue samples of mice. Frozen lung homogenate was thawed on ice and added to the MPO buffer. Samples were placed in a 96 well plate in addition to hydrogen peroxide immediately before running the plate on the SpectraMax Plate reader. The colorimetric change in oxidation of the sample was measured in time periods of 0, 30, 60 and 90 seconds. The colour change observed overtime indicated the activity of the myeloperoxidase enzyme in each of the tested samples.

Multiplex Cytokine Analysis

Plasma was collected from whole blood (Centrifuged for 10 minutes at 20000g) and stored at -80°C until use. The V-Plex Proinflammatory Panel 1 Mouse Kit was used from Meso Scale Discovery to quantify the levels of 10 pro and anti-inflammatory cytokines and chemokines in the plasma of experimental mice. Procedure was followed as outlined in the protocol; no alterations were made.

RT-qPCR

Quantitative PCR was used to quantify the levels of various gene expressions in the lung and liver tissue from experimental mice. Organs were preserved in *RNA*later from ThermoFisher and stored at -80°C until use. RNA was extracted using the RNeasy Mini Kit from Qiagen. PCR was performed on SimpliAmp Thermal Cycler by AppliedBiosystems. cDNA was created using iScript Reverse Transcription Supermix for RT-qPCR. The protocol for qPCR was PowerUp SYBR Green Master Mix. qPCR was performed on Quant Studio 3 and Quant Studio 6. All procedures were followed as outlined in the protocol; no alterations were made.

Statistical Analysis

All data are presented as median values ± range. Statistical analysis was completed through the program Prism by GraphPad, where the degree of significance can be calculated using parametric and nonparametric tests, survival and dose response curves. The results of these analyses are significance values relative to the set significant value of less than 0.05. Specific tests and parameters are indicated in figure legends.

Chapter 3: The Role of Sexual Dimorphism in Sepsis Illness Severity

Introduction

Preclinical studies of bacterial and viral sepsis using animal models have consistently observed greater illness severity in males compared to females[31,71]. However, the underlying mechanisms driving this sex-biased difference in sepsis severity remain unknown. To further understand this male bias in sepsis severity, we first aimed to determine the impact of biological sex on host defence against bacterial infection in mouse models of sepsis. For this, we began by adapting a commonly used model of sepsis, Fecal Induced Peritonitis (FIP), based on previous preliminary data from our collaborators within the National Preclinical Sepsis Platform[8]. These preliminary data demonstrated the induction of severe sepsis in mice, 1.0mg/g intraperitoneal dose of fecal slurry, as shown by an 83% mortality rate in the 72-hours following administration[69]. Consistent with CACC animal ethics guidelines, and recommendations from international sepsis research consensus statements, this project does not investigate mortality endpoints, and instead focused on illness severity as the outcome of interest. The preliminary data from the NPSP study above found that mortalities from a 1.0 mg/g FIP dose began after ~16-hour timepoint, thus we decided to use an earlier timepoint to observe illness severity in live mice. The NPSP FIP model was adapted for our study in two ways: (1) Given our interest in using this model in Germ-Free mice, we utilized mouse cecal contents as the infection inoculum, rather than rat contents as used by NPSP, as this would not be allowed in our GF facility and (2) we optimized the dosage and timepoint for our experiments (as described below). In addition to this polymicrobial model of sepsis, we further validated our results in an additional commonly used model of monomicrobial sepsis induced by *E. coli* peritonitis[50,61,72,73].

Using these models of sepsis, we aimed to define the impact of biological sex on illness severity and host defence during infection. We characterized illness severity differences between

male and female mice using the Murine Sepsis Scoring System (MSS), and studied the differences in infection resistance by quantifying pathogen burden and dissemination after sepsis[69,74]. Further, we studied sex differences in systemic inflammation, neutrophil trafficking, and mitochondrial infection tolerance to understand potential mediators driving the difference between male and female sepsis illness severity.

Results

3.1 Development of Fecal Induced Peritonitis (FIP) Model

For the purpose of our experiments, we defined 'optimal illness' as quantifiable illness severity that enabled measurable biological sex differences, however not severe enough to require humane-endpoint euthanasia prior to the experimental timepoint. Illness severity was quantified using the Murine Sepsis Severity Score (MSS) (Table 2). The MSS is a published and validated behavioural scoring system to quantify the degree of illness severity in infected mice by scoring each animal in seven different categories (Table 2)[74]. We utilized the MSS to track disease progression and ultimately quantify the illness severity of each mouse, higher scores correlate with mice that are more ill (Table 2). For this, we first completed dose finding pilot experiments using various concentrations of cecal slurry ranging from 0.1 mg/g up to 1.0 mg/g, and found that 1.0mg/g induced measurable, illness severity in male and female mice using the Murine Sepsis Severity Index (Figure 1). Next, we conducted timepoint finding experiments and infected mice with 1.0mg/g of slurry to observe illness progression over a 5-day period. We found that within 12 hours of infection, mice were developing severe illness that required euthanasia for humane endpoints (defined by MSS>21, or a score of >3 in any individual category). Therefore, to achieve measurable illness severity while avoiding mortality, we chose an acute timepoint of 6 hours post infection as our experimental endpoint (Figure 2).

Although our study is focused on the impact of biological sex in the septic host, we recognize that the sex of the donor from which cecal contents is used as the infectious inoculum may impact the severity of sepsis in the recipient. To determine whether donor sex impacts severity of sepsis in our FIP model, we collected cecal contents from both male and female donors and injected them into both male and female recipients. At 6 hours after infection, we found that illness severity was independent of donor sex, and instead, was significantly different between recipient sexes (Figure 3). These data therefore indicate that the cecal contents of either sex is acceptable for the induction of severe sepsis in our FIP models. For consistency and practical reasons arising from mouse availability, we chose to use female donors for cecal contents for the remainder of our study.

In summary, these data led us to concluded that 1.0mg/g of female fecal slurry over a timepoint of 6 hours induced optimal illness and we utilized this model for our study[8,69].

Variable	Score and Description
Appearance	0. Coat is smooth 1. Patches of hair piloerected 2. Majority of back is piloerected 3. Piloerection may or may not be present, mouse appears “puffy” 4. Piloerection may or may not be present, mouse appears emaciated
Level of Consciousness	0. Mouse is active 1. Mouse is active but avoids standing upright 2. Mouse activity is noticeably slowed 3. Activity is impaired. Mouse only moves when provoked, movements have a tremor 4. Activity, severely impaired. Mouse remains stationary when provoked, with possible tremor
Activity	0. Normal amount of activity. Mouse is involved in any of the following activities: Eating, drinking, climbing, running, fighting 1. Slightly supressed activity. Mouse is moving around at the bottom of the cage 2. Supressed activity. Mouse is stationary with occasional investigative movements 3. No activity. Mouse is stationary 4. No activity. Mouse is experiencing tremors, particularly in the hind legs
Response to Stimulus	0. Mouse responds immediately to auditory stimulus or touch 1. Slow or no response to auditory stimulus, strong response to touch (moves to escape) 2. No response to auditory stimulus, moderate response to touch (moves a few steps) 3. No response to auditory stimulus, mild response to touch (no locomotion) 4. No response to auditory stimulus. Little or no response to touch. Cannot right itself if pushed over
Eyes	0. Open 1. Eyes not fully open, possibly with secretions 1. Eyes at least half closed, possibly with secretions 2. Eyes half closed or more, possibly with secretions 3. Eyes closed or milky

Respiration Rate	0. Normal, rapid mouse respiration 1. Slightly decreased respiration (rate not quantifiable by eye) 2. Moderately reduced respiration (rate at the upper range of quantifiable by eye) 3. Severely reduced respiration (rate easily countable by eye, 0.5s between breaths) 4. Extremely reduced respiration (>1s between breaths)
Respiration Quality	0. Normal 1. Brief periods of labored breathing 2. Labored breathing, no gasping 3. Labored with intermittent gasping 4. Gasping

Table 1: Murine Sepsis Scoring System (MSS). Table used in illness severity scoring[74].

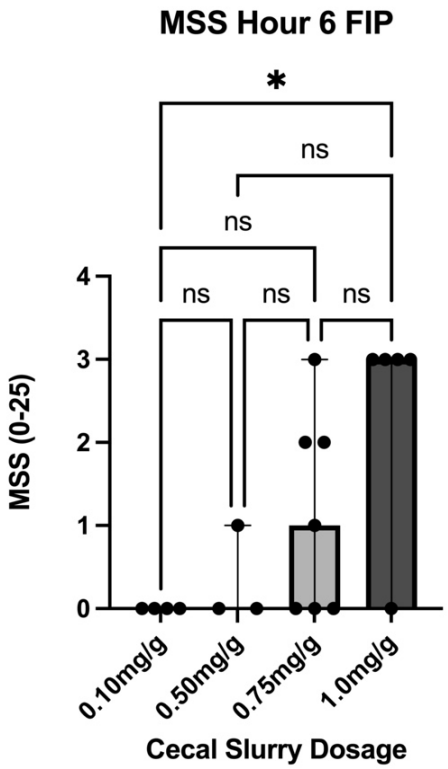

Figure 1: Illness severity during acute polymicrobial sepsis caused by FIP of increasing cecal slurry dosages.
Age-matched male and female C57BL/6 mice were injected intraperitoneally with 0.1, 0.5, 0.75 or 1.0mg/g of cecal slurry to induce sepsis. Illness severity scores were quantified at the 6h endpoint using the Murine Sepsis Scoring System (MSS). Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Kruskal Wallis test and Dunn post-hoc for multiple analysis comparisons. ns, not significant. *($p<0.05$).

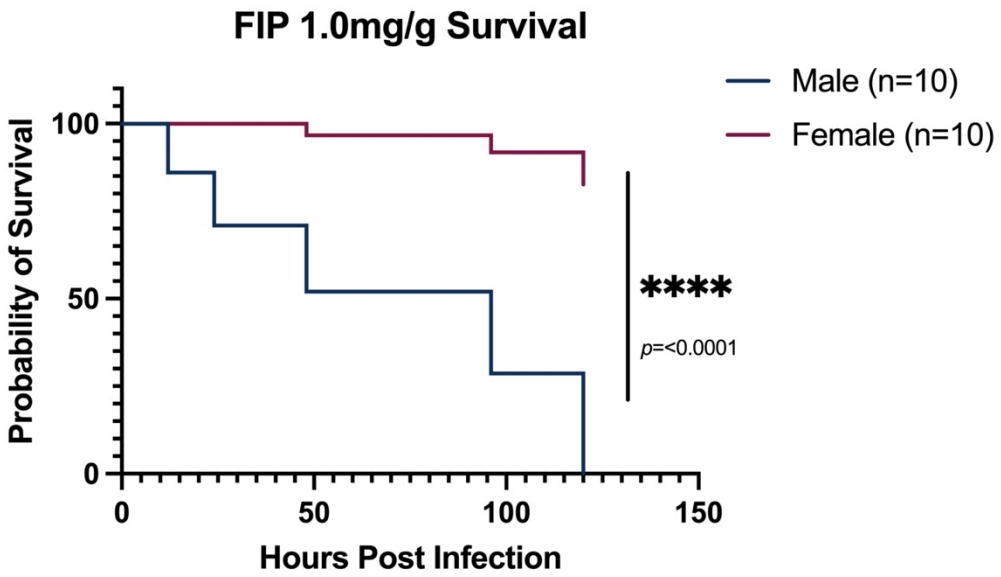

Figure 2: Survival curve during acute polymicrobial sepsis caused by 1.0mg/g FIP. Age-matched male (n=10) and female (n=10) C57BL/6 mice were injected intraperitoneally 1.0mg/g of cecal slurry to induce sepsis over a 5-day (120h) period.. Illness severity scores were quantified at the 6h endpoint using the Murine Sepsis Scoring System (MSS). Deaths were recorded if a mouse was humanely euthanized based on their MSS score or they were found deceased. Survival rates were analyzed using a Kaplan-Meier non-parametric test and p value was calculated using Mantel Cox test. ****($p<0.0001$).

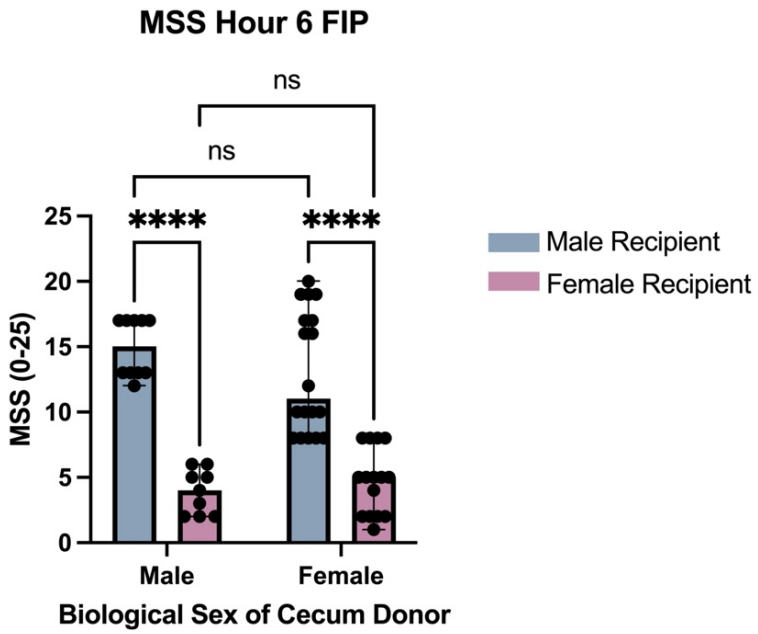

Figure 3. Illness severity during acute polymicrobial sepsis caused by FIP from male or female donors.
Age-matched male and female C57BL/6 mice were injected intraperitoneally with 1.0mg/g of either male or female donor cecal slurry to induce sepsis. Illness severity scores were quantified at the 6h endpoint using the Murine Sepsis Scoring System (MSS). Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Kruskal Wallis test and Dunns test for multiple comparisons. ns, not significant. ****($p<0.0001$).

3.2 Biological Sex and Illness Severity

To determine the impact of biological sex on sepsis severity, we quantified illness severity between female and male mice in response to polymicrobial and monomicrobial sepsis using the Murine Sepsis Severity Index (MSS) described above. First, polymicrobial sepsis was induced by FIP, and illness severity (MSS) was measured every 2 hours up to our 6 hour endpoint (Figure 4). We found that male mice began displaying significantly higher MSS illness severity scores compared to females by 2 hours after infection (Figure 4). The greatest difference between males and females prior to any mortality, was seen at 6 hours post infection (Figure 2 above and 4).

Next, to determine whether this observation of male-biased illness severity was unique to polymicrobial sepsis or generalizable to sepsis caused by other infections, we repeated these experiments using a model of monomicrobial sepsis induced by *E. coli* peritonitis. Consistent with our observations during polymicrobial sepsis, male mice displayed significantly higher illness severity scores compared to females during monomicrobial (*E. coli*) sepsis (Figure 5). Overall, these results demonstrate that male mice develop significantly greater illness severity compared to female mice in response to acute bacterial sepsis, consistently across multiple different infections.

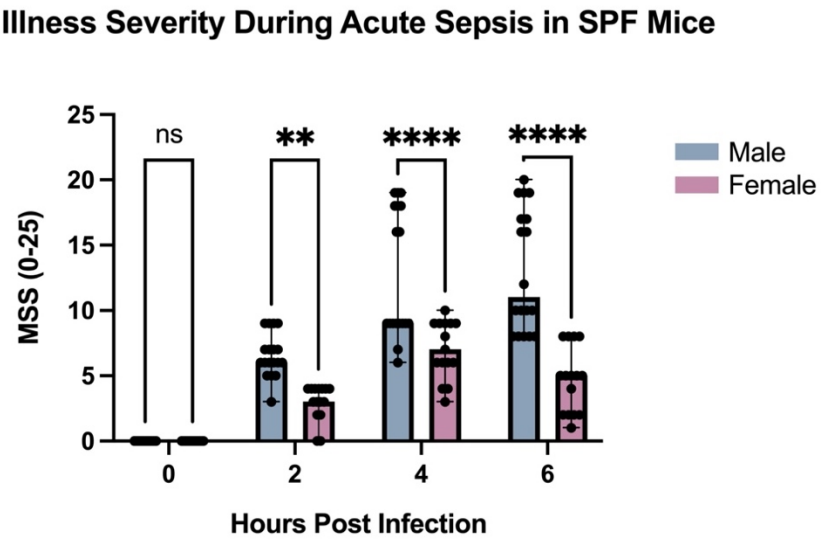

Figure 4. Illness severity during acute polymicrobial sepsis caused by FIP.
Age-matched male (n=18) and female (n=15) C57BL/6 mice were injected intraperitoneally with 1.0mg/g of donor (female) cecal slurry to induce sepsis. Illness severity scores were quantified every two hours up to a 6h endpoint using the Murine Sepsis Scoring System (MSS). Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Šídák's multiple comparisons test. ns, not significant. **($p<0.01$), ****($p<0.0001$).

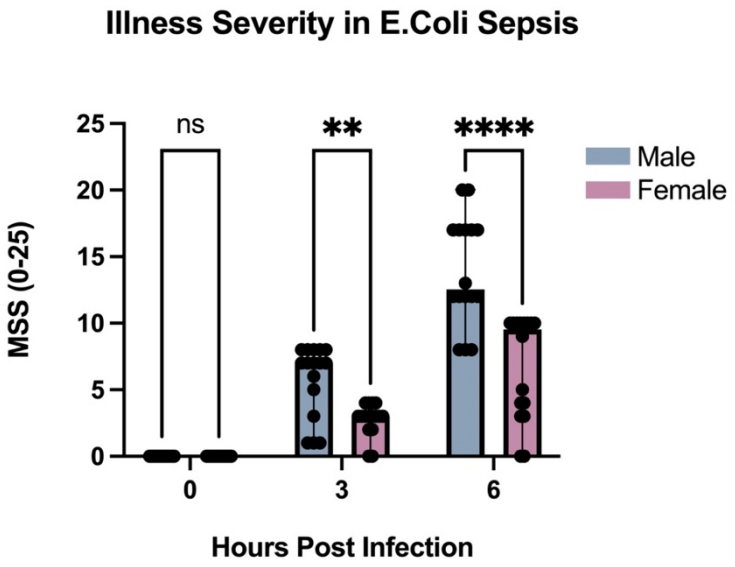

Figure 5. Illness severity during acute monomicrobial sepsis induced by *E. coli* peritonitis. Age-matched male (n=16) and female (n=16) C57BL/6 mice were injected intraperitoneally with with $5x10^7$ CFU of *E. coli* ST131 to induce sepsis. Illness severity was quantified at hours 0, 3 and 6 using the MSS system. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA test with Šídák's multiple comparisons test. ns, not significant. ns, not significant. **($p<0.01$), ****($p<0.0001$).

3.3: Biological Sex and Host Defense Against Infection in Sepsis

Our observation that female mice displayed reduced sepsis severity compared to males may be attributable to either (or both) superior ***infection resistance*** (ie. better ability to combat infecting pathogens, yielding reduced illness severity through reduced pathogen burden), or superior ***infection tolerance*** (ie. better ability to mitigate injury and illness from sepsis, independent of pathogen burden).

To address this, we first investigated whether sex-biased illness severity was linked with differential abilities to control pathogen proliferation and dissemination between males and females (ie. infection resistance). At 6 hours after infection with FIP, we quantified the amount of bacterial pathogens present at the site of infection (peritoneal lavage fluid), as well as the

amount of bacterial pathogens that had disseminated to other body compartments (blood, liver, lung, kidney and spleen) using standard quantitative bacterial culture methodology (expressed as colony forming units, CFU, of bacteria). Of note, preliminary cultures were performed both aerobically and anaerobically and similar results were founds using both methods, therefore, the data below only show aerobic bacterial culture conditions. At the site of infection (peritoneal lavage fluid), both males and females were found to have high CFU values consistent with severe infection, with no difference between sexes (Figure 6a). Assessment of pathogen dissemination to the blood, liver, lung, and spleen similarly revealed no significant differences in the quantity of bacteria at any of the organ sites tested between males and females (Figure 6b-f). Given that both males and females displayed high levels of pathogens and no difference in total pathogen burden in all compartments, we conclude that sex-based difference in illness severity cannot be attributed to differential infection resistance mechanisms.

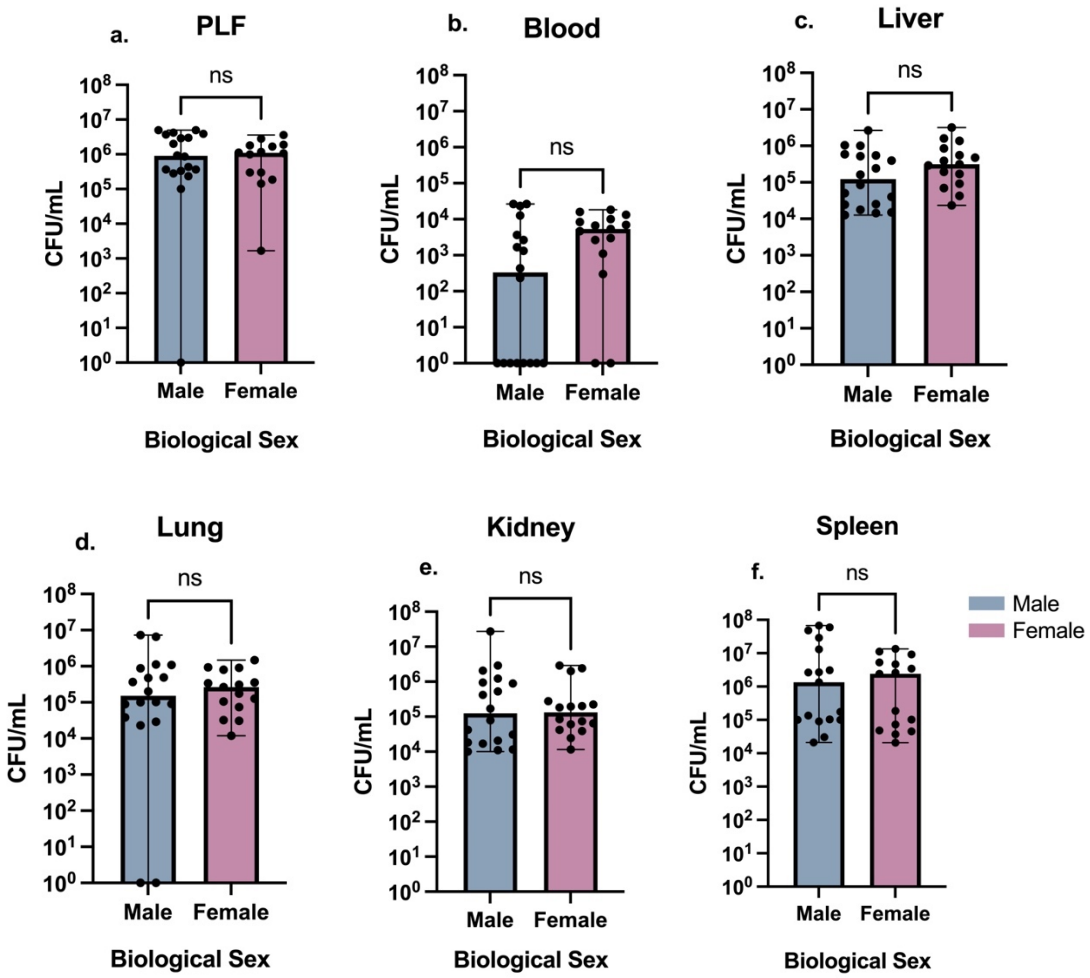

Figure 6: Comparison of pathogen burden (infection resistance) between male and female mice 6 hours post FIP.
a-f: Male and female C57BL/6 mice were injected intraperitoneally with 1.0mg/g of donor female cecal slurry, and tissues were harvested 6 hours post infection to quantify the amount of bacterial pathogens in various body compartments. The colony forming units (CFU) of bacterial pathogens were quantified in the peritoneal lavage fluid (a), blood (b), liver (c), lung (d), kidney (e) and spleen (f) by overnight culture on Brain-Heart Infusion (BHI) agar plates under aerobic conditions. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Mann-Whitney test. ns, not significant.

3.4: Biological Sex and Systemic Inflammation in Sepsis

Sepsis is a heterogeneous disease with various components and factors contributing the severity of the illness. Within the literature, it has been found that several main components of the immune response are known to play an influential role on how an individual responds to infection and the progression of sepsis[13,15]. As noted above, the quantity of infecting pathogens, their virulence mechanisms, as well as their dissemination throughout the host can contribute a higher severity of illness[13,75,76]. However, the magnitude of systemic inflammation that is elicited in response to infection in the host also represents a crucial contributor to illness severity. The systemic inflammatory response of sepsis is a highly complex, multi-system response that involves cellular players from both the innate and adaptive immune system. This response culminates in pathological hyperproduction of both pro- and anti-inflammatory mediators in the circulation, as part of a response that has been termed a "cytokine storm" that contributes to multi-organ damage and illness severity[15,77]. Therefore, we hypothesized that differential illness severity in sepsis between sexes may be due to differential magnitudes of systemic inflammatory responses between males and females. To test this, we measured plasma levels of ten prototypical pro- and anti-inflammatory cytokines and chemokines involved in the 'cytokine storm' of sepsis in male and female mice 6 hours after the induction of FIP using a multiplex chemiluminescence assay panel (Figure 7). No significant differences in levels of key pro-inflammatory cytokines IL-1β, TNF- α, and IL-12p70 between male and female mice were found (Figure 7a-c). However, we did observe that males had significantly higher concentrations of the neutrophil chemokine KC/GRO, as well as pleiotropic proinflammatory cytokine IL-6 compared to females (Figure 7d-e). In contrast, females were found to have increased levels of anti-inflammatory mediators IL-10, and IL-4, as well as immunomodulatory cytokines INF- γ, IL-2, and IL-5 (Figure 7f-j). Altogether, it appears that females were generally exhibiting a more anti-

inflammatory systemic response compared to males, who tended to have more pronounced pro-inflammatory profiles. In general, it should be noted that these differences between males and females are fairly modest and thus further investigations are needed to fully understand the contribution of these differential cytokine profiles towards sexual dimorphism of illness severity.

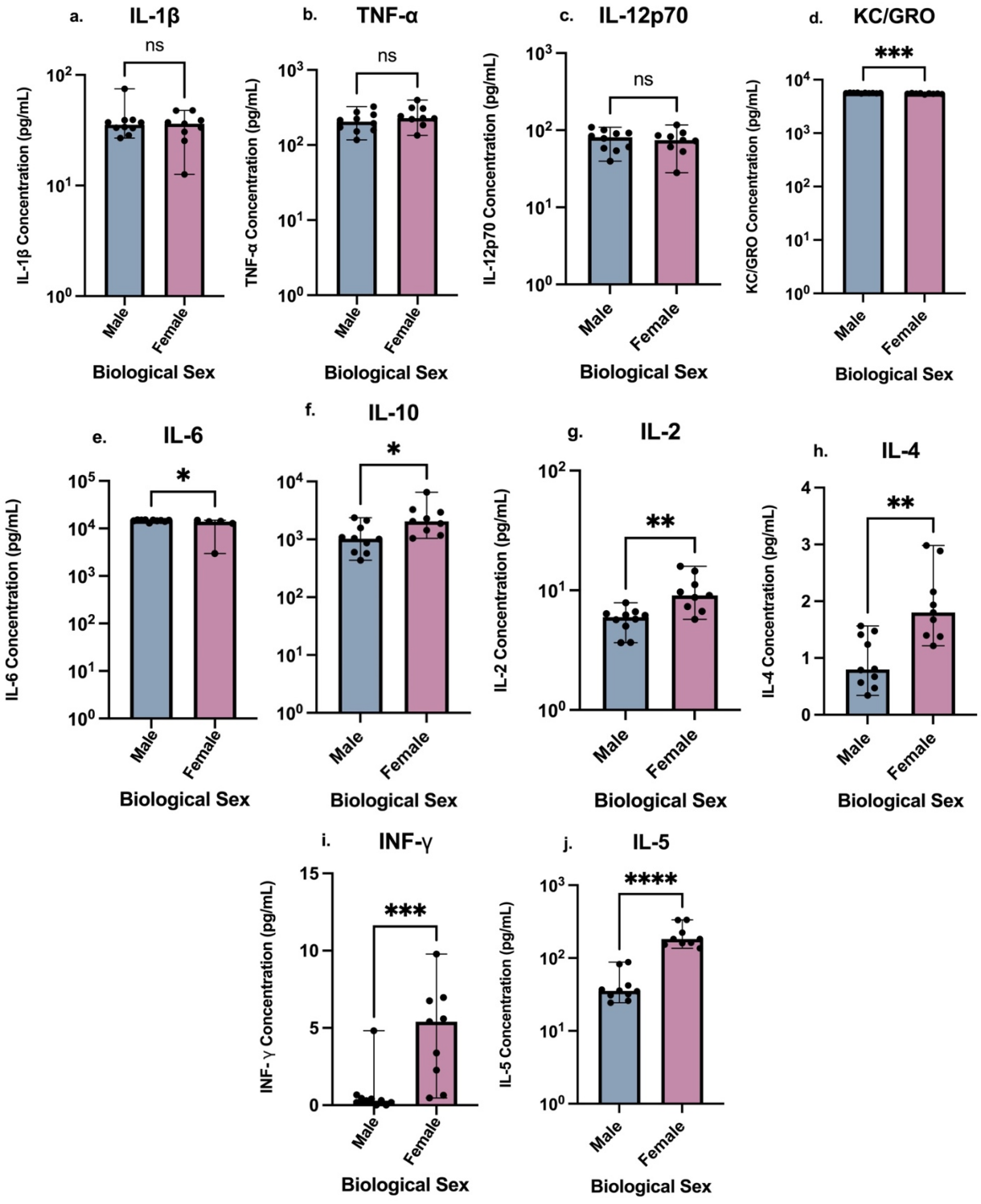

Figure 7: Pro and anti- inflammatory cytokine and chemokine results from SPF mice during FIP.

Cytokine/chemokine results of male and female C57BL/6 SPF mice that were injected intraperitoneally with 1.0mg/g of donor female cecal slurry over a 6-hour FIP experiment.

Plasma results from individual mice are represented in the concentrations of the following proinflammatory cytokines IL-1β (a), TNF- α (b), IL-12p70 (c), KC/GRO (d), IL-6 (e), IL-10 (f), IL-2 (g), IL-4 (h), INF- γ (i), IL-5 (j). Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Mann-Whitney test. ns, not significant. *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$).

3.4: Biological Sex and Neutrophil Infiltration

Neutrophils are a critical component of the innate immune response and have been shown to play an important role in sepsis pathogenesis[50,61,78]. Within a dysregulated immune system, an increased influx of neutrophils to critical organs such as the liver and lung, has been shown to result in organ damage, thus increasing the illness severity of the host [18,60,79]. This infiltration into the lungs and liver represents a core mechanism of inflammatory pathogenesis in sepsis that contributes to illness severity and adverse outcomes. To further investigate potential contributors to differential illness severity between males and females in sepsis, we investigated the extent of neutrophils infiltration into the lungs and liver during sepsis.

First, we began with a simplified model of sepsis/systemic inflammation induced by endotoxemia in C57BL/6 male and female mice by injecting 1.0 mg/kg lipopolysaccharide (LPS) intraperitoneally. At 3 hours after the onset of endotoxemia, mice were euthanized, and lung and liver tissues were collected to quantify neutrophil recruitment using flow cytometry. Neutrophils were identified by gating for CD11b+ Ly6G+ live cells (Figure 8). We found no significant differences in the percentage of neutrophils in the lungs or liver between males and females (Figure 9). This suggested that neutrophil influx into end organs like the lungs and liver was not driving the difference observed sepsis illness severity between males and females. However, due to low sample size we performed a second method of quantifying neutrophil influx into the lungs by performing a myeloperoxidase (MPO) activity assay. MPO is a peroxidase enzyme released

from neutrophils to catalyze the formation of reactive oxygen species, and is commonly used as a surrogate marker to quantify neutrophils within lung tissue[80]. Consistent with our findings using flow cytometry, we found no significant differences in MPO activity in the lungs between males and females (Figure 10). Thus, further indicating that septic male and female mice had similar levels of neutrophil influx in critical organs such as the lung and liver.

To further validate our initial findings of differences in neutrophil infiltration, we next investigated whether blocking neutrophil influx into the lungs and liver during sepsis would impact sex-biased illness severity or pathogen dissemination. For this, we used mutant mice that lack a critical adhesion molecule (Dipeptidease-1, DPEP-1) that has recently been shown to mediate neutrophil influx into the lung and liver during sepsis[65]. First, we quantified DPEP-1 gene expression in the lungs and liver of wild-type mice to determine whether there were sex-based differences at a transcriptional level. Using RT-qPCR for DPEP-1 gene expression (normalized to housekeeping gene GAPDH) in homogenized lungs and liver from wildtype mice post FIP infection, we found no significant difference in DPEP-1 expression in the lungs between males and females, whereas expression was significantly reduced in male livers compared to females (Figure 11). Although statistically significant, this difference represents an average of only ~0.07 cycles greater in male liver samples compared to females, and substantial variability was noted. Therefore, to further corroborate our findings, we decided to test the hypothesis that differences in DPEP-1 quantity or activity mediates the difference in illness severity between males and females. To test this, we compared illness severity and pathogen dissemination between male and female DPEP-1 knockout mice compared to wild-type in response to FIP sepsis. If DPEP-1-mediated neutrophil recruitment contributes to sexual dimorphism of illness severity, we would expect to see equilibration of illness severity between

sexes in DPEP-1 knockout mice. Although mice lacking DPEP-1 (DPEP-1 -/-) had a trend towards reduced illness severity overall compared to wild-type mice, male mice consistently had a higher illness severity score compared to female mice in both DPEP-1-/- and +/+ mice (Figure 12). To assess if the absence of DPEP-1 had a sex-based effect on the host's ability to resist the infection, we cultured blood, liver, lung, spleen, and kidney samples from DPEP-1 -/- and +/+ mice using methods described in Results 3.3. From this we found, no sex-based differences between DPEP-1 -/- and +/+ mice in disseminated body sites (Figure 13).

Therefore, from these data we conclude that DPEP-1 mediated neutrophil influx does not play a role in the sexual dimorphism of illness severity in sepsis.

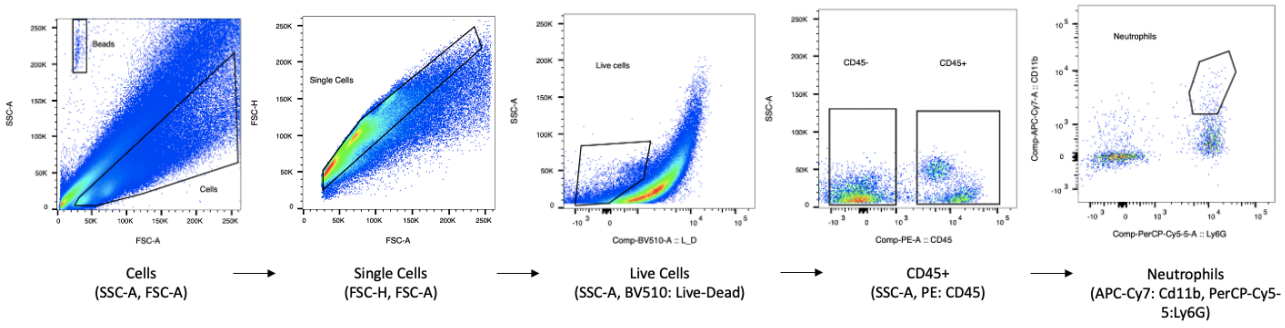

Figure 8: Flow cytometry neutrophil gating strategy
Representative flow cytometry plots demonstrate the gating strategy of neutrophils in the lungs and liver. Compensation beads are gated in SSC-A and FSC-A to set parameters for fluorescent signaling. Neutrophils were gated using BV510-, CD45+, Cd11b+/Ly6G+ single live cells.

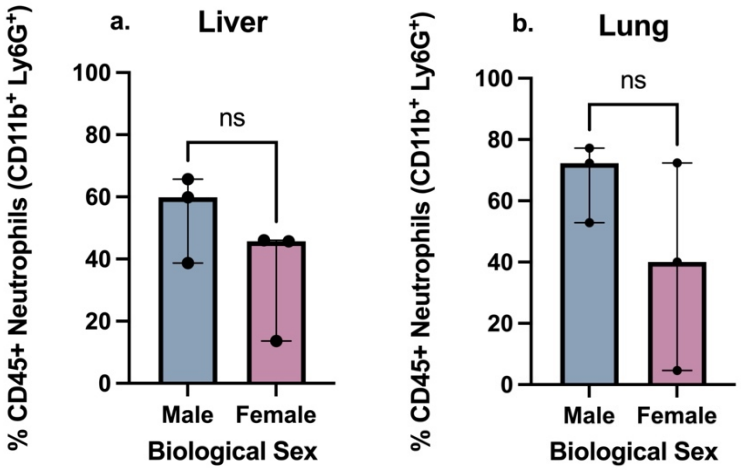

Figure 9: Neutrophil percentage in the liver and lung of mice during FIP.
Liver and lung tissue was removed after 6 hours of FIP Infection. Vasculature neutrophils were eliminated via cardiac perfusion prior to removing tissue for analysis. Flow cytometry gating of CD11b+ and LY6G+ live liver cells identified neutrophil populations within the samples. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Mann-Whitney test. ns, not significant.

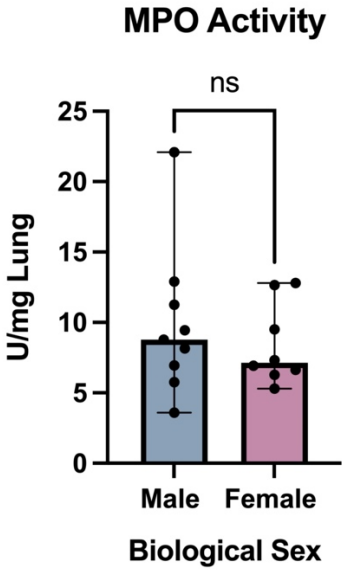

Figure 10: Myeloperoxidase activity from homogenized lung tissue of mice during FIP.
Male and female mouse lung tissue was removed and homogenized 6 hours post FIP infection. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Mann-Whitney test. ns, not significant.

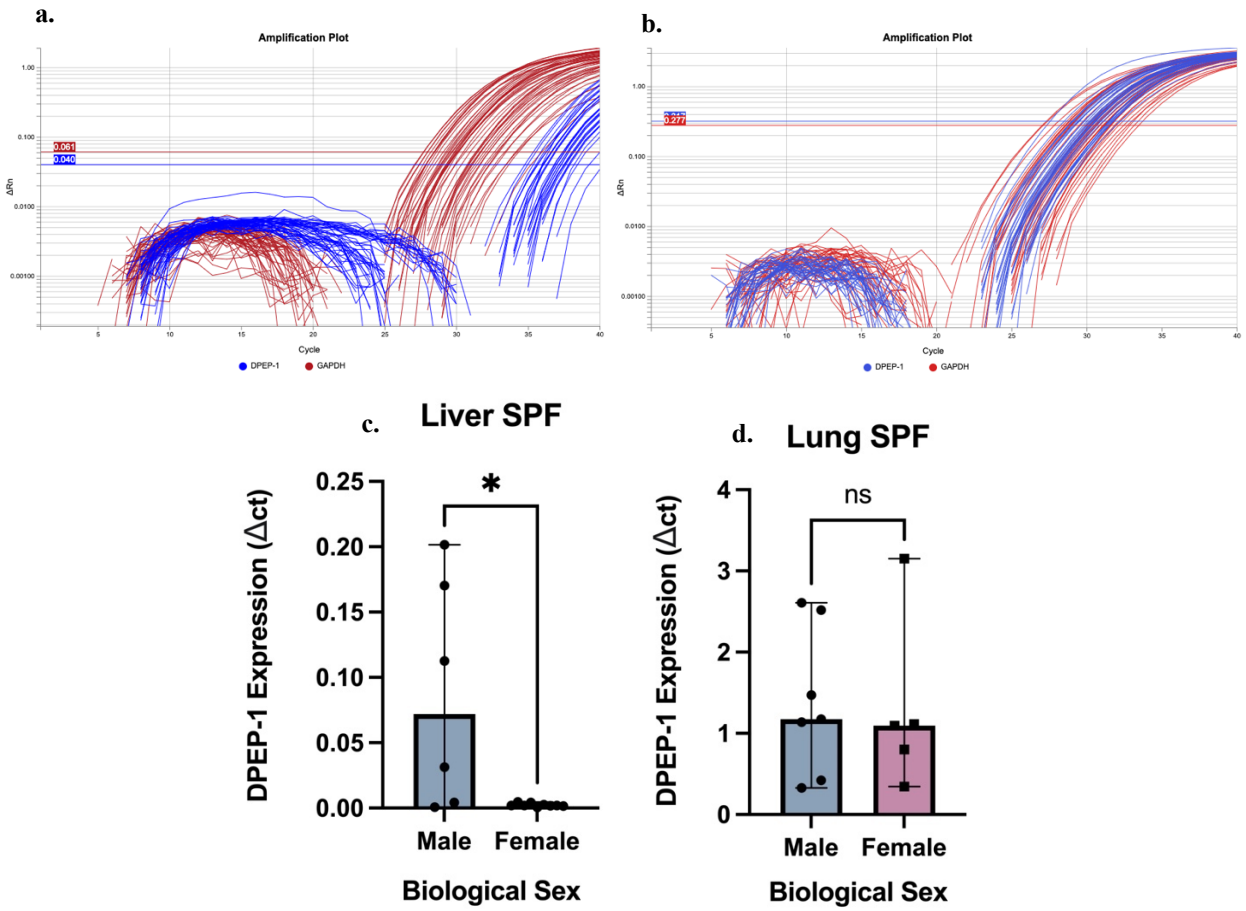

Figure 11: DPEP-1 expression of liver and lung tissue from wildtype male and female mice during FIP. Whole lung and liver tissue was preserved in RNAlater and quantified using qPCR with a GAPDH housekeeping gene. Amplification plots of liver (a) and lung tissue (b) are representative of the target gene of interest (DPEP-1) in blue and the housekeeping gene (GAPDH) in red. DPEP-1 gene expression for liver (c) and lung (d) is represented using ΔCt calculated from DPEP-1 and GAPDH. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Mann-Whitney test. ns, not significant. *($p<0.05$).

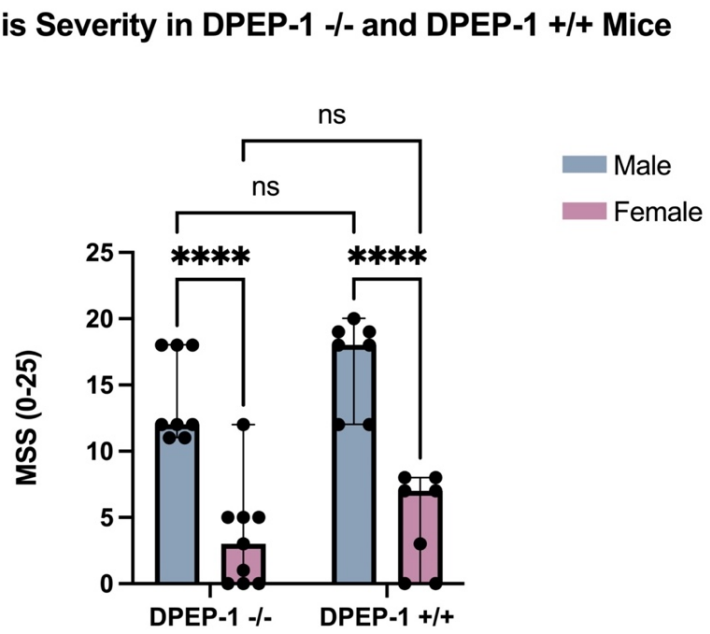

Figure 12. Sepsis illness severity in DPEP-1 wildtype and knockout mice 6 hours post FIP. Illness severity scores at the experimental endpoint quantified using the Murine Sepsis Scoring system. Male and female C57BL/6 SPF DPEP-1 knockout (-/-) and DPEP-1 wildtype (+/+) mice were inoculated with 1.0mg/g of donor female cecal slurry over a 6-hour FIP experiment. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant. ****($p<0.0001$).

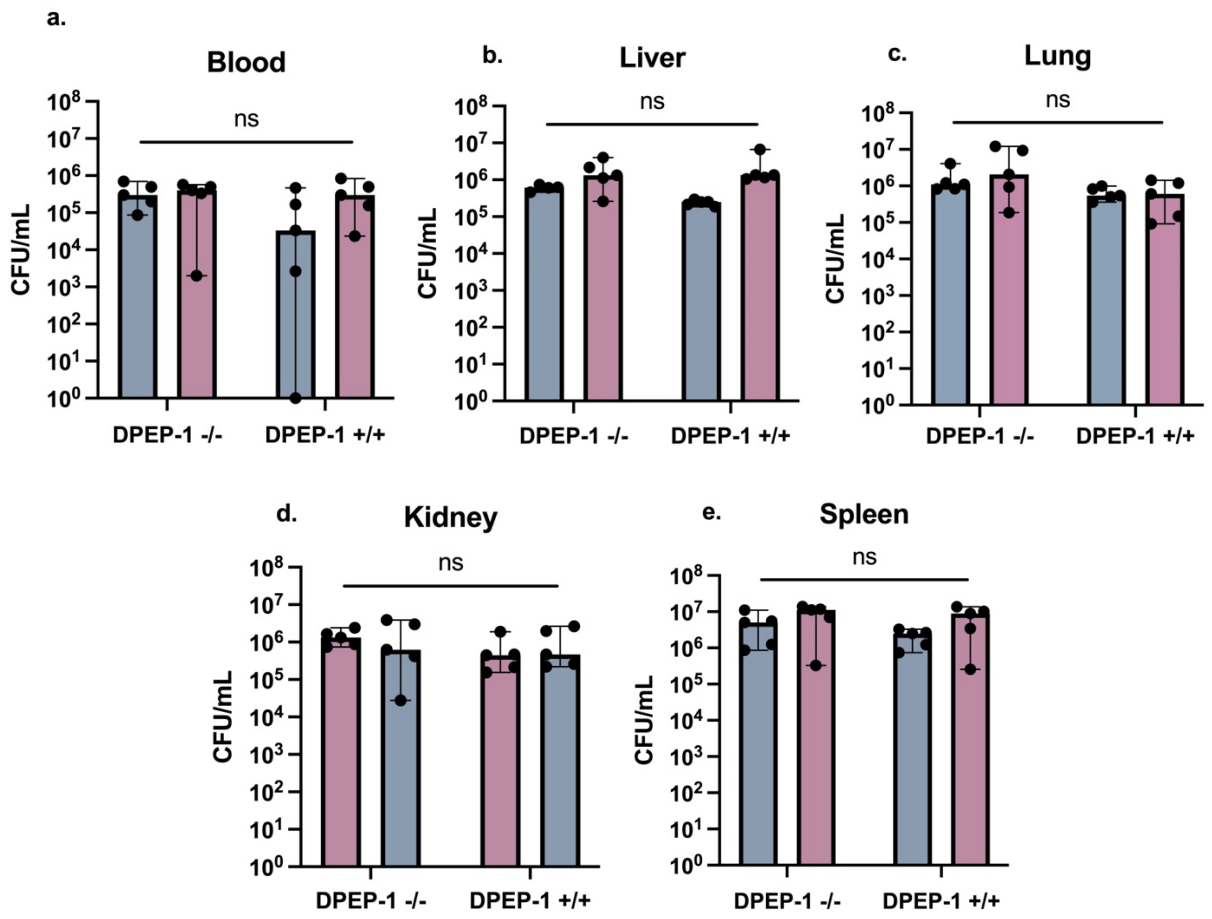

Figure 13: Comparison of pathogen burden (infection resistance) between male and female DPEP-1-/- and +/+ mice during FIP.
a-f: Male and female C57BL/6 SPF DPEP-1 knockout (-/-) and DPEP-1 wildtype (+/+) mice were injected intraperitoneally with 1.0mg/g of donor female cecal slurry, and tissues were harvested 6 hours post infection to quantify the amount of bacterial pathogens in various body compartments. The colony forming units (CFU) of bacterial pathogens were quantified in the blood (a), liver (b), lung (c), kidney (d) and spleen (e) by overnight culture on Brain-Heart Infusion (BHI) agar plates under aerobic conditions. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant.

3.5 Biological Sex and Mitochondrial Infection Tolerance

Our results in sections 3.1-3.4 above reveal that female mice have a lower illness severity than males in response to sepsis, and this sexual dimorphism not explained by differences in infection resistance as both males and females have similar levels of pathogen burden and dissemination. Furthermore, differences in infection tolerance mediated by systemic inflammation and neutrophil infiltration into end organs could not fully explain the marked differences in illness severity observed between males and females. Therefore, we turned our attention to another major mechanism of infection tolerance that has been shown to control illness severity in response to sepsis – mitochondrial tolerance. Mitochondria are critical organelles that provide the organism with ATP through oxidative phosphorylation and regulate various metabolic pathways[81]. In sepsis, cellular metabolism is altered, resulting in disruptive glucose metabolism and increased stress-related signaling (ex. ROS production), causing a lack of energy production and potential organ damage[81,82]. Mitochondrial tolerance is the ability of the mitochondria to alter cellular metabolism, providing energy thorough alterative pathways and limiting excessive damage to the host (ex. ROS), thus allowing the host to tolerate the infection independent of its ability to eliminate the pathogens. Recently within the literature is has been recognized that altering mitochondrial gene expression through particular bioenergetic pathways (ex. increase of fatty acid oxidation) is a crucial mediator of infection tolerance[67,83,84].

Interestingly, work from our lab using bulk RNA sequencing of the liver between septic male and female mice revealed that many of the genes that were highly differentially expressed between males and females were mitochondrial metabolism genes (Figure 14 – data courtesy of Jared Schlechte and Amanda Zucoloto). As many of these genes are involved in mitochondrial tolerance pathways, these data raise the possibility that sex-based differences in illness severity in sepsis may be linked to sexual dimorphism of mitochondrial infection tolerance.

Tetracycline antibiotics have previously been found to inhibit mitochondrial protein synthesis through its selective impact on mitochondrial gene expression, shifting cellular metabolism to provide the host with adequate energy and limit damage to tissues, providing protection to the host independent of its antimicrobial activity[67,81]. However, similar to many studies within the field of immunology, only male mice were reported to have been used. We have found that male mice have a significantly greater sepsis illness severity compared to females (which is not the result of differences in disease resistance), thus leading us to hypothesize that this sexual dimorphism is driven by differences in mitochondrial tolerance. Using tetracycline antibiotics (specifically doxycycline) as a potentiator of mitochondrial tolerance within our *E. coli* sepsis model will allow us to study the impact of biological sex on mitochondrial tolerance within sepsis disease severity. Thus, we carried out an experiment to test the biological sex differences of disease tolerance by infecting mice with *E. coli* ST131, a strain of *E. coli* resistant to doxycycline, to allow us to observe the disease tolerance effects of the drug, separate from its antimicrobial ability. We injected male and female C57BL/6 mice with either doxycycline or a PBS control every 24 hours for 3 days before *E. coli* infection. Mice were infected with *E. coli* and monitored for 6 hours, at which point illness severity was quantified using MSS scoring, and blood/tissues were collected to quantify bacterial burden and dissemination. In the PBS control group, male mice scored higher than female mice as previously observed (Figure 4-5). However, in the doxycycline groups there were no significant differences in the illness severity between biological sexes (Figure 15). This suggests that the sexual dimorphism of illness severity is dependent upon a doxycycline-sensitive mechanism of infection tolerance. As expected, doxycycline treatment did not impact bacterial burden nor dissemination of the doxycycline-resistant *E. coli* infection. Furthermore, pathogen burden and

dissemination were equivalent between males and females in both treatment groups, again demonstrating that illness severity is uncoupled from pathogen burden in this model (Figure 16). Collectively, these data suggest that potentiation of mitochondrial tolerance by doxycycline treatment abrogates sexual dimorphism of illness severity in sepsis. These findings serve as compelling hypothesis-generating data for further interrogation of mitochondrial tolerance pathways as mediators of sexual dimorphism of illness severity in sepsis, however these further investigations are outside of the scope of my Master's thesis.

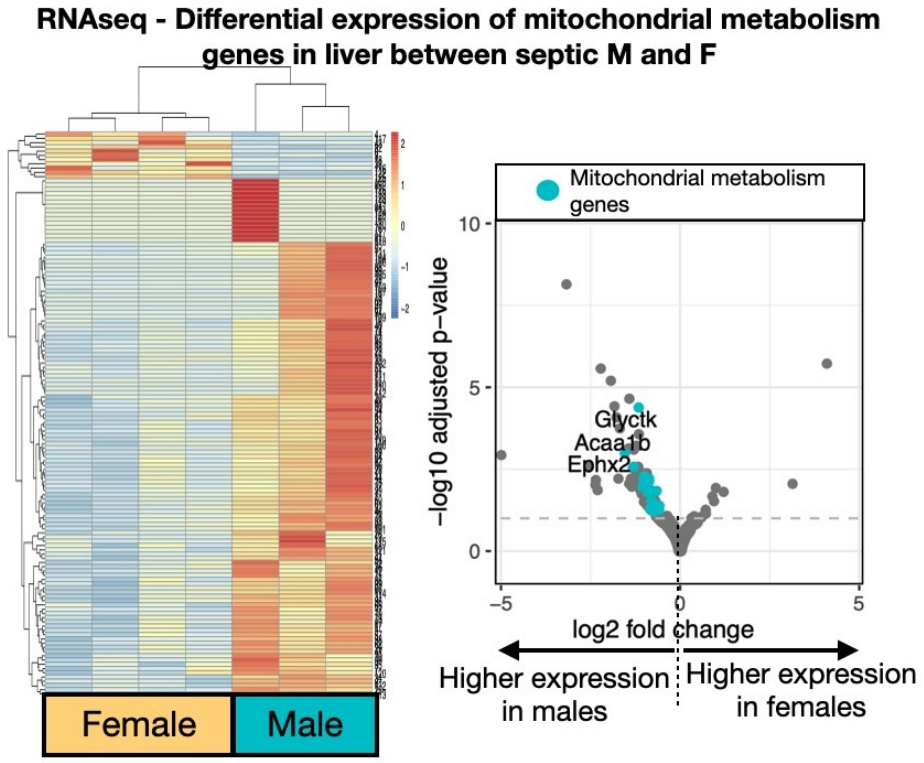

Figure 14: Differential expression of mitochondrial metabolism genes in liver between septic males and females. Transcriptomic analysis of liver from septic mice 6h after infection. Multiple mitochondrial pathways highlighted in the volcano plot reveals extensive transcriptome differences between males and females.

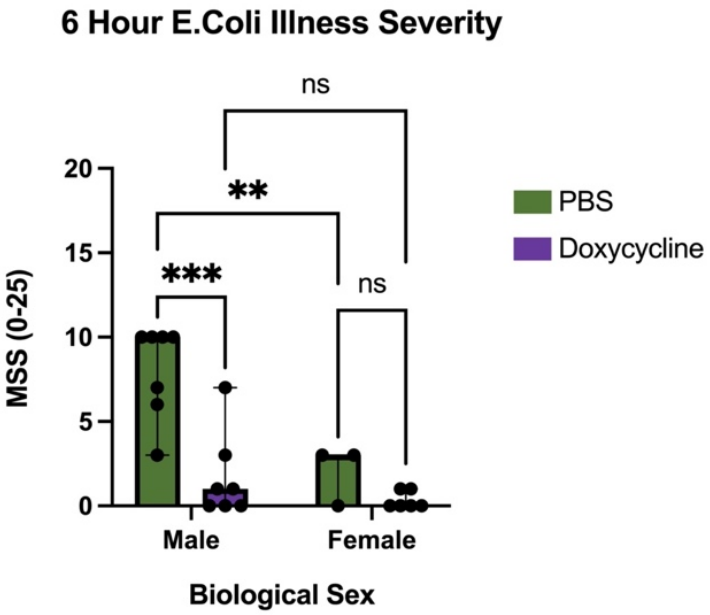

Figure 15: *E. coli* sepsis illness severity for PBS or doxycycline treated mice.
Illness severity quantified using the Murine Sepsis Scoring system 6 hours after *E. coli* infection. Male and female C57BL/6 SPF mice were administered either PBS or 1.75µg/g of Doxycycline 72, 48, 24 and 0 hours prior to infection with $5x10^7$ CFU of *E. coli* ST131. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant. **($p<0.01$), ***($p<0.001$).

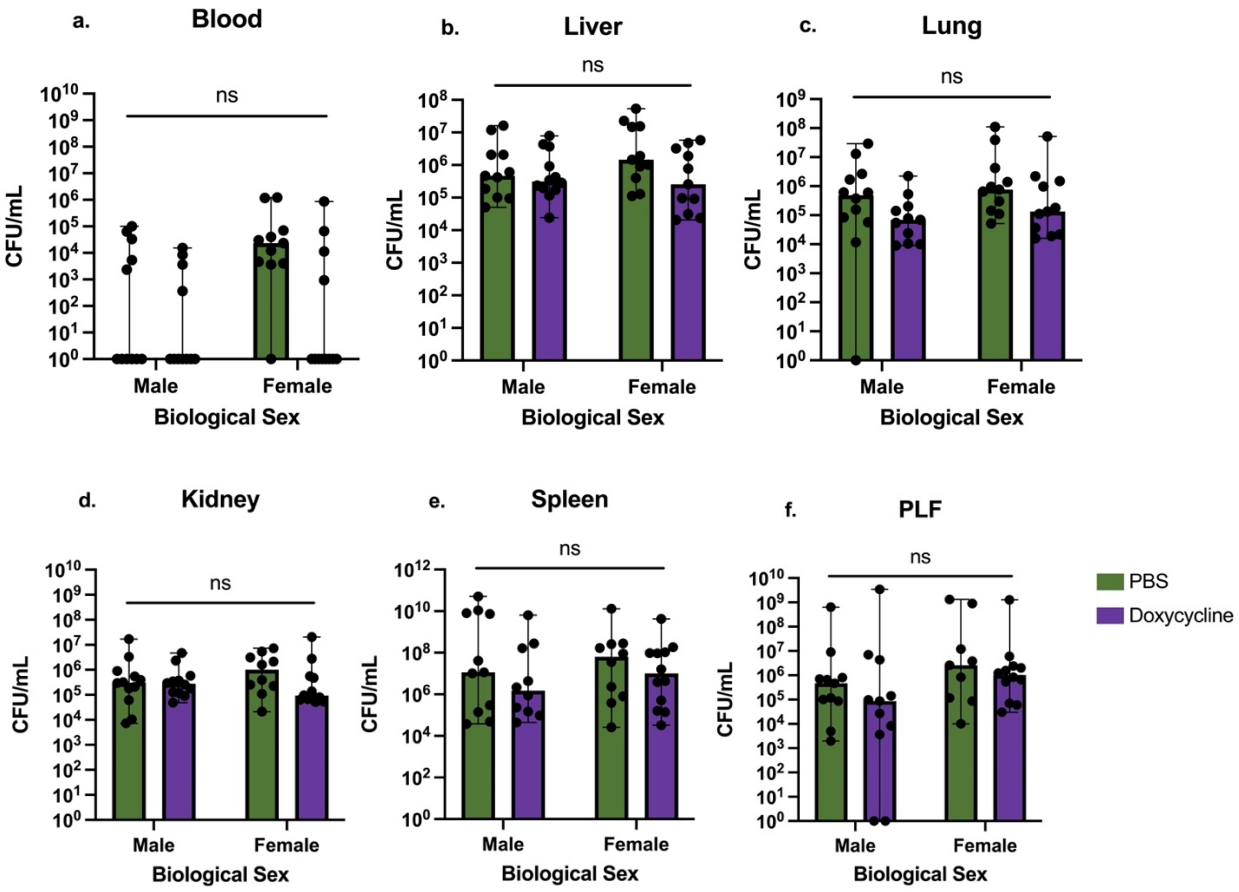

Figure 16: Pathogen clearance in PBS or doxycycline treated mice after 6 hours of *E. coli* sepsis. a-f: Male and female C57BL/6 SPF mice were administered either PBS or Doxycycline 72, 48 and 24 hours prior to infection with $5x10^7$ CFU of *E. coli* ST131. Blood (a), liver (b), lung (c), kidney (d), spleen (e) and PLF (f) were cultured on Lysogeny broth (LB) agar plates. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant.

These results illustrate a clear male sex bias in sepsis illness severity, as males less effectively tolerate polymicrobial and monomicrobial sepsis (Figures 4-5). This sexual dysmorphism cannot be explained by differences in bacterial burden, systemic inflammation, or neutrophil infiltration (Figures 6-7, 9-13). However, when administered doxycycline, this sex bias diminishes, suggesting that the mechanism(s) driving this sexual dimorphism of illness severity are disrupted by the actions of doxycycline (Figures 15-16). These preliminary findings encourage further investigation into the specific mechanisms of sex-based mitochondrial tolerance. The lack of sex-based sepsis therapeutics despite a clear sex-bias within the disease, highlights the clinical significance to fill this critical gap in knowledge and provide insight for novel sepsis therapeutics.

Chapter 4: Results: Mediators of Sexual Dimorphism of Sepsis Severity

Introduction

To deepen our understanding of the mechanisms underlying sexual dimorphism of sepsis severity, we next aimed to decipher the mediators of sex phenotype that contribute to differences in illness severity between male and female mice. For this, we developed experimental approaches to systematically dissect the impact of the 3 fundamental mediators of biological sex phenotypes - sex chromosomes, sex hormones, and the gut microbiota[10,23,85].

As described in detail in the introduction chapter above, X and Y chromosomes contain a number of immune genes (including TLR genes and cytokine receptor genes) that may influence inflammation and disease severity in sepsis. Similarly, sex-determining gonadal hormones are also known to display pleiotropic effects on immune cell functions, including inflammatory response capacity and antimicrobial effector mechanisms. To decipher the contributions of sex chromosomes vs. gonadal sex hormones towards illness severity in sepsis, we employed a powerful transgenic mouse model that uncouples X/Y chromosomes from gonadal sex, called the Four Core Genotype mouse model (4CG). The four genotypes of these mice are: XX with female gonads/ovaries, XY with female gonads/ovaries, XX with male gonads/testes, XY with male gonads/testes[86,87] (see Figure 19 below). This system involves breeding wild-type XX females with fertile transgenic XY males in which the sex-determining *sry* gene is deleted from the Y chromosome, and a sry-transgene is inserted into an autosome[86]. As a result, gonadal sex is driven by the autosomal transgene, and is entirely uncoupled from X and Y chromosome makeup. This system provides the ability to separate and isolate the effects of sex chromosomal and gonadal sex hormones differences through comparison between these four genotypes.

In addition to sex chromosomes and hormones, another key mediator of sexually dimorphic phenotypes is the gut microbiota. The composition of the gut microbiota has been shown to differ between males and females, and these sex-based differences in the microbiota have been found to mediate sexual dimorphism of various immune functions and diseases (ex. female bias of type 1 diabetes)[7,47,55,88]. Given that dysbiosis of the gut microbiota is an important modulator of sepsis pathogenesis in mice and humans, we also sought to determine the potential impact of sex-based differences in the gut microbiome towards illness severity in sepsis by using germ-free (GF) mouse models[7,49,50].

Therefore, we aimed to decipher the underlying mediators of the observed sexual dimorphism in by utilizing GF and 4CG mice within our FIP model of sepsis. After 6 hours of fecal induced peritonitis, we monitored illness severity (using MSS) and bacterial burden to highlight any potential contributions of the gut microbiota, sex chromosomes, and gonadal sex hormones.

Results

4.1 The Role of the Gut Microbiota in Sepsis Illness Severity

To determine the contribution of the gut microbiome towards sexual dimorphism of illness severity in sepsis, we compared male and female mice that have no colonizing microbes (Germ Free, "GF") with mice that have a conventional laboratory microbiota (Specific Pathogen Free, "SPF"). GF mice are born and bred without microorganisms in the International Microbiome Centre, and SPF mice are derived from an ex-GF line that originated in the IMC but have been bred under SPF conditions in our conventional facility (MBU) for multiple generations. Utilizing the FIP sepsis model developed in Results 3.1, we infected SPF and GF mice with 1.0mg/g of fecal slurry, and 6 hours later measured illness severity using MSS score, and harvested blood and tissues to quantify pathogen burden and dissemination. The comparison between mice with and without a microbiome (SPF and GF respectively), allowed us to study the potential contribution of a gut microbiota to sex-based differences in sepsis illness severity.

We found that GF mice and SPF mice showed the same sex bias in illness severity, with male mice in both microbiome conditions demonstrating significantly higher MSS scores compared to females (Figure 17). Consistent with our previous findings, pathogen burden in the blood and tissues (liver, lung, spleen, kidney) were equivalent between males and females under both GF and SPF conditions (Figure 18). Therefore, these findings lead us to conclude that the male sex bias in illness severity in sepsis is independent of the gut microbiome, given that males had higher illness severity than females regardless of the presence (SPF) or absence (GF) of a gut microbiome (Figure 17-18).

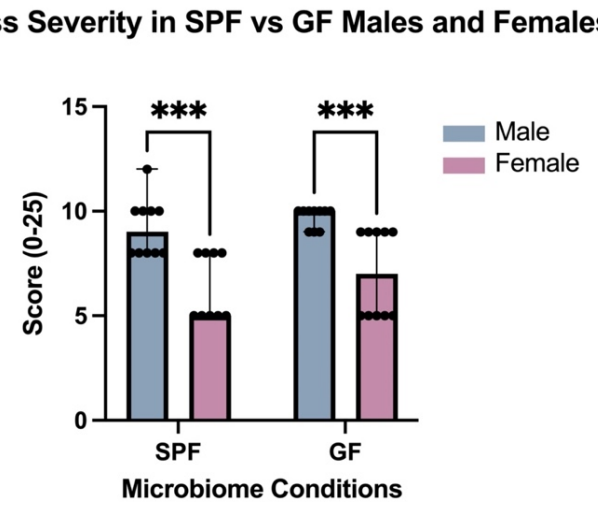

Figure 17: Sepsis illness severity in GF and SPF mice 6 hours post FIP.
Male and female SPF mice were imported from Jackson River. GF were purchased from the IMC and kept in a GF environment prior to the experimental endpoint. All mice were injected intraperitoneally with 1.0 mg/g of a slurry made from the cecal content from donor (SPF) mice. Illness severity was measured using the murine sepsis severity scale (MSS) hourly post infection. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA test with Tukey's multiple comparison test. ***($p<0.001$).

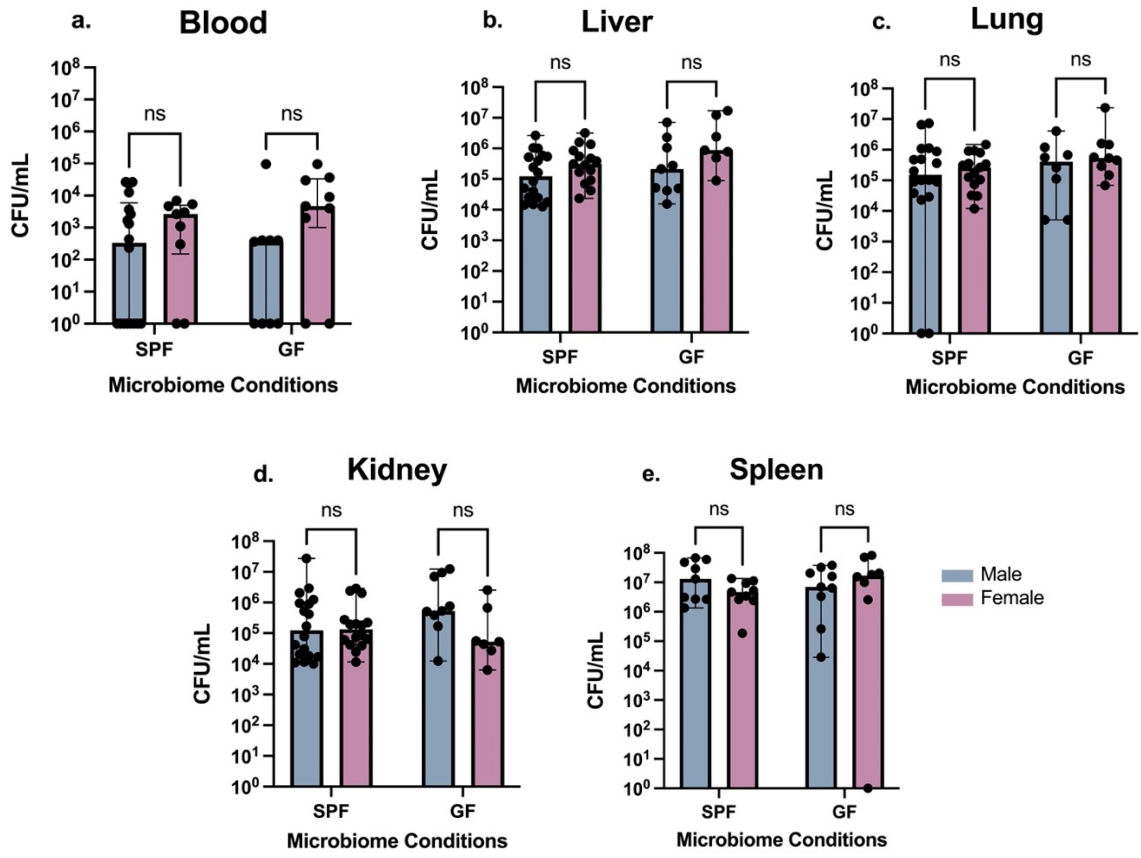

Figure 18: Comparison of pathogen burden (infection resistance) between male and female SPF and GF mice during acute polymicrobial sepsis.
Male and female C57BL/6 SPF and GF mice were injected intraperitoneally with 1.0mg/g of donor female cecal slurry, and tissues were harvested 6 hours post infection to quantify the amount of bacterial pathogens in various body compartments. The colony forming units (CFU) of bacterial pathogens were quantified in the blood (a), liver (b), lung (c), kidney (d) and spleen (e) by overnight culture on Brain-Heart Infusion (BHI) agar plates under aerobic conditions. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant.

4.2: The Role of Gonadal Hormones and Sex Chromosomes in Sepsis Illness Severity

After finding that sex-based differences in illness severity was independent of the gut microbiota, we next turned our attention towards the other two principal mediators of differential host response between males and females: gonads/hormones and sex chromosomes. To understand whether this observed sex bias is driven by X or Y linked genes, or gonadal sex, we utilized the 4CG mouse model to uncouple chromosomal and hormonal influence on the severity of illness during sepsis. The basis of this model is the transgenic manipulation of the *sry* gene (sex determining region of Y), which is deleted from the Y chromosome, and inserted into an autosome[86,89]. Absence of the *sry* gene 'defaults' to ovaries, while the presence of the *sry* gene leads to male gonadal development. Therefore, the presence of the transgene on an autosome results in the development of male gonads, independent of whether the animal has XX or XY chromosomes. Thus, the development of male gonads is not dependent upon a Y chromosome. Breeding a wildtype female (XX with ovaries), with a transgenic XY male with the *sry* gene deleted from the Y chromosome and inserted into an autosome yields 4 different genotypes of offspring: XX with ovaries, XX with testes, XY with ovaries and XY with testes (Figure 19)[87]. Genotyping of all offspring was performed using PCR, as demonstrated in Figures 20-21.

To understand the contributions of sex chromosomes and hormones separately, we carried out a FIP experiment, infecting all four genotypes of mice with 1.0mg/g of cecal slurry and monitoring them over a 6 hour period. Interestingly, we observed that gonadal males had significantly higher MSS scores compared to gonadal females at 6 hours after infection, entirely independent of XX or XY chromosome makeup (Figure 22). When quantifying bacterial burden by culturing tissues, blood and PLF, we found no differences between any of the four core genotypes (Figure 23). While we see no differences in the ability of these mice to resist the infection (Figure 23), the mice that contain female gonads appear to tolerate the illness better as

displayed by their lower illness severity scores compared to gonadal males (Figure 22). These results demonstrate that the sex bias in sepsis illness severity is not dependent upon Y-liked genes but is rather mediated by male gonadal/hormone composition.

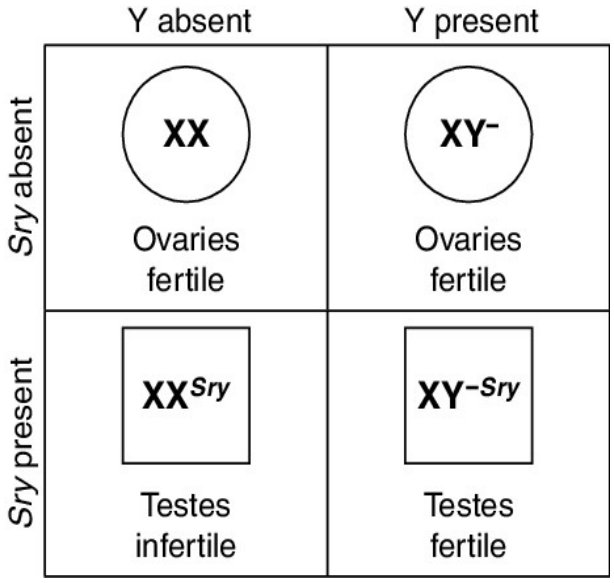

Figure 19: Representation of the four-core genotype mouse model. Circle = female phenotype; Square = male phenotype; XY- = Sry gene deleted from Y-chromosome; XXSry = Sry gene inserted into genome; XY-Sry = Sry gene deleted from Y-chromosome and inserted into an autosome[87].

Cycle Conditions

Denaturation	95°C	3 min	
Denaturation	95°C	30 sec	
Annealing	60°C	1 min	X35
Elongation	72°C	1 min	
Final Elongation	72°C	5 min	
Hold	4°C		

Expected Results
Mutation = 350 bp
Transgene = 420 bp
Internal Positive Control = 200 bp

XX female= 200 bp
XY- female= 350 bp and 200 bp
XX male= 420 bp and 200 bp
XY- male = 420 bp and 350 bp and 200 bp

Reaction Mix	µl/Rxn
H20	13.25
$MgCl_2$	0.5
5X running buff (7.5mM MgCl)	5
primer 1	0.5
primer 2	0.5
primer 3	0.5
Primer 4	0.5
primer 5	0.5
primer 6	0.5
dNTPs	1
Taq Polymerase	0.25
DNA	2
total volume	25

Figure 20: PCR cycling conditions for 4CG genotyping
Conditions of PCR cycling using mouse ear skin for 4CG genotyping. PCR was completed by a laboratory technician and expected band size correlated with mouse genotype was provided by Jackson River Laboratories where the mice embryos were purchased from.

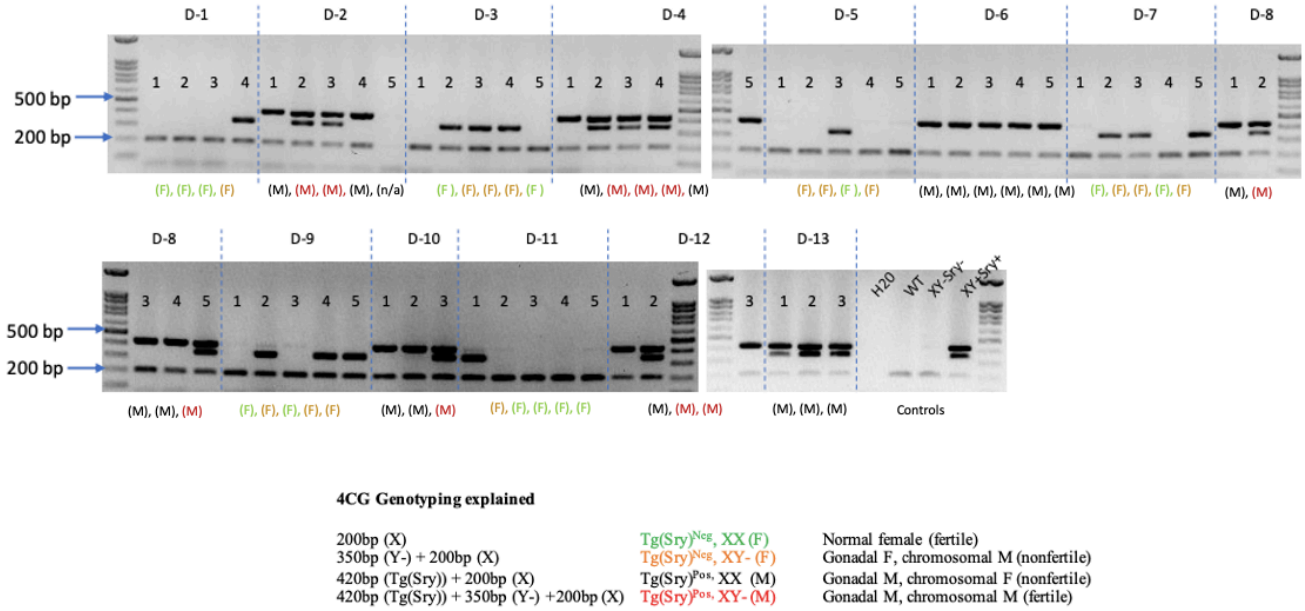

Figure 21: Genotyping results from 4CG mice.
Genotype identity was confirmed by a laboratory technician by using ear skin samples from each mouse in PCR and then running the samples on a gel electrophoresis apparatus. Band size is correlated with the expected genotype of each mouse.

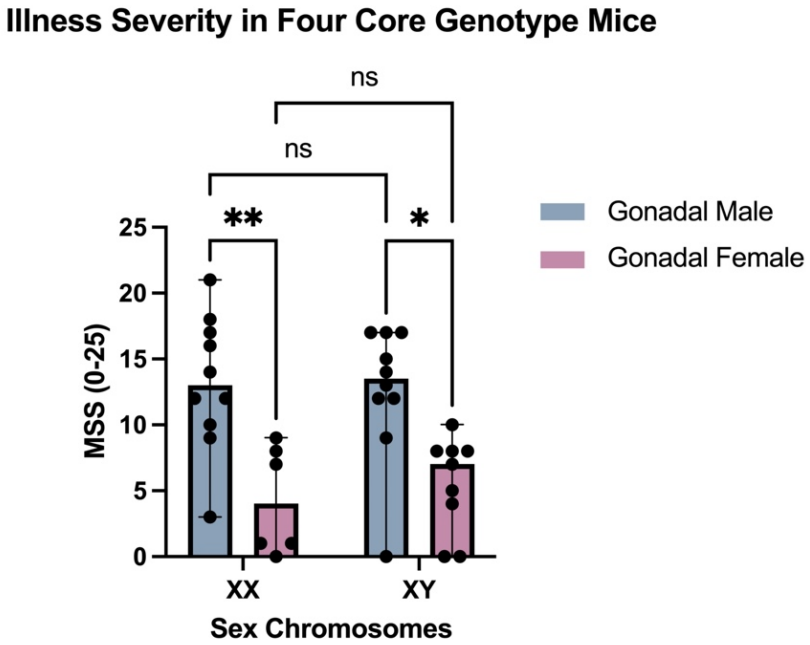

Figure 22: Sepsis illness severity in 4CG mice 6 hours post FIP.
Male and female transgenic 4CG mice were injected intraperitoneally with 1.0 mg/g of a slurry over a period of 6 hours. Illness severity was measured using the murine sepsis severity scale (MSS) 6 hours post infection. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA test with Tukey's multiple comparison test. ns, not significant. *($p<0.05$), **($p<0.01$).

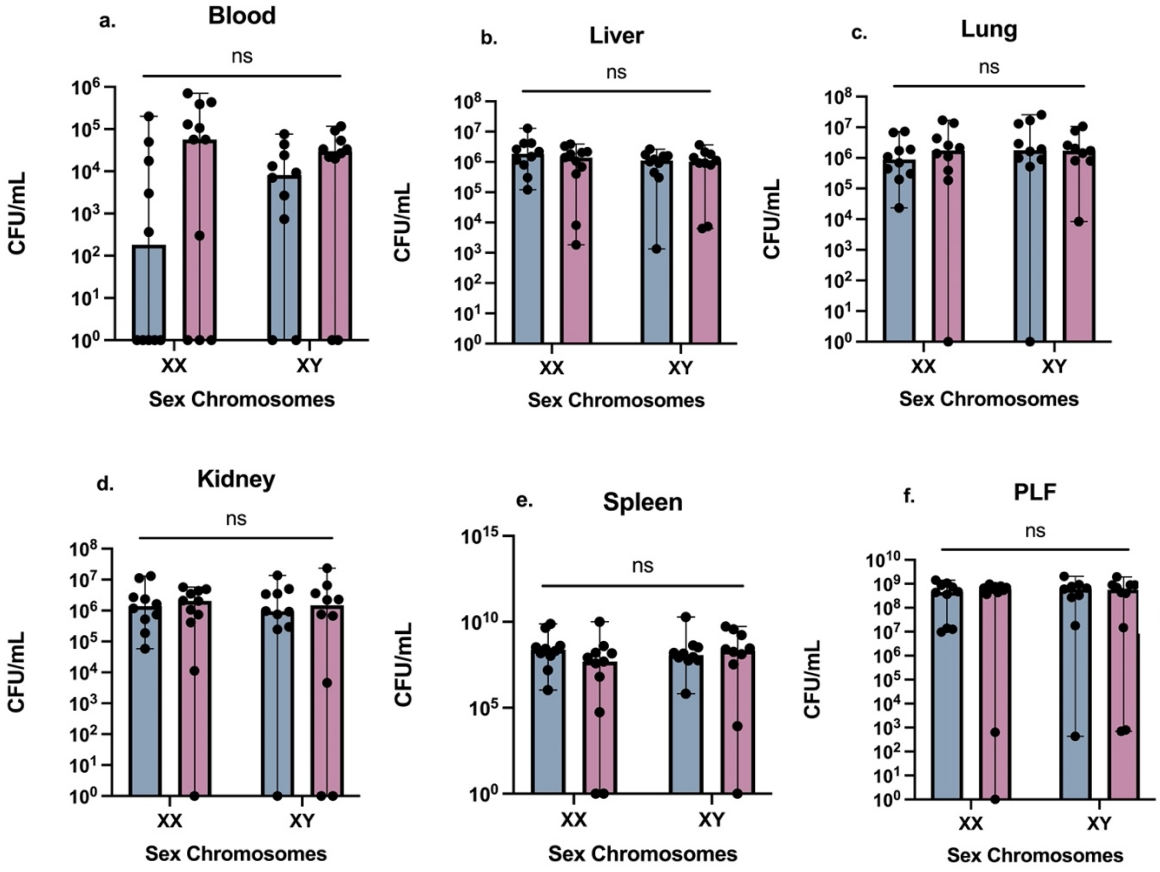

Figure 23: Comparison of pathogen burden (infection resistance) between 4CG mice during acute polymicrobial sepsis.
Male and female transgenic 4CG mice were injected intraperitoneally with 1.0mg/g of donor female cecal slurry, and tissues were harvested 6 hours post infection to quantify the amount of bacterial pathogen in various body compartments. The colony forming units (CFU) of bacterial pathogens were quantified in the blood (a), liver (b), lung (c), kidney (d), spleen (e) and PLF (f) by overnight culture on Brain-Heart Infusion (BHI) agar plates under aerobic conditions. Points represent individual values, bars represent median, error bars represent range. Data were analyzed using a two-way ANOVA with Tukey's multiple comparison test. ns, not significant.

In summary, in Chapter 4 we systematically determine the contributions of the gut microbiome, sex chromosomes, and gonadal sex hormones towards illness severity in our mouse models o FIP sepsis. These data clearly show that sexual dimorphism of illness severity is independent of the gut microbiome, as male-biased illness severity was observed in both SPF and GF mice (Figure 17). Furthermore, we uncoupled the other two key mediators: gonads/sex hormones and sex chromosomes using the 4CG mouse model. We found that the gonadal male mice maintained a higher illness severity during sepsis, independent of this sex chromosome composition (Figure 22). Thus, concluding that of the three biological sex mediators in immune responses, male gonad/sex hormone influence is a stronger driving factor in in the severity of sepsis than Y-linked genes and the gut microbiota. These data open exciting new directions into the understanding the mechanisms driving the sex bias in sepsis. Further research will be needed to delineate the hormones, pathways, and effector cells/mechanisms that underly higher sepsis severity in males (and/or protective impact of female hormones) will help to address the lack of knowledge in the field of biological sex and sepsis.

Chapter 5: Discussion

Summary and Main Conclusions

In this thesis, we explored how biological sex impacts illness severity in sepsis, the contribution of infection resistance and tolerance mechanisms, as well as the roles of the three principal mediators of biological sex phenotypes between males and females. We found that illness severity was significantly higher in males compared to females in response to both polymicrobial and monomicrobial models of sepsis in mice. Interestingly, this sexual dimorphism of illness severity was independent of pathogen burden, as males and females had equivalent levels of pathogens both at the site of infection and disseminated to the blood and distant organs. Instead, our data pointed to a putative contribution of differential infections tolerance mechanisms between males and females. Specifically, we found differences in the systemic inflammatory response between males and females, wherein males had higher circulating levels of pro-inflammatory mediators (IL-6, KC/GRO), while females had higher levels of anti-inflammatory (IL-10, IL-4) and immunomodulatory (IFN- γ, IL-5). Furthermore, potentiation of mitochondrial tolerance pathways through administration of doxycycline reduced and eliminated the differential illness severity between septic male and female mice. Therefore, females appear to demonstrate superior infection tolerance, putatively mediated by enhanced mitochondrial tolerance mechanisms and advantageous systemic inflammatory response, compared to males. Lastly, we systematically interrogated the contributions of the three key mediators of sex phenotype towards sex-biased illness severity in sepsis (sex chromosomes, gonadal sex hormones, and the microbiome). Comparing germ-free mice to SPF mice, we found that male-biased illness severity was independent of the gut microbiome, and using the transgenic 4CG mouse model we demonstrated that male sex-biased illness severity was

independent of XY chromosome composition, and instead mediated by gonadal/sex hormone phenotype. Collectively, this thesis project has revealed key mechanistic underpinning of sexual dimorphism of illness severity in sepsis that have been entirely overlooked in the sepsis literature and may have important implications for our understanding of sepsis pathogenesis, and treatment responses in sepsis.

5.1 Biological Sex, Gender, and Sepsis Severity

The clear influence of biological sex on immune function is perhaps best demonstrated by the overwhelming female predominance in many autoimmune diseases, as ~80% of autoimmune diseases are female predominant[10]. Despite this, and other clear demonstrations of differences in immune responses between males and females, less than 10% of immunology studies report biological sex as a variable in their analysis[90]. In the sepsis field, epidemiological studies of human sepsis have consistently reported sex-biased outcomes – specifically, that males have a higher incidence, higher severity, and higher mortality compared to females[2–5,29,30,71,91,92]. Despite these epidemiological data in humans, little attention has been paid to the cross-talk between biological sex and mechanisms of pathogenesis in pre-clinical sepsis research. A recent systematic review of animal studies of sepsis revealed very few studies of sepsis pathogenesis, and even fewer studies of sepsis treatments/interventions that included biological sex as a factor in their analysis[7]. This clear gap in the field of biological sex and sepsis, inspired our investigation into the factors that drive this differential response between males and females during sepsis. It is important to note that we focused our attention on differences in biological sex, not gender. Gender is specific to humans and is socially constructed around a multitude of factors including behaviours, activities, environment, and culture[10]. We acknowledge that gender

influences immune response within humans (ex. differential access to healthcare, lifestyle choices), however, because we use mice to address our research questions, gender cannot be studied within this context[22].

One of the greatest limitations to using animal models is their translation to human diseases, as laboratory mice lack the genetic differences, pre-existing conditions and environmental and societal influence that humans face[8]. Nevertheless, murine models have been, and continue to be one of our most powerful tools to define the mechanistic basis of diseases including sepsis, and for the discovery and testing of novel treatment targets. Indeed, murine models serve as a useful tool to interrogate the mechanistic contributions of biological sex to disease pathogenesis. In this context, our study made use of common, well characterized models of sepsis that have been used for many years in the field (fecal peritonitis, and monomicrobial peritonitis with *E. coli*) in order to understand difference in illness severity between males and females. It is important to note that several other sepsis models have been developed, including: endotoxemia, Cecal Ligation and Puncture (CLP), monomicrobial infections by a variety of other pathogens, as well as organ-specific infection models (pneumonia, cellulitis, meningitis, etc.)[70]. The endotoxemia model uses an intraperitoneal injection of lipopolysaccharide (a single component of the gram-negative bacterial membrane), that triggers only one TLR pathway and neglects the complex microbial interactions of gram positive bacteria and polymicrobial infections[70]. CLP requires mice to undergo surgery to ligate the cecum allowing for cecal contents to be released. This model commonly results in high amounts of variability in the quantity of cecal contents released into the peritoneal cavity, resulting in large inter-experimental variability[70]. Furthermore, CLP would not be feasible for our particular investigation given our use of GF mice, which would lack gut bacteria to induce a septic response. Therefore, we elected

to use the FIP model of sepsis as opposed to endotoxemia or CLP, in order to encompass a polymicrobial infection, while achieving very rigorous standardization and reproducibility that could be used across the various mouse models that we utilized, including GF mice[70,74,93]. In addition, we went further and confirmed our results in an additional, more controlled model of sepsis induced by a monomicrobial intraperitoneal infection with *E. coli* to confirm that our findings are relevant across multiple infections that cause sepsis. However, we recognize that other routes of infection including intratracheal, intravenous, intradermal, etc., as well as other pathogens, could potentially yield different contributions of biological sex based on unique mechanisms of pathogenesis, however it would not be feasible to study all of the permutations of sepsis-inducing infections in this thesis.

To be rigorous in our development of an optimized FIP model for our study, we first completed dosage and timepoint finding experiments. Based on our own adaptations and the foundation of the model laid by the National Preclinical Sepsis Platform (NPSP), we found 1.0mg/g of cecal slurry over a timepoint of 6 hours induced measurable, severe sepsis without requiring euthanasia prior to the experimental timepoint (Results 3.1)[69]. During infection we monitored mice using the Murine Sepsis Scoring system (MSS) and found that male mice became significantly more ill than female mice. This observation aligned with male sepsis bias historically reported in the literature for both animal models and clinical findings[11,94]. Of note, the inoculum used to infect mice in FIP was weight adjusted (1.0mg/g), therefore, due to female mice generally weighing less than males, females received a smaller dosage of inoculum. In contrast, our monomicrobial models of *E. coli* peritonitis utilized a fix inoculum in both males and females ($5x10^7$ CFU of *E. coli* ST131). Regardless, we found equivalent results in both models, with males showing higher MSS scores than females, while achieving equivalent levels

of pathogen replication and dissemination in the peritoneal cavity, blood, and distant organs. Therefore, our findings that male mice have a greater sepsis illness severity than females, remained consistent across the monomicrobial and polymicrobial model of sepsis.

While this study focuses on bacterial sepsis, it should be noted that there are other forms of sepsis including fungal and viral, with various mechanisms of action that were not assessed in our FIP model [95]. Bacterial infections (specifically pneumonia) are currently the leading cause of sepsis in North America, however in light of the recent SARS-CoV2 pandemic, viral sepsis has significantly increased[95–98]. Global Health 50/50 found similar rates of SARS-CoV2 infections across males and females, however males were more likely to be hospitalized and admitted to the ICU[99]. Additionally, it has been found in severe SARS-CoV-2 infections, that males and females had a differential neutrophil response, with males having increased IFN^{active} neutrophils, mirroring differential outcomes between males and females[100].

Overall, in line with epidemiological studies of human sepsis showing that males have an increased incidence, severity and mortality rate in sepsis compared to females, our results using 2 different mouse models of sepsis confirm that males display higher illness severity in response to septic infections compared to females[7,43]. As such, these models served as an ideal foundation for us to further investigate the potential mechanisms and mediators underlying this sexual dimorphism of sepsis severity.

5.2: The impact of Biological Sex on Infection Tolerance, and Infection Resistance

The severity of an infection is dictated by the balance between two core host responses – infection resistance (ie. the host's ability to supress pathogen growth and dissemination), and infection tolerance (the host's ability to mitigate damage and sickness caused by the infection, independent of the pathogen load)[101]. Various infections have been shown to demonstrate a sex

bias in disease tolerance and resistance. For example, in the asymptomatic period of HIV infections, women have a shorter duration of infection (stronger resistance) and men have greater viral loads (stronger tolerance)[102]. Sepsis is fundamentally an illness of impaired infection resistance and infection tolerance – there is a severe uncontrolled infection (ie. impaired resistance), as well as dysregulated host immune response to infection that causes collateral damage to host cells and tissues (ie. impaired tolerance). Therefore, to understand the mechanisms that mediate sexual dimorphism of illness severity in sepsis, we explored whether higher illness severity in males was the results of impaired infection resistance, infection tolerance, or both.

The host resistance strategy is primary destruction and elimination of pathogens, therefore a strong infection resistance would act to minimize the quantity of bacteria throughout the body[103]. To determine whether male biased illness severity was due to impaired infection resistance we collected blood, peritoneal lavage fluid and organ tissue samples from male and female mice 6 hours after infection with FIP or *E. coli*, and quantified the pathogen burden at the site of infection (peritoneum) and disseminated (blood and tissues). At the site of infection (peritoneum), within the blood and throughout disseminated body sites, both males and females had high amounts of pathogens, and there were no differences in the quantity of pathogens between sexes. If a lack of disease resistance was giving rise to male's poor disease tolerance, we would expect to see greater CFU values compared to females. However, this was not the case as both males and females demonstrated equivalent pathogen burden. It is important to note that all samples were collected at the 6 hour timepoint, thus this only represents the infection resistance within the hyperacute phase of sepsis. In a study of bacterial sepsis using *C. burnetii,* mice were infected for a longer timepoint, and it was found that females cleared the bacteria more

effectively than males[104]. Therefore, it is possible that longer endpoints post infection (ex. 24h) may uncover differences in infection resistance between males and females that are not apparent in the early phase of infection. Nevertheless, based on the observation that males were significantly sicker than females despite equivalent pathogen burden, this leads us to conclude that sexual dimorphism of illness severity in acute sepsis is not mediated by differences in infection resistance.

We next turned our attention to mechanisms of infection tolerance. A core mechanism of infection tolerance that is impaired in sepsis involves the development of an excessive systemic inflammatory response, which leads to damage to host tissues and organs[105]. Therefore, we investigated whether there were sex-based differences in the systemic inflammatory response to sepsis by measuring plasma levels of ten prototypical cytokines/chemokines involved in the "cytokine storm" of sepsis in males and females during FIP sepsis. We hypothesized that a greater systemic inflammatory response and excessive activation of pro-inflammatory cytokines (ie. "cytokine storm") in males may underly their higher illness severity compared to females. First, our results showed widespread upregulation of both pro- (TNFa, IL-1b, IL-6, KC/GRO), anti- (IL-10, IL-4), and immunomodulatory (IFN-γ, IL-2, IL-5) cytokines, confirming that our mice were indeed developing a "cytokine storm". When comparing males and females, although there were no significant differences in levels of TNF- α, or IL-1b, males did have small but significantly increased levels of pro-inflammatory cytokine IL-6 and the pro-inflammatory neutrophil chemokine KC. In contrast, females had a greater concentration of anti-inflammatory IL-10 and IL-4, as well as immunomodulatory cytokines including INF-γ and IL-5. Therefore, overall, it appears that females were mounting a more anti-inflammatory/immunomodulatory systemic inflammatory response compared to males, which tended to have more robust pro-

inflammatory profiles. However, we note that the differences were relatively small for most mediators, and therefore, it is unlikely that these subtle differences can fully explain the stark differences in illness severity between males and females. The only mediator that showed major differences in expression between males and females was IFN-γ, which was markedly higher in females compared to males. As a classical macrophage activating cytokine, it is somewhat difficult to attribute lower illness severity in females to a higher IFN-γ response, however it is possible that modulating macrophage function systemically may impart advantages to the host in terms of other downstream infection tolerance mechanisms. Therefore, it will be of interest to conduct future experiments to test this hypothesis through use of INF-γ -/- mice in the FIP model.

In addition to the systemic cytokine storm, another important mechanism of pathological systemic inflammation driving tissue and organ damage in sepsis is excessive neutrophil influx into end organs like the lung and liver. Neutrophils play a critical role in protecting the host within the innate immune response, however an excessive influx of these cells and their effector functions into vital organs like the lungs and liver, can cause severe tissue damage and organ dysfunction[61,79].

Differences in the activation of certain leukocytes have been noted between males and females in recent literature that may provide insight to this difference. Adullah *et al*. found that males have a greater frequency of Natural Killer cells compared to females[106]. Conversely, septic females were found to have a greater number of resident macrophages specifically in the peritoneal cavity during sepsis[107]. Differential response in neutrophil recruitment was noted in SARS-CoV-2 infection, as females had less immature neutrophil expansion compared to males[100]. It has also been found in an endotoxemia model that female macrophages and

neutrophils have a higher phagocytic activity[108]. The potential difference in innate cell recruitment, specifically first responders of infection, neutrophils, led us to investigate these sex-based differences.

To do this, we quantified neutrophil influx into lung and liver tissue from mice post FIP using flow cytometry and found no significant difference in the percentage of neutrophils in either lungs or liver between male and female mice during sepsis. It should be noted that there were only three mice per group, thus to increase the strength of this finding, increasing the number of mice used would strengthen this conclusion. However, similar to our flow data, no significant differences in MPO activity in the lungs was noted between males and females. It should be noted that we did not measure MPO activity in the liver, as the antioxidant content within the liver rapidly neutralizes free radical species, rendering this assay unreliable in liver tissue. Nevertheless, our results did not demonstrate meaningful differences in neutrophil recruitment to the lungs and liver between males and females. In future studies, it would be of interest to investigate other effector functions of neutrophils that may differ between males and females, including neutrophil phagocytosis, ROS, secondary granule release[109].

To further corroborate our findings, we targeted a key molecular mechanism of neutrophil recruitment in the lung and liver, DPEP-1, to determine whether disruption of neutrophil influx into these organs diminished the sexual dimorphism of sepsis illness severity. DPEP-1 was recently shown to play a critical role in mediating the influx of neutrophils into tissues, facilitating organ damage during an inflammatory response[65]. We first quantified the DPEP-1 gene expression at the transcriptional level using RT-qPCR, and found a significant difference between male and female DPEP-1 expression in the liver but not in the lungs. It should be noted that DPEP-1 gene expression was greater by an average of only ~0.07 Ct cycles

in male liver compared to females, with variability across samples. Indeed, comparing DPEP-1 knockout mice to wildtype, we found that males continued to have a greater illness severity than females. Because the knockout mice demonstrated the same sex bias in illness severity compared to the wildtype group, this suggests that the presence of DPEP-1, and further the lack of neutrophil infiltration, was not influencing this sexual dimorphism within illness severity. Overall, our results suggest that the sexual dimorphism of illness severity in sepsis cannot be explained by overt differences in systemic inflammation, including neutrophil influx into the lungs and liver.

As systemic inflammation and neutrophil influx into end organs could not fully explain the differences in illness severity between males and females, we next turned our attention to the core mechanisms of impaired disease tolerance in sepsis – mitochondrial tolerance. Mitochondria undergo a number of transcriptional and functional changes during infection to counteract infection induced cell damage and cell death, including upregulation of alternative pathways for ATP pathways (eg. shunting from oxidative phosphorylation towards fatty acid oxidation), as well as mechanisms to reduce reactive oxygen species generation – collectively, these processes are termed mitochondrial tolerance[81,82,110]. Recent studies have highlighted the importance of these mitochondrial tolerance mechanisms towards host fitness during infections, as disruption of mitochondrial tolerance pathways results in exacerbated inflammation, tissue damage, and death during infection[67,81,110]. Mitochondrial dysfunction through impaired mechanisms of mitochondrial tolerance is well established as a core mechanism of sepsis pathogenesis[67,81,111,112]. Interestingly, multiple studies have recently uncovered strategies to therapeutically potentiate mitochondrial tolerance during infection, which has been shown to improve outcomes in animal models of sepsis[67,110,113]. One of the most effective strategies to potentiate mitochondrial

tolerance is through treatment with the tetracycline antibiotic, doxycycline, which selectively acts on mitochondrial ribosomes to induce metabolic reprograming of mitochondria[67]. Colaço *et al*. recently found that doxycycline is a potentiator of mitochondrial tolerance, providing host protection from *E. coli* infection, independent of its antibiotic abilities (through the use of doxycycline-resistant pathogens)[67]. Doxycycline was shown to modulate mitochondrial gene expression, upregulating beneficial bioenergetic pathways and limiting host tissue damage (ex. increasing fatty acid oxidation and glucocorticoid sensitivity)[67]. However, like the majority of immunology studies, this study only evaluated mitochondrial tolerance and doxycycline treatment in male mice. Together, these data led us to hypothesize that differences in sepsis severity may be related to differences in mitochondrial tolerance between males and females, and testing this hypothesis by determining whether potentiation of mitochondrial tolerance with doxycycline could abrogate differential illness severity between males and females.

We found that in the PBS control groups, male mice had a significantly greater illness severity as found previously with our *E. coli* infections. However, when mice were given doxycycline prior to the infection, this bias diminished, indicating that the underlying mechanism driving the difference in illness severity is one disrupted by the effect of doxycycline (independent of its antimicrobial effect as we used doxycycline-resistant *E. coli*, and confirmed that both groups have equivalent severe disseminated infection). Given the extensive literature around doxycycline and potentiation of mitochondrial tolerance, these results suggest that differences in illness severity between males and females may be mediated by sex-based differences in mitochondrial tolerance. Further dissection of mitochondrial tolerance pathways between males and females will be the critical next step in this work, however this is outside the scope of the current thesis. Nevertheless, future experiments we aim to look at differences in

mitochondrial quantity, and activity through mitroROS production, and mitochondrial metabolism between males and females. Studying the mechanistic differences in mitochondrial function between males and females in sepsis opens critical investigation into potential sex-based therapeutics for sepsis.

While these initial experiments provide direction towards a potential mediator of the observed sexual dimorphism of disease tolerance, it's important to acknowledge that these experiments were limited to a single strain of a gram-negative bacteria, *E. coli* (ST131), to induce sepsis given the need for a doxycycline-resistant pathogens for these experiments. Because sepsis is a heterogeneous disease with various infections that may contribute to its onset, other models of infection (ex. viral) could be utilized in future studies to see if a similar protection is afforded to the host. Therefore, our preliminary results dive further into the mechanisms of disease tolerance show that doxycycline may mitigate this sexual dimorphism and highlight potential mechanisms for therapeutic pathways.

Overall, these results demonstrate that male mice have a higher illness severity as a result of impaired infection tolerance compared to females. Sex-based differenced in infection tolerance could not be fully explained by differences in systemic inflammation or neutrophil influx, but instead may be mediated by differential mitochondrial tolerance between males and females. These findings therefore open the door for further investigation and potential sex-based therapeutic options that target mitochondrial tolerance mechanisms.

5.3 Mediators of Sepsis Illness Severity

There are three key mediators that drive phenotypic differences between males and females, including sex chromosomes (XY), gonadal sex hormones, and the gut microbiota. We investigated each of these mediators and their potential impact on sepsis illness severity.

Gut Microbiota

It has been shown in recent years that the gut microbiome composition influences host defense again infection, and the dysbiosis of the microbiome can exacerbate illness during sepsis[47,114]. Additionally, composition and abundance differences between male and female microbiota have been noted to drive various immune diseases, including type-1 diabetes, multiple sclerosis, and others[55,56]. However, despite these findings, it remains unclear if these differences play a role in mediating the sexual dimorphism of sepsis disease tolerance. To test this, we compared GF and SPF mice and found that the male bias in illness severity observed equivalently in both SPF and GF mice. Therefore, this finding disproved our initial hypothesis that the gut microbiota was driving this difference in illness severity. It is important to note that we only studied the impact of the SPF microbiome, which is different in composition and abundance compared to the human microbiota. The gut microbiome composition of SPF mice is known to be less rich and diverse (in addition to being pathogen free) which is very different from humans, potentially limiting the clinical translation of findings of SPF microbiome. However, it has been found that mice with a wild microbiome phenocopy human responses to infection much more effectively than SPF mice[115]. Therefore, future studies may consider using wild microbiome mice (specifically re-wilded GF mice) to study the interplay between biological sex and the microbiome in sepsis, as this may reveal additional learnings about the contribution of sex-based microbiome differences in the context of a more complex microbiome and would also strengthen the translational potential of the findings.

Sex Chromosomes and Hormones

Given that the gut microbiota did not appear to be mediating difference in sepsis illness severity between males and females, we next looked to sex chromosomes and hormones. Firstly, the X chromosome has been found to play an important role in immune function, based on the

vast number and type of genes it encodes. Critical genes for immune surveillance are encoded on the X chromosome, and it has been proposed that the strong female bias for autoimmune disorders may be related to an increase in X gene dosage during XCI escape[116]. Alternatively, the production of different sex hormones (ex. androgens and estrogens) has been found to modulate the inflammatory responses and underlie the bias of specific immune disorders. For example, estrogens have been found to play a protective role during acute illness by inhibiting inflammatory pathways limiting an excessive immune response[41,117].

To decipher the contributions of sex chromosomes vs gonadal sex hormones, we employed a powerful transgenic mouse model, the four core genotypes (4CG) mouse model, that allows us to uncouple genotype from gonadal/hormonal phenotype[86]. We infected all four genotypes of these mice with FIP, and found that gonadal males displayed significantly higher illness severity compared to gonadal females, regardless of the sex chromosome makeup. These findings very clearly demonstrate that differences in illness severity are not driven by XY-linked genes, but instead are mediated by gonadal sex hormones. This finding pivots the attention to sex hormones as the potential mediator of sepsis illness severity.

The next steps would be to first understand if either male or female hormones are causing this sexual dimorphism. To investigate this, male and female mice could be gonadectomized to see if the illness severity phenotype is diminished[118]. Secondly, specific receptors could be targeted to block the function of sex steroid hormones to investigate their role in illness severity. Antagonists for estrogen receptor alpha (ERα) and/or androgen receptor antagonists could be utilized within the FIP model to understand how the elimination of sex steroid hormones impacts the immune response effects in sepsis[118,119].

Additionally, an important caveat of the 4CG mouse models is that is has a limited ability to inform on the effect of X-linked gene dosage. In the 4CG model, differences between XX and XY mice with the same gonads can be attributed to either the dosage of X chromosomes (two X vs one), or the absence of the Y chromosome[89]. Thus, because we did not see a difference between XX and XY mice with the same gonads, we are not able to definitively conclude that X linked genes do not contribute, only that Y linked genes are not involved. To mitigate this challenge, we could investigate the contribution of X linked gene dosage by implementing the XY* mouse model. The XY* model is used to detect the impact of X chromosome number through four genotypes of mice are: XX (ovaries), XY (testes), XO (ovaries) and XXY (testes)[86]. Implementing this model into our FIP experiment would provide further insight to the effects of X linked genes on sexual dimorphism of sepsis illness severity.

Overall, our investigations into the mediators of disease tolerance in sepsis provided valuable insight to the potential driving factors underlying the sex bias in illness severity. We were able to conclude that sexual dimorphism of illness severity is independent of the gut microbiota and sex chromosomes (within the limits described above), but rather dependent on gonadal sex hormone. This provides much needed insight into the field of biological sex and sepsis, furthering the investigation into the clear male sex bias that is severely understudied in humans and animal models.

5.4 Therapeutic and Translational Implications

Our findings within this project, in addition to the recent literature, highlights the critical importance of including male and female subjects in pre-clinical studies and clinical trials, and further demonstrates the critical importance of understanding how biological sex can impact

disease pathogenesis, and treatment responses. Given that the field of sepsis continue to be plagued by failed clinical trials of targeted therapeutics, our data lead us to posit that some of these failures may have been impacted by the fact that these trials failed to account for the heterogeneity of disease pathogenesis between patients, including the differences between males and females.

Our findings suggests that key differences in sex hormones may influence disease pathogenesis between males and females. Female subjects have notoriously been neglected in preclinical research studies despite the evidence that biological sex influences the prevalence, progression and outcome of many diseases including sepsis[90]. The strong male sex bias within sepsis demands that male and female subjects not only need to be included in pre-clinical studies, but specific attention be paid to differences arising from biological sex that may help understand this underlying difference. Therefore, given the sex bias demonstrated within our findings, preclinical research testing for novel therapies must include male and female subjects to ensure that sex-biased treatments are not applied through the translational pipeline.

It is crucial to learn from past oversights within this area, for example the failure to include women in clinical trials for cardiovascular disease that resulted in a sex biased understanding of illness presentation and ineffective treatments for women[120]. When designing clinical trials, it is important to consider the sample size of both male and female populations. Potential sex-specific mechanisms of action or treatment outcomes within the study may be overlooked if the sample size is split in half for a sex-disaggregated analysis. To combat this issue moving forward, we must be diligent about including appropriate sample sizes of both males and females in clinical trial design and further analyze and disaggregate the results by biological sex.

Our results align with clinical sepsis data, indicating that males have a greater sepsis illness severity than females[7]. The lower illness severity in females despite the same infection and similar levels of disease resistance, may result in the delayed treatment of women with sepsis in healthcare. Our findings show that when males and females are given the same infection and have similar abilities to clear the bacteria, males present with a worse illness. This bias may lead to women seeking treatment later than men despite the same level of infection, thus allowing the infection to further disseminate leading to more critical disease outcomes. Again, referring to one of the hallmark examples of overlooked sex bias in medicine; cardiovascular disease preclinical and clinical trials neglected females, leading to a classification and treatment design tailored to a male disease response[120]. This oversight has led to a plethora of complications as women presented with 'atypical' symptoms, thus they sought treatment later than men and received treatment designed for men. It is vital to apply these learnings to sepsis studies; acknowledging that sex bias is an important determinant of clinical disease phenotype, and this must be considered in the translation to mitigate biased treatment.

Conclusion

Throughout this thesis, we investigated the impact of biological sex in sepsis illness through three key mediators of sex-based immune response differences. We disproved our initial hypothesis that differences in the gut microbiota were driving the sexual dimorphism of illness severity. We uncoupled the contributions of sex hormones and chromosomes to find that gonadal hormones may be underlying this difference.

Despite the lack of difference found in infection resistance, we identified a greater infection tolerance for females within bacterial peritonitis. Preliminary data using doxycycline as a potentiator of mitochondrial tolerance, uncovered the potential of sex-based differences in metabolic mechanisms to be driving this difference in infection tolerance. Altogether, these results demonstrate that there are notable sex-based differences in infection tolerance and this difference is independent of differences in disease resistance, the gut microbiota or Y-linked chromosomes. Further, gonadal hormones were highlighted as a potential mediator of this bias and should be further investigated. Additionally, our preliminary results suggest that the sex bias in illness severity may be partially mediated by mitochondrial tolerance and provides insight for the next steps of much needed sex-specific therapeutics.

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