A ROLE PLAYED BY SEX IN INNATE IMMUNE MEMORY AND DISEASE SEVERITY

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled:

A ROLE PLAYED BY SEX IN INNATE IMMUNE MEMORY AND DISEASE SEVERITY

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a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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Dedicated to my friends, my parents, and some who are no longer here. Thank you all.

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Acetyl-CoA	Acetyl coenzyme A
Akt	Protein kinase B (“Ak” comes from mouse strain AKR)
ANOVA	Analysis of variance
AR	Androgen receptor
ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guérin
Bcl10	B cell lymphoma/leukemia 10
BCSA	*Burkholderia cepacia* specific agar
BM-MNCs	Bone marrow mononuclear cells
CARD9	Caspase recruitment domain-containing protein 9
CD	Cluster of differentiation
CLR	C-type lectin receptor
COVID-19	Coronavirus disease 2019
CpG	Cytosine-phosphate-guanine DNA motif
CRD	Carbohydrate recognition domain
csFBS	Charcoal stripped fetal bovine serum
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif ligand
DAMP	Damage associated molecular pattern
DCs	Dendritic cells
DMEM	Dulbecco’s modified Eagle’s medium

DMSO	Dimethyl sulfoxide
E2	17β-estradiol
ECAR	Extracellular acidification rate
EFR	EF-Tu receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FiO2	Fraction of inspired oxygen
FLS2	Flagellin sensor 2
GC	Germinal center
GLUT1	Glucose transporter 1
H3	Histone protein 3
HC	Heavy chain
HIF-1α	Hypoxia-inducible factor 1-alpha
HKCA	Heat-killed *Candida albicans*
HK2	Hexokinase 2
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
IPL	Immune priming lncRNA
JNK	c-Jun N-terminal kinase

KDM	Lysine demethylase
LB	Luria-Bertani
LC	Light chain
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
M1	Type 1 macrophage
M2	Type 2 macrophage
MALT1	Mucosa-associated lymphoid tissue translocation protein 1
MiRNA	Micro-RNA
MPO	Myeloperoxidase
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate H
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-kappa B
NK	Natural killer
oxLDL	Oxidized low-density lipoprotein
P4	Progesterone
PAMP	Pathogen associated molecular pattern
PaO2	Pressure of arterial blood oxygen
PASC	Post-acute sequelae of SARS-CoV-2
PBS	Phosphate buffered saline
PDK	Pyruvate dehydrogenase kinase

PI3K	Phosphoinositide 3-kinase
PMNs	Polymorphonuclear neutrophils
Poly(I:C)	Polyinosinic:polycytidylic acid
PR	Progesterone receptor
PRR	Pattern recognition receptor
RelB	Oncogene-related B
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (medium)
SA	Salicylic acid
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
SRY	Sex-determining region Y protein
TAD	Topologically associating domain
TCA	Tricarboxylic acid
Tfh	T follicular helper
Th	T helper
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNFa	Tumor necrosis factor-alpha
TRIF	TIR-domain-containing adaptor-inducing interferon
UMLILO	Upstream master lncRNA of the inflammatory chemokine locus

ABSTRACT

While immunological memory has traditionally been understood to be solely within the purview of adaptive immunity, recent evidence has shown that a type of “memory” exists in innate immune cells, as a prior insult can lead to enhanced subsequent pro-inflammatory activity, such as cytokine release, against a broad range of secondary stimuli. However, innate immune responses can differ between females and males, as sex hormones can augment or reduce activity. This dissertation examines the influence of the female sex hormones 17β-estradiol and progesterone on innate immune memory, or ‘trained immunity’. We hypothesized that progesterone would attenuate trained secondary responses. To this end, we found that infection by the opportunistic bacterium *Burkholderia gladioli* decreased survival in trained female adaptive immune-deficient mice, and that female mouse serum significantly attenuated trained secondary responses. This effect varied with serum from the different stages of the mouse estrous cycle and correlated with higher progesterone levels. We further showed that exogenous progesterone decreased trained responses, and glycolytic activity underlying trained immunity, while blocking its cognate nuclear receptor restored activity and rescued trained female mice from infection to the level of protection seen in males. This work suggests that the naturally cycling variability in progesterone levels may affect trained immunity and even protection against infections, possibly even during pregnancy though further studies are needed. This is the basis for a mechanism by which males can produce greater

average trained immune responses than females, which protected males from opportunistic *B. gladioli* infection, but oppositely, might correlate with increased male morbidity from coronavirus disease 2019 (COVID-19).

In early 2020, the COVID-19 viral pandemic shut down schools worldwide, although classes resumed in the Fall semester. We sought to track antibody seropositivity in the University of Missouri population during that semester, paying special attention to demographics including sex. As expected, antibody-positive cases increased throughout the duration of the semester, particularly among students. Older individuals, especially older men, had higher antibody concentrations after infection compared to women, although these levels declined more rapidly than those of women over time. We can correlate these observations with worldwide studies concluding that during the pandemic, men were shown to be more susceptible to severe morbidity and mortality than women, on average.

We proposed application of an existing drug to attempt to alleviate morbidity in the most severe COVID-19 cases. COVID-19 is primarily a respiratory disease caused by a viral pathogen. Many severe respiratory viral infections, including influenza, are often characterized by high neutrophil infiltration into the lungs and production of neutrophil extracellular traps (NETs), comprised of DNA and inflammatory enzymes, which can clog the respiratory tracts. We hypothesized that severe COVID-19 patients had high lung neutrophil counts and NETs; and that treatment with the cystic fibrosis drug, dornase alfa, which breaks apart these clogging NETs, would improve disease outcomes.

Indeed, we found that dornase alfa decreased NETs in the lungs and also improved lung characteristics in treated patients. Though we did not design the clinical trial to assess sex differences in our small cohort (n = 10 patients), together with the sex differences observed in the large data set sampling the MU community, and those reported worldwide, these findings imply an outsized role for innate immunity in severe respiratory infections. Although unexplored here, it is conceivable that training of innate cells and their precursors may have influenced immune hyperresponsiveness in COVID-19.

PART I:

Sex and Innate Immune Memory

Chapter 1

Introduction: Innate Immunity, Memory, and Sex

I. Overview of Innate Immunity

The vertebrate immune system is comprised of two primary arms: innate and adaptive immunity. Both are characterized by distinct functions and mechanisms, yet they work together in an integrated fashion to defend the host against invasive pathogens. The main distinguishing aspect of adaptive immunity is its ability to develop immunological memory against specific pathogens while, traditionally, the innate system is believed to lack this feature. Innate immunity acts as the first line of defense against invasive pathogens, operating through different cellular and molecular components. Among these are physical and chemical barriers intended to prevent unwanted microbial invasion into the host, and also a wide variety of professional immune and non-immune cells. This arm of immunity generally recognizes different classes of evolutionarily conserved molecular structures on or produced by pathogens through pattern recognition receptors (PRRs) (Akira, Uematsu & Takeuchi 2006). It is “innate” in that it provides broad, generalized, rapid responses that are inherent to these cells without the need to be primed by prior exposure to specific pathogens. Therefore, innate immunity has historically been believed to have broad pathogen applicability while lacking pathogen-specific memory.

In contrast to the innate system, adaptive immunity is generally characterized by highly pathogen-specific responses. Adaptive immune cells,

called lymphocytes and primarily comprised of B and T cells, have the ability to "remember" previous encounters with pathogens to yield rapid and robust responses upon re-exposure that are intended to kill the invading target. These functions are facilitated through the generation of memory lymphocytes that persist within the host long after an initial infection has been eliminated. During development, adaptive lymphocytes rearrange specific genetic loci, leading to unique receptors on each B and T cell specific to one of potentially trillions or more possible antigens which they could recognize. With each T and B cell adopting one antigen specificity, the population of millions of these lymphocytes cover the millions of reactivities needed for normal, healthy immune protection. B cell antigen receptors have a secreted form, antibodies, well known for binding antigens in bloodstream, mucosal sites, and throughout the body. Often dependent on interaction with T cells in a process termed "T cell help", adaptive B cell memory and secreted antibody serves as the primary basis of the disease control strategies most elicited by vaccination. Interestingly, both innate and adaptive immunity appear to show sexually dimorphic activities, with genetics and hormones playing an influential role (Klein & Flanagan 2016).

Recent evidence, however, shows that even innate immunity may develop some primitive form of "memory" akin to long-term priming (Netea et al., 2012). This emergent concept posits that after exposure to an initial stimulus, innate immune cells exhibit altered (usually enhanced) responsivity to later encounters with pathogens that may be unrelated to the first one. Lacking the pathogen specificity that hallmarks adaptive immune cells, this innate memory formation is

called immune training, generally creating immune primed sites where enhanced responses to diverse secondary stimuli can be induced. This emerging aspect of innate immunity adds a new dimension to the understanding of immunology, with potential for multifarious avenues of research including evaluating the influence of sex. Few studies have examined this, and as such, the focus of this section is to investigate the role of sex in innate immune memory.

A. Components of the innate immune system

Innate immune cells

Innate cells are characterized by a diverse array of functions that activate rapidly to kill pathogens and prevent establishment of infection and pathogenic colonization by microbes. Among these are the ability to phagocytize (or engulf and destroy) microbes; induce pro-inflammatory and microbicidal responses; and to present microbial components to cells of the adaptive immune system to engage adaptive effector and memory functions. The professional phagocytes are divided into two main classes: mononuclear cells, including monocytes and macrophages, and neutrophils (or polymorphonuclear neutrophils, PMNs). These cells specialize in the engulfment and degradation of foreign materials and microbes (Amulic et al., 2012). The classical monocytes are predominantly phagocytic but also synthesize inflammatory mediators and readily differentiate into two primary classes of macrophages after migration into tissues (Serbina et al., 2008; Guilliams, Mildner & Yona 2014). Classical

macrophages, or M1 cell, are phagocytic and produce pro-inflammatory mediators; while alternative macrophages, or M2 cells, aid in tissue repair post-infection and inflammation. Nonclassical monocytes also share functional properties with M2 cells, in that they promote wound healing (Murray et al., 2014). Additionally, PMNs can release their nuclear material and microbicidal proteins, such as myeloperoxidase and neutrophil elastase, to form neutrophil extracellular traps (NETs) which function like a web to ensnare and destroy pathogens (Brinkmann et al., 2004). NETs are highly pro-inflammatory and can also damage host cells.

Other mostly pro-inflammatory innate cell types include basophils, eosinophils, and mast cells, these being granulocytes that contain abundant numbers of internal granules that are released when activated primarily by parasites and allergens, resulting in strong inflammatory activity (Voehringer 2013; Klion & Nutman 2004; Lu & Huang 2017). As with PMNs, these responses can also damage the host. Meanwhile, dendritic cells (DCs) are unique. Generally divided into three major subgroups, DCs function to produce inflammatory mediators to help cells kill viruses, while they also capture foreign antigens to present them to adaptive cells in order to initiate adaptive effector and memory functions (Banchereau et al., 2000). Another lymphocyte type are the natural killer (NK) cells that act as an early defense against tumors and viruses by secreting proteins to induce cytotoxicity and producing cytokines such as IFN-γ (Vivier et al., 2008). Though generally overlooked as an immune

system component, epithelial cells are also able to mount limited innate immune responses upon recognition of or infection by pathogens (Chustz et al., 2011; Steinbrecher et al., 2008).

Soluble factors

Aside from phagocytosing pathogens, innate immune cells also exert functions via the production and release of soluble mediators which go on to affect other cell activities. Among these are the cytokines, the major types of which include interleukins (ILs), interferons (IFNs), and tumor necrosis factors (TNFs). These bind to specific receptors on the surfaces of immune cells that activate signaling mechanisms pivotal to immune functions (Villarino et al., 2015). For instance, ligation of cytokine receptors induces Janus kinase (JAKs) activity leading to the recruitment and functions of signal transducers and activators of transcription (STATs), which then go on to modulate gene expression leading to variable outcomes such as cell survival, differentiation, and metabolic activity (Villarino et al., 2015). Certain cytokines also work as chemokines (chemotactic cytokines) to direct the migration of immune cells to the site of microbe invasion or injury. As examples, CCL2 recruits monocytes, dendritic cells, and some adaptive cells to sites of inflammation, while CXCL8 (or, IL-8) functions in a similar manner to recruit PMNs (Xu et al., 1996; Baggiolini, Walz & Kunkel 1989). These chemokines can, in turn, also induce the production of other inflammatory cytokines. Furthermore, other signaling mechanisms work alongside JAK-STAT, such as the

mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-Kinase (PI3K)/Akt pathways to affect cellular activities such as survival, proliferation, metabolism, and elaboration of inflammation (Arthur & Ley 2013).

B. Pathogen recognition and signal transduction

Pathogen recognition receptors

The core functions of the innate immune system rely on pattern recognition receptors (PRRs), specialized proteins that identify certain molecular markers known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These PRRs are comprised of a wide array of proteins, including the C-type lectin receptors (CLRs), toll-like receptors (TLRs), and other classes such as the NOD-like and RIG-I-like receptors which will not be discussed here. These PRRs recognize distinct microbial components such as carbohydrates, lipopolysaccharides (LPS), and nucleic acids, acting as an important surveillance system by which innate cells monitor presence of infection and/or tissue destruction. The CLRs mostly recognize carbohydrate structures on pathogens, thus forming a substantial line of defense against certain bacterial and fungal infections (Pyz et al., 2006). Meanwhile, TLRs recognize a different diverse set of PAMPs, including lipopolysaccharides (LPS) from Gram-negative bacteria, bacterial flagellin and other microbial components such as nucleic acids (Poltorak et al.,

1998; Hemmi et al., 2000; Hayashi et al., 2001). DAMPs, markers released by damaged or dying host cells, are also recognized by many TLRs, including TLR9 in the case of extracellular CpG-rich host DNA (Bauer et al., 2001; Lund et al., 2003). This interplay between PAMPs and DAMPs activates certain signaling mechanisms culminating in phagocytic activity and the production of pro- and sometimes anti-inflammatory cytokines (Akira & Takeda 2004). However, PRRs in innate immune cells also function as a bridge to adaptive immunity, as activation facilitates the expression of co-stimulatory molecules that help to enable adaptive immune responses to specific pathogens (e Sousa 2004). Thus, the complex functions of PRRs in innate immunity generally help to ensure a comprehensive first defense against a dynamic landscape of pathogens that may break through initial body barriers.

C-type lectin receptors

The CLRs comprise a family of PRRs important to innate immune recognition of ligands including carbohydrate moiety-based PAMPs and DAMPs, with calcium-dependent functions. While CLRs play a significant role in host defense, their activation can also lead to metabolic changes in innate cells. CLRs are classified into several groups, primarily based on conserved structural features such as carbohydrate recognition domains (CRDs). The most well characterized are the first two of these groups. The group I CLRs generally possess two or more CRDs or CRD-like domains, comprising receptors such as macrophage mannose receptor, which binds

to mannose-containing ligands (Mullin, Hitchen & Taylor 1997). Group II CLRs, however, typically utilize just one CRD, with receptors such as Dectin-1 and Dectin-2 that bind to β-glucans in bacterial and fungal cell walls and high-density mannose structures, respectively (Brown 2003; Saijo et al., 2010). Ahead, we will see that receptors such as Dectin-1 play leading roles initiating the signals that induce innate immune training. The signaling mechanisms of CLR activation are varied, involving the recruitment of adaptor molecules like Syk or CARD9, ultimately engaging certain inflammatory pathways. For instance, the ITAM-like motif of Dectin-1 recruits Syk, activating the CARD9-Bcl10-MALT1 complex, resulting in IKK phosphorylation of IkBα and freeing the nuclear regulatory factor NF-kB, allowing its translocation into the nucleus to induce pro-inflammatory gene transcription (Gross et al., 2006). This CARD9-Bcl10-MALT1 complex also activates MAPK pathways, leading to ERK, JNK, and p38 movement into the nucleus, contributing to inflammatory cytokine production (Strasser et al., 2012; Saijo et al., 2010). This signaling complexity also leads to activation of the NADPH oxidase complex, leading to reactive oxygen species (ROS) generation. ROS play vital roles in autophagy regulation, pathogen killing, and modulation of inflammatory responses including those of NF-kB functions.

Toll-like receptors

Perhaps the most well-studied PRRs are the TLRs, evolutionarily conserved type I transmembrane proteins characterized by leucine-rich

repeat domains which recognize different PAMPs and DAMPs, and cytoplasmic TIR domains responsible for initiating downstream signaling (Akira, Uematsu & Takeuci 2006; Janssens & Beyaert 2003). There are at least 13 different TLRs identified in humans and other mammals that can be divided into two groups based on their cellular localization. The first comprises TLR1, 2, 4, 5, 6, and 10, which are typically expressed on the plasma membrane and mostly function by recognizing bacterial and fungal cell wall components. The second group includes TLR3, 7, 8, and 9, which are usually found within endosomes and primarily detect bacterial and viral, although sometimes also host, nucleic acids (Kawai & Akira 2010). However, both TLR3 and 9 can also be found in low abundance on the plasma membrane. For instance, TLR4 recognizes LPS from Gram-negative bacteria (Poltorak et al., 1998), while TLR3 detects double-stranded RNA (dsRNA) commonly associated with viral infections (Alexopoulou et al., 2001). Each of these typically forms homodimers to recognize their cognate ligands, although TLR4 homodimers can also utilize CD14 to facilitate activity (Jiang et al., 2000).

Upon ligation, TLRs typically undergo conformational changes enabling the recruitment of specific adaptor proteins containing TIR-like domains such as MyD88 and TRIF. The MyD88-dependent pathway, utilized by all TLRs except TLR3 and exclusively by TLR5, 7, 8, and 9, leads to the activation of MAPKs and NF-kB through the recruitment and phosphorylation of IRAKs and TRAF6 (Kawai & Akira 2011). This

promotes the transcription of pro-inflammatory genes like IL-6 and TNFa. The TRIF-dependent pathway is engaged exclusively by TLR3 and also TLR4, leading to the activation of IRF3 and IRF7, both of which translocate to the nucleus leading to the production of type I IFNs which are vital for antiviral responses (Yamamoto et al., 2003).

It is therefore clear that rapid recognition of a broad range of pathogenic microbes via the PRRs is a vital feature of innate immune function. Ahead, we will see that responses through these receptors can be enhanced by prior innate immune training. The engagement of such receptors activates multiple different signaling cascades, leading to the activity of various transcription factors, notably NF-κB, that help initiate inflammatory responses (Takeuchi & Akira 2010). While the different PRR classes utilize overlapping signaling mechanisms, it is their ability to recognize a wide variety of pathogen markers that sets them apart and defines their essential role in innate immunity (Kawai & Akira 2011). For instance, signaling inducing NF-κB activity, perhaps the most common intermediate downstream of PRRs, acts as a converging point for these various pathways, leading to the expression of pro-inflammatory cytokines and other mediators of immune response (Hayden & Ghosh 2012; Ghosh & Hayden 2012). This recognition system is also not exclusive to professional innate immune cells. Epithelial cells, which comprise the lining of various organs such as the intestines, lungs, and uterus, also exhibit primitive immune activity. They also extensively rely on PRR-

mediated activation of signaling pathways such as NF-κB to induce cytokine production, thus aiding in their functions to alert neighboring cells to the presence of pathogens and even inducing apoptosis when necessary.

II. Innate Immune Memory

Historically, the innate immune system was thought to provide broad, generalized responses against pathogens without any form of memory. However, this perspective began to shift with the emergence of the concept of heterologous immunity, whereby exposure to one pathogen leads to some level of protection against unrelated pathogens, although one finds both innate and adaptive components to heterologous immunity such that the previous literature on this theme did not isolate a memory-type response to innate cells. Indeed, B and T cells may significantly contribute to heterologous immunity via antigen cross-reactivity, which can allow an otherwise naïve response to include contributions from memory lymphocytes previously expanded during an unrelated historical infection. Ultimately, the concept of innate immune memory was formally recognized and defined by Netea in 2011, termed trained immunity based on the apparent “training” of innate cells by an initial insult to yield significantly enhanced responses upon subsequent encounter of a second immune insult (Netea, Quintin & Van Der Meer 2011).

A. Trained immunity

Earliest evidence

Evidence for innate immune memory was later recognized as having accumulated decades before its formal recognition as a distinct aspect of innate immunity. Previous studies found that immunocompetent mice vaccinated with bacillus Calmette-Guérin (BCG, a live-attenuated strain of *Mycobacterium bovis*) against tuberculosis hindered the progression of transplanted tumors (Old, Clarke & Benacerraf 1959). Furthermore, later studies showed that BCG administration in similar mice protected against subsequent infections with diverse pathogens such as *Listeria monocytogenes* and *Salmonella typhimurium* (Mackaness 1964; Blanden, Mackaness & Collins 1966). Notably, these prior studies utilized mice with intact adaptive immune systems, which may indicate at least some role for adaptive immunity in these results. However, this BCG-dependent protective effect was then found in adaptive lymphocyte-deficient mice following administration of a lethal dose of *Candida albicans* (Welsh & Selin 2002).

Evidence from plants and invertebrates

Such effects are not limited to vertebrates, or even the animal kingdom. Certain plant species develop a form of antigen-nonspecific innate memory. For instance, bacterial challenge in *Arabidopsis thaliana* engages the EF-Tu receptor (EFR) and flagellin sensor 2 (FLS2) PRRs which results in enhanced activity against subsequent infections, even in

completely different parts of the plant (Sticher, Mauch-Mani & Métraux 1997; Luna et al., 2012). Such memory-like characteristics appear to be the result of epigenetic changes, with histone modifications playing a vital role (Jaskiewicz, Conrath & Peterhänsel 2011). Plants also exhibit a type of immune memory termed *systemic acquired resistance*, whereby activation of PRRs leads to the accumulation of salicylic acid (SA), a hormone which enables greater responsiveness to later infections after subsequent PRR engagement (Moreau, Tian & Klessig 2012; An & Mou 2011). This buildup of SA also alters the epigenetic landscape to help increase secondary responses (Latzel et al., 2012).

Some forms of innate memory are also present in insects, such as the common fruit fly *Drosophila melanogaster* and the mosquito *Anopheles gambiae*. Importantly, insects do not have adaptive immunity. In *Drosophila*, activation of phagocytic hemocytes via Toll, a PRR believed to be a precursor to mammalian TLRs and for which TLRs were named, results in protection against reinfection. However, this effect appears to be pathogen-specific as infection with *Streptococcus pneumoniae* confers resistance to the same bacterium and is mediated by accumulation of thioester-containing proteins enabling enhanced activity (Pham et al., 2007). *Anopheles* mosquitoes, after an initial exposure to an attenuated form of the malarial parasite *Plasmodium falciparum*, subsequently show greater responses to bacterial and other malarial infections (Rodrigues et al., 2010). Such protective activities seem to be primarily mediated by

hemocytes, also evident in mealworm beetles, but epithelial cells may also play a role (Roth & Kurtz 2009; Pham et al., 2007; Rodrigues et al., 2010). The persistence of such findings across invertebrate and vertebrate organisms, and even kingdoms suggests that innate immune memory may be an evolutionarily conserved phenomenon.

Trained immunity in vertebrates

A landmark human clinical study from 2011 found that administration of the BCG vaccine against tuberculosis reduced all-cause mortality in infants, particularly from many different infectious diseases (Aaby et al., 2011). These effects are also long-lasting, as BCG augments off-target immune activity up to one-year post-vaccination (Kleinnijenhuis et al., 2014). This long-term protection may be attributable to bone marrow hematopoietic progenitors, leading to monocytes and neutrophils capable of enhanced activity in mice and humans (Kaufmann et al., 2018; Cirovic et al., 2020). However, this does not rule out the role of adaptive immune activity, as cross-reactivity from such aspects as epitope sharing may contribute to this protective effect. Aside from BCG, other vaccines also appear to elicit pathogen-nonspecific protective effects. Immunization of children against measles significantly reduces all-cause mortality by approximately 30% (Mina et al., 2015). Meanwhile, other live attenuated vaccines exhibit enhanced pathogen-nonspecific protection, as oral polio and smallpox immunization diminishes overall childhood mortality (Andersen et al., 2018; Sørup et al., 2011).

The development of trained immunity is orchestrated through a series of well-coordinated steps, the first of which is the priming phase. This involves the engagement of PRRs such as Dectin-1 or TLR4 by broadly different stimuli, setting off intracellular signaling cascades utilizing nuclear regulatory factors like NF-κB leading to transcriptional changes favoring pro-inflammatory activity, resulting in the cellular metabolic and epigenetic reprogramming characteristic of the second step, which will be discussed in detail, below (Arts et al., 2018). Although this is how typical immune responses are initiated, what sets trained immunity apart is mostly the timing of the evaluated response. Many studies examine immune activity at the time of primary stimulation, whereas assessment of trained immunity focuses on subsequent secondary responses. However, the dose of the initiating stimulus also plays a key role in later activity. High concentrations of LPS induce a “tolerant” state in monocytes, characterized by unresponsiveness to additional stimuli, while low LPS doses result in enhanced subsequent activity (Ifrim et al., 2014). Such dose-dependent effects appear to be important in the induction of trained immunity by other stimuli, as low doses enhance later responses while high doses lead to “tolerance” (Ifrim et al., 2014).

Induction of training was first shown with the fungal pathogen *C. albicans*, as a non-lethal infection enhanced survival against a subsequent fatal dose in adaptive immune-deficient mice (Quintin et al., 2012). This effect was pinpointed to the 1,3-β-glucan component of the fungal cell wall

as the stimulus binding Dectin-1 to initiate training. PRR engagement of many different pathogenic stimuli have since been shown to induce trained immunity. Bacterial cell wall components such as muramyl dipeptides, LPS, and flagellin initiate training via TLR2, TLR4, and TLR5 engagement, respectively, while the malarial parasite *P. falciparum* and herpesviruses protect mice against later pathogen-nonspecific infections (Schrum et al., 2018; Barton et al., 2007). However, training stimuli are not limited to pathogens, as endogenous DAMPs also play a role. Oxidized low-density lipoprotein (oxLDL), a major component of cardiovascular disease-promoting cholesterol plaques in blood vessels, induces trained immunity by engaging the innate cell surface protein CD36 with TLR2, 4, or 6 of circulating monocytes (Bekkering et al., 2014). Meanwhile, certain host homeostatic components like aldosterone, which regulates blood mineral levels and pressure; and even host factors related to infection, such as extracellular heme, can directly initiate training by triggering cytoplasmic factors (Jentho et al., 2021).

The culmination of innate immune training is enhanced pro-inflammatory responses after subsequent activation. Upon restimulation with various PAMPs like *C. albicans*, β-glucan, *Escherichia coli*, or LPS after prior training with heat-inactivated *C. albicans*, mouse and human monocytes exhibit significantly increased production of various pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, CXCL1, CXCL2, type I IFNs, and TNFa (Quintin et al., 2012; Arts et al., 2018; Kaufmann et

al., 2018). This enhancement in cytokine and chemokine output upon secondary stimulation does not encompass the full breadth of training-induced changes, as ROS activity in trained monocytes is significantly upregulated (van der Heijden et al., 2020). Meanwhile, phagocytic capacity is altered in trained cells, although there is discordance between studies: some have found enhanced phagocytosis after induction of training, while others show decreased activity (Sun et al., 2021; Pan et al., 2022). These effects are not solely limited to the trained cells, as they also exhibit increased output of cytokines like IL-12 and IL-6 leading to the differentiation of pro-inflammatory CD4 T helper cells (Kleinnijenhuis et al., 2014). This goes both ways, however, as the induction of training in alveolar macrophages appears to require CD8 T cell production of IFN-γ (Yao et al., 2018).

Trained immunity is most well-described in monocytes and macrophages, mostly within circulation although some studies have focused on resident macrophages within specific anatomic niches (Zahalka et al., 2022; Jeyanathan et al., 2022; Wang et al., 2023). However, other innate cells can be trained by initiating stimuli. For instance, conventional dendritic cells exposed to the fungal pathogen *Cryptococcus neoformans* exhibit enhanced protection against subsequent infectious insults, a process mediated by greater IFN production compared against untrained cells (Hole et al., 2019). It is possible this increased activity also helps to modulate adaptive cells to

mount more profound responses. Granulocytes, myeloid-lineage cells initially believed to not be capable of training due to their exceptionally short lifespan compared to other innate cells, can also be trained. One study found that pretreatment of mice with β-glucan enhanced neutrophil activity against transplanted tumors, and adoptive transfer of these neutrophils leads to greater activity against tumors in untreated mice (Kalafati et al., 2020). BCG vaccination in humans and mice also results in long-term changes in neutrophils, enabling enhanced responsiveness against nonspecific infections (Moorlag et al., 2020). This training effect in both humans and mice was found to be due to cellular changes in bone marrow myeloid progenitors which give rise to most innate immune cells (Cirovic et al., 2020; Kalafati et al., 2020; Moorlag et al., 2020). Indeed, training of cells in the bone marrow via vascular and not subcutaneous immunization leads to enhanced responsiveness in other innate cell types such as monocytes and macrophages (Kaufmann et al., 2018). Such findings suggest that training is not confined to circulating cells but can also be imparted from precursors and may be a more important aspect of the long-term consequences of trained immunity compared to cells in circulation. Other innate cell types, like NK cells and innate lymphoid cells, are also capable of developing trained immunity.

B. Mechanistic basis of trained immunity

Metabolic rewiring

The induction of trained immunity begins with the initiating stimulus acting on its cognate PRR, the most well-studied of which is the ligation of the dectin-1 receptor by 1,3-β-glucan. This sets off intracellular signaling cascades that alter metabolic activity away from oxidative phosphorylation in favor of aerobic glycolysis enabling rapid, enhanced pro-inflammatory responses upon later insults, in a process similar to the Warburg effect in cancer cells. Ligation leads to activation of PI3K-Akt, which induces the expression of GLUT1, allowing for excess glucose transport into the cell, and enzymes like hexokinase 2 (HK2) which initiate glycolytic activity (Su et al., 2021). PI3K-Akt signaling further activates mTOR which, in turn, leads to the activation of the transcription factor HIF-1α that goes on to increase the expression of glycolysis-related genes and some which suppress activity of the TCA cycle, like *GLUT1* and *HK2*, and *PDK*, respectively (Semenza 2003; Cairns, Harris & Mak 2011; Ward & Thompson 2012). Although glycolysis is less efficient than oxidative phosphorylation in producing ATP for cellular energy, it is a much more rapid process that enables fast energetic activity that aids in the enhanced production of pro-inflammatory mediators characteristic of trained immunity.

Pyruvate, the final byproduct of glycolysis, goes on to the TCA cycle for further metabolic breakdown. However, the production of TCA cycle

suppressors like PDK during the initial phase of trained immunity induction leads to altered activity which allows for the accumulation of multiple metabolic intermediates like acetyl-CoA, citrate, succinate, and fumarate, all of which further contribute to the development and maintenance of trained immunity (Keating et al., 2020; Sohrabi et al., 2020; Verberk et al., 2021; Paris et al., 2020; Arts et al., 2016). Most of these, such as citrate which is further converted to acetyl-CoA, itaconate, and fumarate play vital roles in epigenetic changes which open up promoter and enhancer sequences of pro-inflammatory genes and will be further discussed, below (Verberk et al., 2021; Arts et al., 2016). Meanwhile, succinate helps to stabilize HIF-1α activity leading to further maintenance of glycolytic activity (Tannahill et al., 2013).

Other metabolic activities aside from altered glycolysis and TCA cycle functions also play a vital role in trained immunity. Glutaminolysis, the sequential breakdown of the amino acid glutamine into various components such as glutamate, aspartate, pyruvate, lactate, alanine, and citrate, is a requirement for the induction of training (Arts et al., 2016). As mentioned previously, pyruvate and citrate are both metabolized into acetyl-CoA, providing more substrate for epigenetic modifications. Meanwhile, the amino acids produced by the breakdown of glutamine provide some of the building blocks for the enhanced production of pro-inflammatory markers like IL-1β, IL-6, IL-8, CXCL1 and CXCL2, and TNFa (de Oliveira et al., 2016). Similarly, glutaminolysis leads to the production

of excess α-ketoglutarate, another TCA cycle intermediate that contributes to the epigenetic remodeling required for training (Martínez-Reyes & Chandel 2020).

Epigenetic changes

Changes to specific epigenetic markers are also required for the induction of trained immunity. Typically, deposition of methyl groups on cytosine residues in DNA inhibits transcription of nearby genes; however, methylation of lysine residues found on histone proteins opens specific genetic regions enabling increased transcriptional activity. This is mostly achieved by trimethylation of the lysine 4 residue on the histone H3 protein (H3K4me3) and is a vital epigenetic component of trained immunity (Quintin et al., 2012; Saeed et al., 2014). Importantly, deposition of H3K4me3 markers near the promoter regions of *IL1B*, *IL6*, and *TNF* after the initiation of training in monocytes aids in the enhanced transcription of these genes after secondary stimulation (Quintin et al., 2012; Saeed et al., 2014; Mitroulis et al., 2018). This is also true at the promoter regions of the CXCL chemokine locus for the *IL8*, *CXCL1*, *CXCL2*, and *CXCL3* genes (Flores-Concha & Oñate 2020). Interestingly, monomethylation of the lysine 4 residue on histone H3 (H3K4me1) also enables greater activation of these genes (Quintin et al., 2012; Arts et al., 2018; Mitroulis et al., 2018). Similarly, the deposition of acetyl groups on histone protein H3 also promotes greater transcription of nearby genes, particularly monoacetylation of lysine 27 (H3K27ac) (Quintin et al., 2012;

Arts et al., 2018; Mitroulis et al., 2018). This marker aids in the increased activation of the aforementioned genes, although they are found not in promoter but instead enhancer regions in order to readily facilitate the binding of transcription factors to promoters leading to greater activity (Saeed et al., 2014). H3K27ac markers also play a role in the increased expression of PRRs in trained monocytes (Saeed et al., 2014).

The rewiring of metabolic functions leads to the accumulation of metabolites vital to some of these long-term epigenetic changes in trained immunity. Acetyl-CoA can translocate to the nucleus and acts as a donor for acetylation of the histone markers described previously; citrate is also metabolized into acetyl-CoA (Verberk et al., 2021). Meanwhile, fumarate aids in the acetylation process for H3K27, but also inhibits activity of the lysine demethylases KDM4 and 5 allowing for greater accumulation of H3K4me1 and H3K4me3 markers near pro-inflammatory genes (Arts et al., 2016). The breakdown of glutamine also plays an important role in training-related epigenetic modifications, as its metabolite α-ketoglutarate acts as a cofactor for DNA cytosine demethylases allowing for greater transcription of nearby genes (Martínez-Reyes & Chandal 2020).

Much of the eukaryotic genome is separated into physical regions in which different parts of the same chromosome, or even different chromosomes, come into contact with each other known as topologically associating domains (TADs) (Fanucchi et al., 2013). These TADs form three-dimensional structures such as chromosomal loops which bring

different components such as DNA and transcriptional regulators such as various lncRNAs into close proximity (Fanucchi et al., 2019). A newly described class, called immune priming lncRNAs (IPLs), play important roles in the epigenetic modifications required for trained immunity (Fanucci et al., 2019). One specific IPL, known as UMLILO (or, upstream master lncRNA of the inflammatory chemokine locus) helps to regulate the deposition of H3K4me3 markers at the promoter sites for the *IL8*, *CXCL1*, *CXCL2*, and *CXCL3* genes by bringing the mixed-lineage leukemia methyltransferase into contact of this locus (Fanucchi et al., 2019; Moorlag et al., 2020). Another, IPL-IL1, interacts with UMLILO to aid in the trimethylation of the promoter sequence for *IL1B* (Moorlag et al., 2020). Taken together, these cellular changes involving long-term metabolic rewiring and epigenetic modifications provide the mechanistic underpinnings required for the enhancement of pro-inflammatory responses upon secondary stimulation. The basic mechanism of trained immunity via Dectin-1 activation is outlined in Figure 1.1.

III. Influence of Sex on Innate Immunity

Sex appears to influence the functions of innate immunity in a nuanced manner. Traditional views have held that females typically mount more complex, even stronger responses against pathogens when compared to males. These differences are mediated by numerous factors, such as chromosome-linked gene regulation and hormonal activity. However, these effects are not necessarily as

well-defined as tradition suggests and can be highly variable, which will be discussed in the following sections.

Such differences may have evolved as adaptations to the reproductive roles and pressures of the sexes. Females may have evolved more complex, heightened responses to protect their developing offspring. Meanwhile, the reproductive strategy of males is believed to favor the allocation of energy toward the development of secondary sexual characteristics, such as increased muscle mass and hair growth in mammals, over immune functions. Such energy reallocation has been called the “immunocompetence handicap” which hypothesizes that testosterone-driven development of these characteristics comes at the cost of greater immune function (Folstad & Karter 1992). This is evidenced in other animal taxa such as birds, where male peafowls have reduced immune activities while developing elaborate tail feathers, a testosterone-driven trait (Roberts, Buchanan & Evans 2004). Such dimorphic functions may also be related to pathogen susceptibility, as males generally have greater parasitic burden (Zuk & McKean 1996). Males are also more susceptible to developing and succumbing to sepsis (Lakbar et al., 2023). They are more likely to mount hyperinflammatory responses to infection common in sepsis, which damages host tissue in order to kill the invading pathogen, releasing DAMPs and recruiting more immune cells (Cao, Yu & Chai 2019). Due to this hyperactivity, these cells can lose their abilities to respond or die off via apoptosis, while macrophages shift to M2 polarization to repair damaged tissue, likely allowing surviving microbes to proliferate and harm or kill the host (Chen et

al., 2021). These M2 cells also help recruit more anti-inflammatory T cells, which can result in immune suppression. Testosterone also enhances degranulation in neutrophils while also promoting NETosis (Deitch et al., 2006). Thus, its role in immunity is more nuanced than being simply suppressive and will not be discussed further as it is not the focus of this work.

A. Genetic factors

X chromosome and genes

Some genes instrumental in innate immune function are found on the sex chromosomes. In particular, the X chromosome contains several involved in TLR signaling and innate cell function and differentiation including the genes for TLR7, TLR8, and also subunits of the cytokine receptors for IL-2 and IL-13 (Souyris et al., 2018; Fish et al., 2008; Libert, Dejager & Pinheiro 2010; Spolarics et al., 2017). Inactivation of one of the two X chromosomes may contribute to the complexity of immune responses in females. This inactivation event does not occur homogenously in all cells and results in a mosaic-like pattern of X-linked genes, some of which retain bi-allelic expression, possibly providing females with some increased immunological potential (Ober, Loisel & Gilad 2008; Libert, Dejager & Pinheiro 2010; Spolarics et al., 2017). Meanwhile, males only possess one X chromosome, and are thus more likely to suffer greater consequences due to mutations in X-linked immune

genes (Fish 2008). These effects could explain some observed sex-based disparities in immune functions.

Consequently, females often exhibit augmented expression of these genetic markers, thereby enhancing their related functions. For instance, the increased TLR7- and TLR8-mediated signaling activity in female immune cells leads to enhanced production of related IFNs and activity against viruses (Berghöfer et al., 2006; Spolarics et al., 2017). Furthermore, females demonstrate elevated M2 and anti-inflammatory lymphocyte counts, which tend to promote tissue repair after an initial immune response (Oertelt-Prigione 2012).

Non-coding RNAs

Genetic factors contributing to sex differential immune activity extends beyond protein-coding genes. Much of the mammalian genome does not encode functional genes but instead regulatory RNA transcripts that do not produce proteins. These include microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), which are now known to play vital roles in the regulation of many genes related to myriad functions, including innate immunity. MiRNAs generally silence gene expression, either via direct repression of transcription or by inhibiting translation of mRNA into functional proteins (Bartel 2009). In contrast, lncRNAs may either down- or upregulate gene expression, and even affect epigenetic modifications, albeit through incompletely understood mechanisms (Atianand, Caffrey & Fitzgerald 2017). Interestingly, a high percentage of these regulatory

RNAs are found on the X chromosome, while only two of them are present on the Y chromosome. Such distribution suggests that females may express these at higher levels, contributing to differential immune activities between the sexes (Pinheiro, Dejager & Libert 2011).

Sexual dimorphism in innate immunity, however, is not exclusively confined to gene loci. Activation of many different genes, and even epigenetic regulation of them, are also affected by sex hormone signaling mechanisms. Receptors for these hormones bind to homologous DNA elements to either down- or upregulate gene expression, including many involved in immune functions.

B. Hormonal influences

Sex hormones and cyclic changes

Sex hormones, namely 17β-estradiol (E2), progesterone (P4), and testosterone primarily exert function through activation of their respective nuclear receptors: the estrogen receptors (ERα and ERβ) for E2 derived from two separate genes (*Esr1* and *Esr2*); the main progesterone receptor isoforms (PR-A and PR-B) for P4 from a single gene (*Pgr*); and the androgen receptor (AR) for testosterone, also derived from one gene (*Ar*). Each of these are nuclear receptors which, when activated by their respective hormone ligands, bind to homologous DNA regions known as hormone response elements (HREs) to regulate gene transcription. They mostly affect aspects related to the cell cycle such as proliferation and

senescence, including regulating metabolic functions to support these activities (Mangelsdorf et al., 1995). This enables development of the germline cells responsible for reproduction, female ova and male sperm, while also controlling reproductive cycles (Klinge 2000).

From puberty and throughout reproductive adulthood, E2 and P4 act on the development of female primary and secondary sexual characteristics. Specifically, increased levels during this time result in breast development, bone growth resulting in pelvic widening, enhanced distribution of fat deposits in the buttocks, hips, and thighs, and increased sex drive (Karastergiou et al., 2012). These hormone levels also play vital roles in shaping the uterine environment to enable reproduction. During the human menstrual cycle (Figure 1.2A), E2 concentrations increase during the first, or follicular, phase which results in a hormonal feedback loop that also increases both follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Hillier 1994). This enables development of the ovarian follicle to eventually release mature ova into the fallopian tubes for fertilization (Edson, Nagaraja & Matzuk 2009). During this phase, increased E2 levels lead to the proliferation of a new endometrial layer, or uterine lining, which helps to enable implantation of a fertilized ovum. Greater E2 also increases cervical and fallopian mucus, creating a more hospitable environment for sperm and ultimately fertilization (Katz, Slade & Nakajima 1997). After ovulation, the luteal phase begins whereby rapid increases in P4 levels stop endometrial proliferation to preserve the

uterine lining for implantation and survival of the fertilized ovum, while also thickening the vaginal wall to prevent improper sperm infiltration into the epithelium (Lydon et al., 1995). P4 also acts on the uterine musculature to reduce contractility, which helps to prevent flushing out the endometrial lining that is necessary for implantation (Sanborn, Weisbrodt & Sherwood 1981). If implantation does not occur, the process starts over where decreasing P4 and increasing follicular phase hormones allows contractility of the uterine muscles leading to removal of the uterine lining. A homologous pattern called the estrous cycle occurs in other mammalian species, including mice, with varying hormone levels throughout four different phases, with similar uterine changes as seen in human menstruation, although instead of shedding the uterine lining it is thinned if pregnancy does not occur (Figure 1.2B) (Bronson 1989). However, during pregnancy, E2 and P4 levels gradually increase until after birth (Figure 1.2C), and it is believed their effects on immune function may partly be responsible for the survival of the fetus until parturition (Mor et al., 2011).

17β-estradiol (E2) and progesterone (P4)

Throughout pregnancy until labor, female immunity switches toward progressively anti-inflammatory states correlating with a gradual increase in E2 and P4 levels. These hormones exhibit similar or differential effects on innate immunity depending on certain factors including concentration and cell type. Low concentrations of E2 appear to enhance monocyte and macrophage pro-inflammatory activity, with increased output of IL-1β, IL-6,

and TNFa after stimulation, while high E2 concentrations are suppressive leading to decreased production of these cytokines (Bouman, Heineman & Faas 2005; Straub 2007). This does not appear to be cell-intrinsic to monocytes and/or macrophages of one sex over another, as both female and male cells exhibit these concentration-dependent responses upon exposure (Laffont, Seillet & Guéry 2017). Greater E2 levels also appear to bias macrophages toward the alternative, or M2, phenotype which is markedly less pro-inflammatory than classical M1 cells and instead promotes tissue repair and regeneration (Villa et al., 2015). This effect is also apparent in conventional dendritic cells, with enhanced output of more anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (Laffont, Seillet & Guéry 2017). However, E2 enhances pro-inflammatory cytokine production in plasmacytoid dendritic cells, which may help to explain the maintenance of anti-viral responses throughout pregnancy (Seillet et al., 2012). E2 appears to decrease transcription of pro-inflammatory markers in PMNs and even reduces migration to sites of injury or infection while, conversely, it increases production of NETs (Oertelt-Prigione 2012).

Unlike E2, P4 is almost universally immunosuppressive. Both low and high concentrations significantly decrease transcription and output of pro-inflammatory cytokines such as IL-6, IL-12, and TNFa in both monocytes and macrophages, an effect that occurs in female and male cells upon exposure (Lee et al., 2011). However, some studies have shown that low concentrations may increase IL-1β production and thus

pro-inflammatory activity, though others refute these findings (Piccinni et al., 1995). Macrophages exposed exclusively to P4 at any concentration favor the M2 phenotype. In concordance with such findings, M1/M2 cell ratios are significantly decreased during pregnancy, with a commensurate increase in anti-inflammatory T lymphocytes and related cytokines (Giron-Gonzalez, et al., 2000; Chen, Liu & Sytwu 2012; Zhang et al., 2017). P4 suppresses the overall pro-inflammatory activity of other innate cell types including dendritic cells, eosinophils, and mast cells by decreasing cytokine transcription and degranulation (Gonzalez, Romero & Girardi 2013). It also dampens neutrophil responses, including the release of NETs (Gomez-Lopez et al., 2017). Thus, it is clear the primary female sex hormones E2 and P4 exert similar yet divergent effects on innate immune function depending on factors such as concentration and cell type (Szekeres-Bartho et al., 2001a; Szekeres-Bartho et al., 2001b). Their similarities are most evident during the later stages of pregnancy, as they both readily suppress innate responses. However, these hormones do not exert effects solely on professional immune cells, as epithelial and endothelial cells also exhibit sex hormone-dependent modulation of immune activity (Alvarez et al., 1997; Foidart et al., 1998; Vázquez et al., 1999; Lam et al., 2014).

It is apparent that sex hormones may influence innate immune function, especially the female hormones E2 and P4, as inflammatory responses to pathogens are altered during pregnancy, which may lead to

a greater susceptibility of pregnant women to infections (Jamieson, Theiler & Rasmussen 2006; Sappenfield, Jamieson & Kourtis 2013; Kourtis, Read & Jamieson 2014). This suppression of inflammatory immune activity also occurs during certain stages of the estrous or menstrual cycles (Kalo-Klein & Witkin 1989; Teepe et al., 1990; Butts et al., 2010; Hughes et al., 2022). Adaptive immune suppression would also be vital for the growth and maintenance of the fetus during pregnancy, as its unique antigen repertoire could result in immune responses leading to miscarriage or spontaneous abortion. There are, however, newly recognized aspects of innate immunity for which the influence of sex is mostly unknown.

ER and PR effects on innate immune function

While the correlation of E2 and P4 with immune responses are generally known, their directly causal effects on immune function are incompletely understood. Evidence suggests that much of the suppressive activity of E2 in epithelial and immune cells is due to the nuclear ER acting against NF-κB-signaled gene induction (Bouman, Heineman & Faas 2005; Ackerman 2006; Heldrig et al., 2007). This occurs either by directly inhibiting NF-κB binding to DNA response elements, or indirectly by regulating the activity of the miRNAs let-7a and miR-125b to interfere with NF-κB binding DNA (Murphy, Guyre & Pioli 2010). Similarly, P4 activation of PRs has been shown to inhibit NF-κB activity by increasing production of IκBα which prevents binding to target DNA elements and downregulates transcription of pro-inflammatory markers (Hardy et al., 2006). PRs also

suppress pro-inflammatory activity by enhancing production of SOCS1, which inhibits phosphorylation-dependent inactivation of IκBα and subsequent translocation of NF-κB to the nucleus (Prele, Woodward & Hart 2007; Su et al., 2009). Some evidence also suggests that P4, via PRs and/or other nuclear receptors, can downregulate expression of TLR4, although this is not conclusive (Su et al., 2009; Zhu et al., 2013).

There is ongoing debate regarding the expression of nuclear PR isoforms in mature immune cells. While at least one of the ERs is found within most immune cells, the nuclear PR isoforms may or may not be present in the same cells (Mansour, Reznikoff-Etievant & Netter 1994). Some studies have found that most circulating mature innate immune cells do not express nuclear PRs, although evidence does support expression in immature bone marrow and early circulating immune cells (Mulac-Jericevic et al., 2000; Merlino et al., 2007; Dosiou et al., 2008; Ndiaye et al., 2012). This contrasts with the wealth of evidence supporting P4-mediated suppression of innate immune function. This may be due to P4's ability to activate the nuclear glucocorticoid receptor (GR), which downregulates pro-inflammatory immune responses by interfering with the transcription of related genes, mainly by acting against NF-κB activity (Li et al., 2003; Lei et al., 2012). P4 may also function in immune cells through membrane-bound PRs (mPRs). These do not directly affect gene transcription but instead influence cytoplasmic processes that may interfere with NF-κB translocation to the nucleus, or even alter metabolic

activity that affects the epigenetic landscape surrounding immune genes (Dressing et al., 2011). However, CD33+ (human) and CD34+(human and mouse) bone marrow hematopoietic cells, which give rise to myeloid lineage innate cells, express the nuclear PR (Mierzejewska et al., 2015; Bongiovanni et al., 2002).

C. Sex and trained immunity

Sex effects in trained immunity

While sexual dimorphism in traditional innate immune activity is generally well-described, prior evidence for sex-based effects on trained immunity is sparse, and what does pre-exist the present work is contradictory. In one study, *ex vivo* concomitant exposure of female and male adult human monocytes with varying levels of E2 or dihydrotestosterone hormones, and BCG to induce training resulted in decreased output of pro-inflammatory cytokines IL-6 and TNFa after 24 hours (De Bree et al., 2018). However, a separate human trial found that BCG vaccination yielded significantly increased production of various pro-inflammatory markers by male over female monocytes after a secondary exposure 24 hours later, and although this was cited as evidence for heterologous immunity, it was also interpreted as basis for supposing innate immune training may have been involved (Koeken et al., 2020). Meanwhile, a conflicting study found that E2 instead promotes trained immunity in bone-marrow derived macrophages isolated from C57BL/B

wild-type mice, polarizing them toward the M1 phenotype leading to enhanced output of IL-6 and TNFa by blocking nuclear translocation of the non-canonical NF-κB subunit RelB (Sun et al., 2020). Previous to the present work, only one study focused on the effect of P4 on trained immunity. Adult human circulating monocytes exposed to P4 during oxLDL-induced training *in vitro* resulted in significantly reduced output of IL-6 and TNFa after secondary stimulation with LPS 6 days later, an effect pinpointed to P4 engagement of the nuclear glucocorticoid receptor (Groh et al., 2022). Thus, it appeared that sex hormones indeed affected training in stimulation experiments *in vitro*, but observation that sex differences existed *in vivo* was not evident from *ex vivo* analysis.

This dissertation

Given the wealth of evidence supporting sexual dimorphism in innate immune activity, yet the scarcity of studies examining the influence of sex on trained immunity; this dissertation section will investigate how sex hormones, particularly E2 and P4, affect trained immunity by way of pro-inflammatory cytokine output, metabolic activity, and survival from an opportunistic infection. We hypothesized that female sex hormones, particularly P4, attenuate trained immune responses leading to decreased female survival from a subsequent systemic infection. Importantly, we do not focus on training in circulating innate cells but instead bone marrow cells, which express all known sex hormone nuclear receptors at high levels. This work will better our understanding of how sex affects trained

immunity, while future work in this area may apply lessons learned to translational therapies.

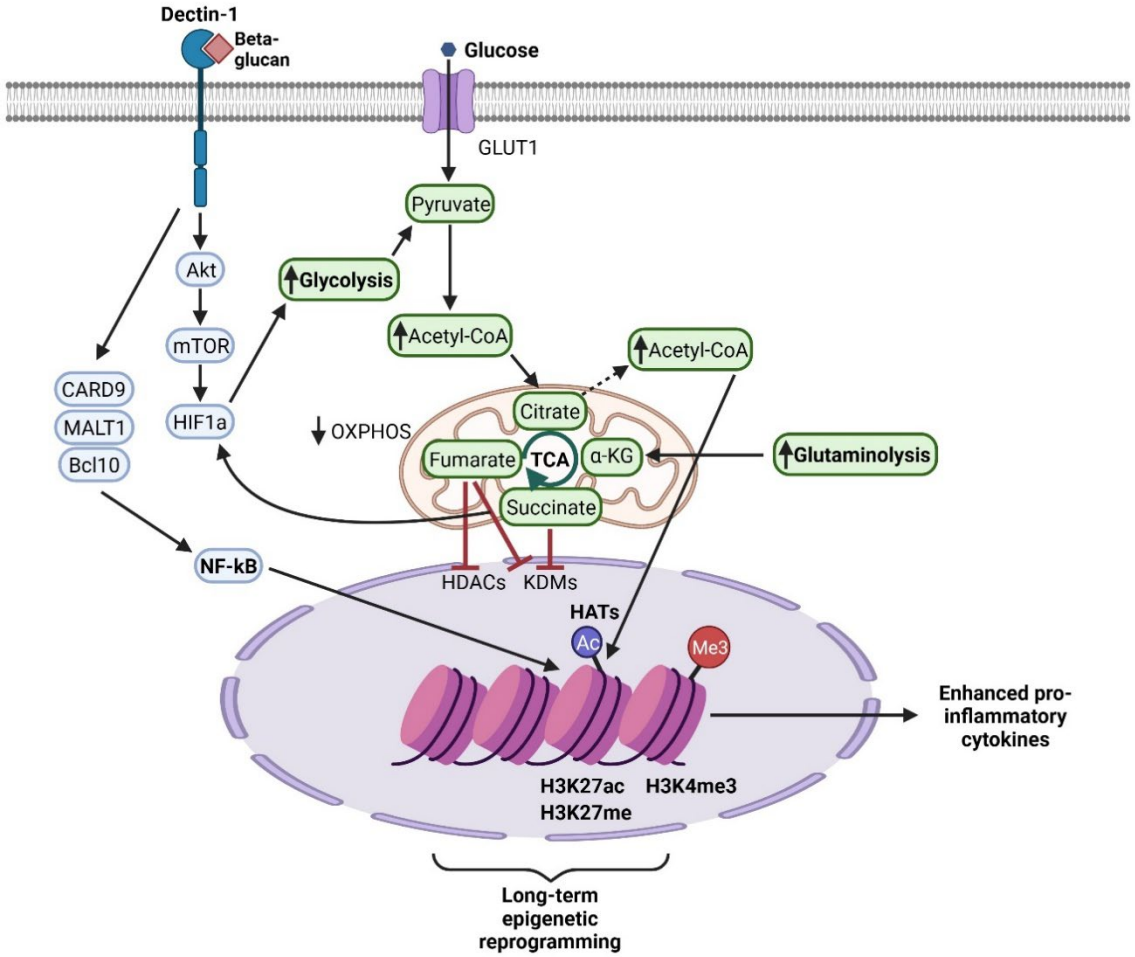
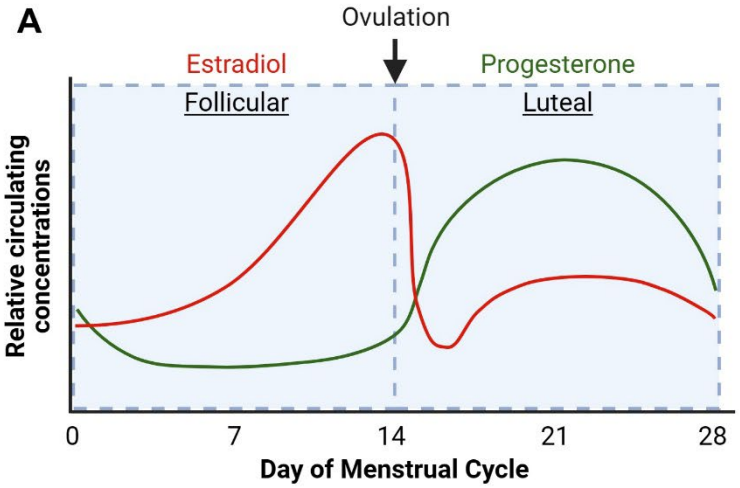


Figure 1.1: Diagram of trained immunity induction by Dectin-1 signaling. Ligation of Dectin-1 by molecules such as β-glucan leads to activation of the CARD9/MALT1/Bcl10 complex and subsequent NF-κB translocation to the nucleus; and of the Akt-mTOR-HIF1α axis which enhances glycolysis, resulting in an altered TCA cycle with accumulation of intermediates, reduction in oxidative phosphorylation, and increased glutaminolysis, which aid in epigenetic reprogramming leading to enhanced secondary responses by output of pro-inflammatory cytokines. Adapted from Drummer et al., 2020, *Arteriosclerosis, Thrombosis, & Vascular Biology*. Created with BioRender.com.


A
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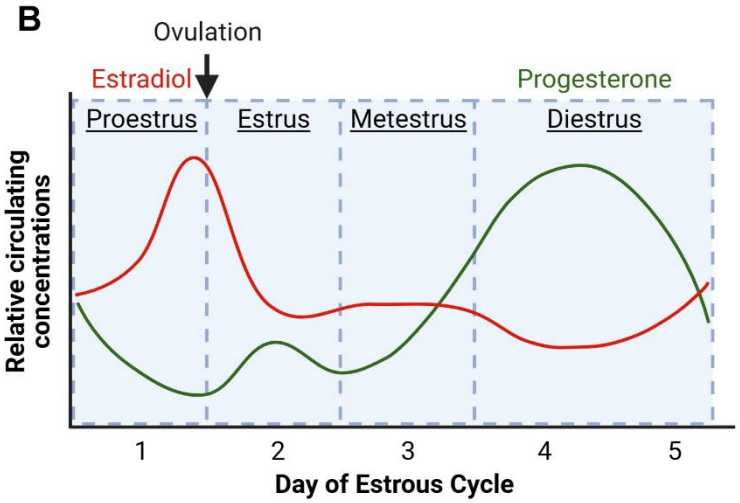
28
Day of Menstrual Cycle


B
Ovulation
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Proestrus
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Metestrus
Diestrus
Relative circulating concentrations
1
2
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Day of Estrous Cycle

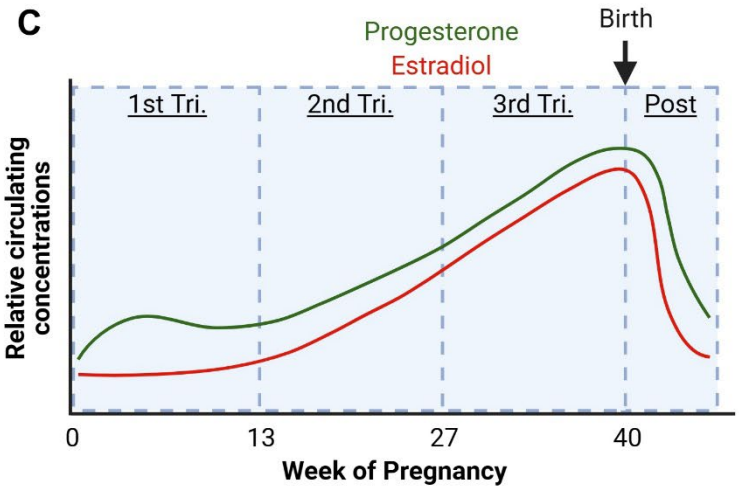
C
Progesterone
Estradiol
Birth
1st Tri.
2nd Tri.
3rd Tri.
Post
Relative circulating concentrations
0
13
27
40
Week of Pregnancy

Figure 1.2: Diagram of female E2 and P4 circulating levels during cyclical changes. (A) Changes during the human menstrual cycle highlighting the point of ovulation. **(B)** Changes throughout the mouse estrous cycle. **(C)** Increasing concentrations of E2 and P4 during the course of human pregnancy, similar to changes in mice. Tri. = Trimester. Adapted from Hong & Choi, 2018, *BMB Reports*. Created with BioRender.com.

Chapter 2

Lower female survival from an opportunistic infection reveals progesterone-driven sex bias in trained immunity

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I: Abstract

Immune responses differ between females and males, although such sex-based variance is incompletely understood. Observing that bacteremia of the opportunistic pathogen, *Burkholderia gladioli*, cause many more deaths of female than male mice bearing genetic deficiencies in adaptive immunity, we demonstrate that this is associated with sex bias in the innate immune memory response called trained immunity. Female attenuation of trained immunity varies with estrous cycle stage and correlates with serum progesterone, a hormone that decreases glycolytic and recall cytokine secretion induced by antigen non-specific stimuli. Progesterone receptor antagonism rescues female trained immune responses and survival from controlled *B. gladioli* infection to magnitudes similar to those of males. These data demonstrate progesterone-dependent sex bias in trained immunity where attenuation of female responses is associated with survival outcomes from opportunistic infection.

II: Introduction

Differences in immune responses in males and females can sometimes favor one or the other sex (Klein & Flanagan 2016; Jaillon, Berthenet & Garlanda 2019). The recent COVID-19 pandemic reported immune hyperresponsiveness in males with increased morbidity and severity (Liu et al., 2020; Penna et al., 2020; Li et al., 2020). In contrast, it has been proposed that females may suffer greater morbidity and severity from many other infectious agents (Maher 2013). Female reproductive cycle and pregnancy have long been described to reduce

inflammatory immune intensity (Butts et al., 2010; Mor, Aldo & Alvero 2017). A well-documented female bias in autoimmune syndromes is described to intersect with reproductive biology and possibly infectious triggers or mimicry in incompletely understood ways (Whitacre 2001; Gleicher & Barad 2007). Most studies of sex impacting immunity have focused on animals and people with complete immune systems, while the possibility that sex might impact protection of immune-compromised individuals is a concept less well developed. Some microorganisms present little threat to individuals with intact immune systems but are harmful to hosts with inborn or acquired immune deficiencies (Gleicher & Barad 2007; Riccardi, Rutolo & Castagnola 2019). Such opportunistic infections may increase in incidence as populations age, and as immunosuppressive therapies multiply (Lin, Lin & Katz 2019; Motolese et al., 2022).

Trained immunity was first described as a form of innate immune memory (Netea, Quintin & van der Meer 2011; Quintin et al., 2012; Saeed et al., 2014), which can be active when adaptive immunity is either intact or compromised. Unlike adaptive immune memory that relies on antigen-specific T cells and B cells, trained immunity primes innate immune cells for enhanced activity against different types of secondary insults that may be unrelated to the first one. In the present study, we demonstrate that survival from an opportunistic infection can be influenced by sex and trained immune responses.

III. Results

A. *In vivo* trained immunity enhances survival in infected male over female mice

Burkholderia gladioli is a soil bacterium and opportunistic pathogen known to be harmful in cystic fibrosis and immune compromised human patients (Christenson et al., 1989; Kennedy et al., 2007). Breakouts of the bacterium are also problematic in immunodeficient mouse strains in comparative medicine research facilities (Foley, LiPuma & Feldman 2004; Collymore, Giuliano & Banks 2019). In our specific-pathogen-free colony of multiple adaptive-immune compromised mouse strains, an unintentional spread of *B. gladioli* was found to be associated with bacteremia and death or morbidity meeting euthanasia criteria. In such mice these results from an opportunistic pathogen would not be surprising, except that the mice that died were overwhelmingly female (89.5%, Supplemental Figure 2.1) even breeding females that had been co-housed with males and offspring that survived. This pattern of mortality implied that rather than a major difference in pathogen exposure, there might instead have been a difference in innate protection, perhaps involving immune training. To test this hypothesis, we used a previously described strategy to assess systemic immune training as an isolated variable, injecting adaptive immune-deficient mice intravenously with either phosphate-buffered saline (PBS) negative control or heat-killed *Candida albicans* ($1x10^5$ colony forming units (cfu)/mouse) to stimulate innate immune training [15]. Seven

days later, control-untrained or exogenously (exog-)trained mice were subjected to systemic *B. gladioli* experimental infection from our clinical isolates, intravenously injected with a lethal dose ($3x10^4$ cfu/mouse) and monitored for survival over 120 days (Figure 2.1A). Using recombination activating gene-1 knockout mice (*Rag1*$^{-/-}$) lacking T and B lymphocytes (Mombaerts et al., 1992), we observed that survival from systemic *B. gladioli* infection was responsive to exogenously stimulated innate immune training, as exog-trained mice showed mean survival significantly increased over control-untrained mice (Supplemental Figure 2.2A). Separating the sexes, we found that male survival exceeded that of females, which matched the pattern observed during the previous unintentional breakout and was evident in presence or absence of exog-training (Figure 2.1B). Exog-training increased average survival of both sexes (Figure 2.1C) with a greater effect in males evident from the survival curves, and further determined as follows. By matching the ranks of survival times between control-untrained and exog-trained mice, we estimated that female mice survived on average 23 days longer in response to exog-training; in contrast, exog-training allowed the first two males that succumbed to survive 31 and 34 days longer than the first to control-untrained males, and three trained males did not succumb over 120 days (Figure 2.1D). This showed that immune training could result in male survival over the observed time course, an outcome in common with the previous unintended breakout pattern of mortality. We conclude that

trained immunity imparted to males an increased survival benefit relative to females from *B. gladioli* opportunistic infection.

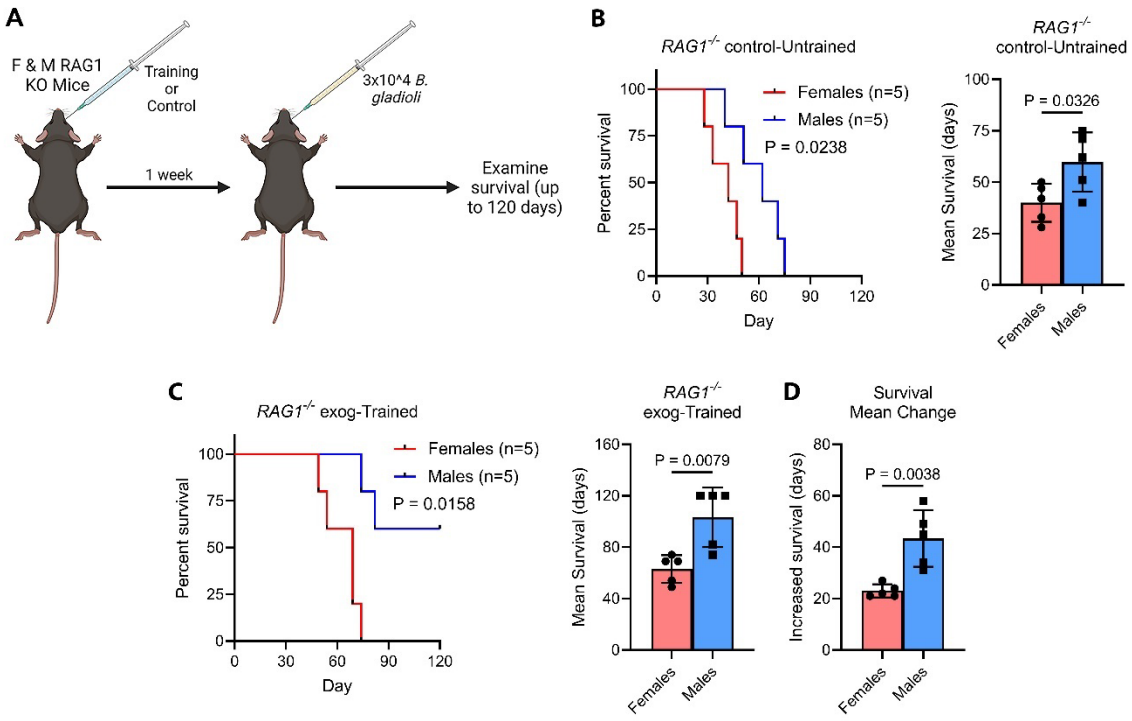

Figure 2.1. Trained immunity imparts an enhanced survival advantage to male mice infected with *B. gladioli*.

(A) Experimental set-up. Female (F) and male (M) mice of *Rag1*$^{-/-}$ genotype (RAG1 KO) were injected retro-orbitally with either PBS (no training) or heat-killed *C. albicans* (exogenous training). After one week, mice were injected with live *B. gladioli* bacteria. Created with BioRender.

(B) Survival curves (left) comparing control-untrained female vs. control-untrained male *Rag1*$^{-/-}$ mice following *B. gladioli* infection, and comparison of mean survival days (right).

(C) Survival curves (left) comparing exog-trained female vs. exog-trained male *Rag1*$^{-/-}$ mice following *B. gladioli* infection, and comparison of mean survival days (right).

(D) Survival mean change (increase) in days provided by immune training as estimated by assessing the survival difference at matched ordered ranks as described in Methods.

Survival curves were analyzed by Kaplan-Meier two-sided log rank statistics with P-values obtained as described in Methods. Log rank P-value 0.0238 has standard deviation of 1.53×10^{-4}, and log rank P-value 0.0158 has standard deviation of 1.91×10^{-4}. Mean survival (days) and Survival mean change were compared with two-sided Mann Whitney test of means. Each dot represents a mouse, $n = 5$ for each group, with mean ± SD shown. P-values < 0.05 are significant.

See also Supplemental Figures 2.1 and 2.2.

B. Female serum attenuates secondary trained immune responses in mouse BM-MNCs

Trained immunity results in the enhanced output of pro-inflammatory mediators following non-antigen specific secondary stimulation (Quintin et al., 2012; Kleinnijenhuis et al., 2012). We studied this property isolating *Rag1*$^{-/-}$ primary bone marrow mononuclear cells (BM-MNC), a major subset that is exposed and responds to systemic stimuli including those of training and bacteremia (Mitroulis et al., 2018; Cirovic et al., 2020; Kleinnijenhuis et al., 2014). We followed a previously described regimen to induce immune training for subsequent recall stimulation and cytokine secretion, *in vitro* (Quintin et al., 2012). BM-MNCs of either sex were trained with the dectin-1 agonist 1,3-β-glucan (β-glucan) or vehicle control for 24 hours, washed, and after 6 days restimulated for 24 hours with bacterial toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), or TLR3 agonist polyinosinic:polycytidylic acid (poly(I:C)) as surrogate for double-stranded RNA stimulation from viral infection (Figure 2.2A). Multiplex enzyme-linked immunosorbent assay (ELISA) of cell supernatant was performed to measure release of cytokine IL-6 and chemokine CXCL2. We reproduced the predicted effect of immune training *in vitro*, where restimulation induced an increased level of the assessed inflammatory mediators in supernatants from *Rag1*$^{-/-}$ BM-MNCs (Supplemental Figure 2.2B). We also found that using heat-killed *B. gladioli* as a candidate exogenous training agent resulted in significantly

increased cytokine levels upon restimulation with LPS and poly(I:C) (Supplemental Figure 2.2C,D). Therefore, we do not consider any infected mouse in our dataset to be “untrained”, since the infection itself can stimulate the innate immune training response. For this reason, the present work distinguishes groups based on experimental manipulation as control-untrained versus exogenously-trained, while noting that control-untrained infected mice represent a category that likely still has some positive level of innate immune training.

We sought to determine if the *in vitro* immune training system would detect sex-based differences. However, assessing male versus female BM-MNCs in this system, no differences in immune training were detected when tissue culture media was supplemented with fetal bovine serum (FBS), where commercial batches pool female and male serum together, or charcoal-stripped (cs) FBS, which reduces concentrations of steroid-based compounds (Cao et al., 2009) (Supplemental Figre 2.3A,B). In contrast, when the experiments used sex-matched *Rag1*$^{-/-}$ BM-MNCs and mouse serum, we observed female attenuation of the innate immune trained response, as follows. First, male and female BM-MNCs never exposed to exogenous β-glucan were cultured according to the timeline (Figure 2.2A) and produced in response to LPS or poly(I:C) amounts of IL-6 and CXCL2 with no sex-based statistical difference (Supplemental Figure 2.3C). In parallel, prior training by exogenous β-glucan that was followed by LPS or poly(I:C) restimulation showed female BM-MNCs in

female serum producing significantly lower IL-6 and CXCL2 compared to male cells trained in male serum (Figure 2.2B). When comparing the enhancement of each sex's exog-training response versus its response without β-glucan exposure, males showed the greatest increase in trained immune cytokine secretion (Supplemental Figure 2.3D). When we reversed the serum sex during training of female and male cells, this biased response from the cells showed a similarly reversed trend: female BM-MNCs trained in male serum now produced higher amounts of IL-6 and CXCL2 upon restimulation than did male BM-MNCs trained in female serum (Figure 2.2C). From the same dataset, we assessed whether the response pattern supported a model where male serum provided training-enhancing product(s), or female serum provided training attenuation product(s). We found that for female BM-MNCs, male serum did not enhance responses above female serum during training (Figure 2.2D). In contrast, however, male cells trained with female serum produced significantly less IL-6 and CXCL2 compared to those trained with male serum after restimulation (Figure 2.2E). In addition to IL-6 and CXCL2, we also found that the pro-inflammatory cytokines IL-1β and IL-18 were produced as part of a higher trained response from male than female BM-MNCs in sex-matched serum (Supplemental Figure 2.3E,F). These data favored the hypothesis that some factor(s) in female serum may be responsible for attenuating trained secondary responses.

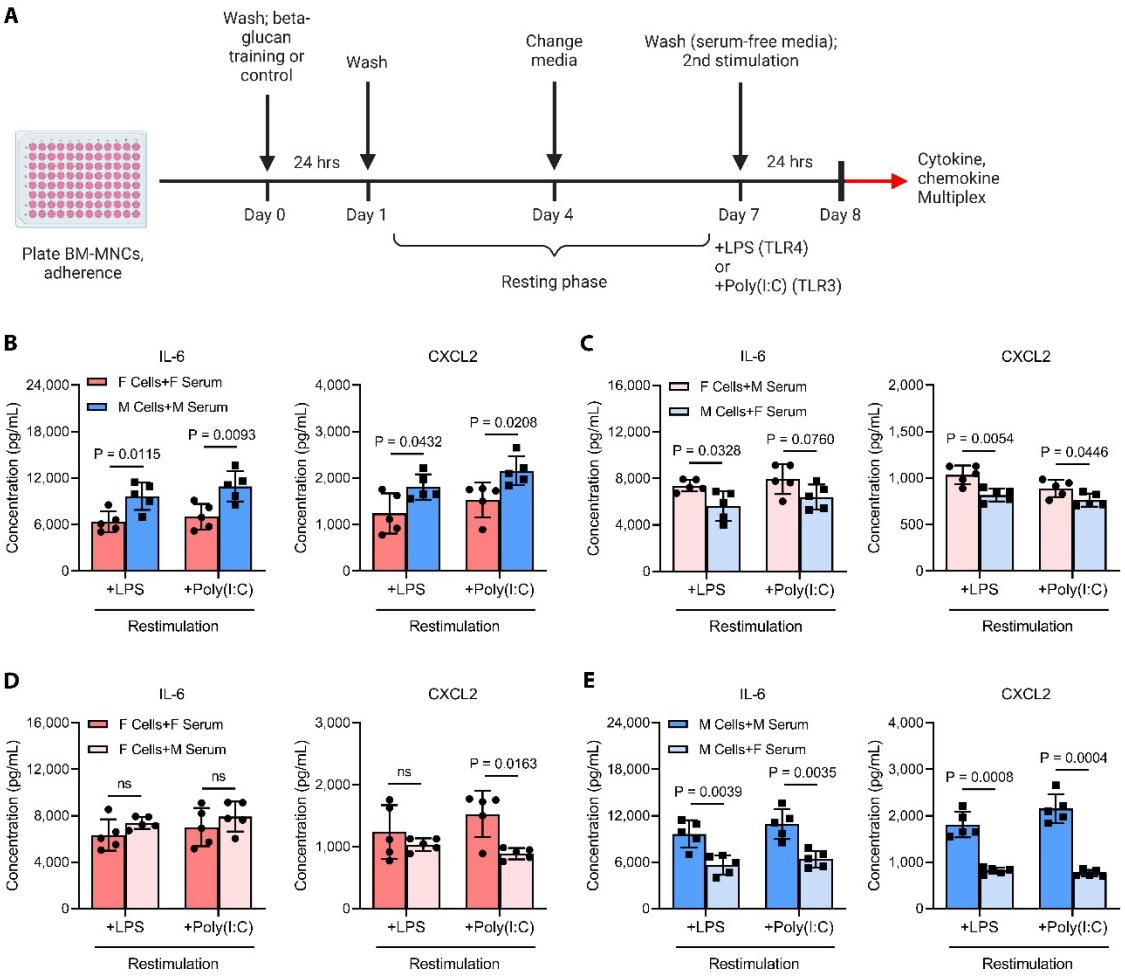

Figure 2.2. Training is attenuated in mouse BM-MNCs when exposed to female serum.

(A) Tissue culture experimental timeline. Created with BioRender.

(B) Supernatant levels of IL-6 and CXCL2 after LPS or poly(I:C) restimulation for 24 hours. Cells used are female and male *Rag1*$^{-/-}$ BM-MNCs that had been b-glucan-trained with sex-matched serum.

(C) IL-6 and CXCL2 levels after restimulation of female and male cells trained with opposite sex serum.

(D) Comparison of IL-6 and CXCL2 levels from female cells trained with matched versus opposite sex serum.

(E) Comparison of IL-6 and CXCL2 levels from male cells trained with matched versus opposite sex serum.

Means were compared with two-sided Mann Whitney test with Holm-Šidák correction for multiple hypothesis testing. Each dot plotted represents a mouse, *n* = 5 for each group with mean ± SD shown. P-values < 0.05 are significant.

See also Supplemental Figures 2.2 and 2.3.

C. Estrous cycle influences trained immunity

The main female sex hormone groups, estrogens and progestins, can have differential effects on innate and adaptive immune function (Klein & Flanagan 2016; Jaillon, Berthenet & Garlanda 2019). The primary estrogen, 17β-estradiol (E2) has varied complex roles, either influencing pro- or anti-inflammatory responses such as those associated with autoimmunity or during the resolution of inflammation, respectively (Panchanathan, Liu & Choubey 2013; Villa et al., 2015; Mukai et al., 2014). Progesterone (P4) is mostly considered anti-inflammatory and has been reported to interfere with nuclear translocation of NF-κB, transcription factor binding to pro-inflammatory genes, and immune training of human circulating mononuclear cells, *in vitro* (Kalkoven et al., 1996; Hardy et al., 2006; Su et al., 2009; Butts et al., 2007; Groh et al., 2022). Despite this prior knowledge, with males also expressing estrogens and progestins (Butts et al., 2007), equivalent responses from both sexes to immune training of human peripheral blood monocytes were previously reported (de Bree et al., 2018). We chose to focus on E2 or P4 as preliminary candidate upstream controllers of the sex biased responses we observed. To begin to address possible roles for E2 or P4, we examined the effects that the 4-stage, 4-to-5-day mouse estrous cycle might have on immune training. We staged sexually mature, male bedding-exposed, female *Rag1*$^{-/-}$ mice via vaginal cytology and blood serum ELISA for E2 and P4, finding highest E2 in proestrus, highest P4 in

diestrus stages, and intermediate results for other stages matching expectations (Walmer et al., 1992; Fata, Chaudhary & Khoka 2001; Wood et al., 2007) (Figure 2.3A, top and bottom). Female cells were then trained in the presence of stage-specific serum, followed by secondary stimulation with LPS or poly(I:C). Compared against cells trained with male serum, those from the diestrus stages when P4 was maximal produced significantly less IL-6 and CXCL2 upon restimulation, with a trend toward this effect noted when P4 was rising in metestrus, and when P4 was downregulating in proestrus stages (Figure 2.3B,C). These data suggested that training attenuation correlated more with serum P4 than with serum E2.

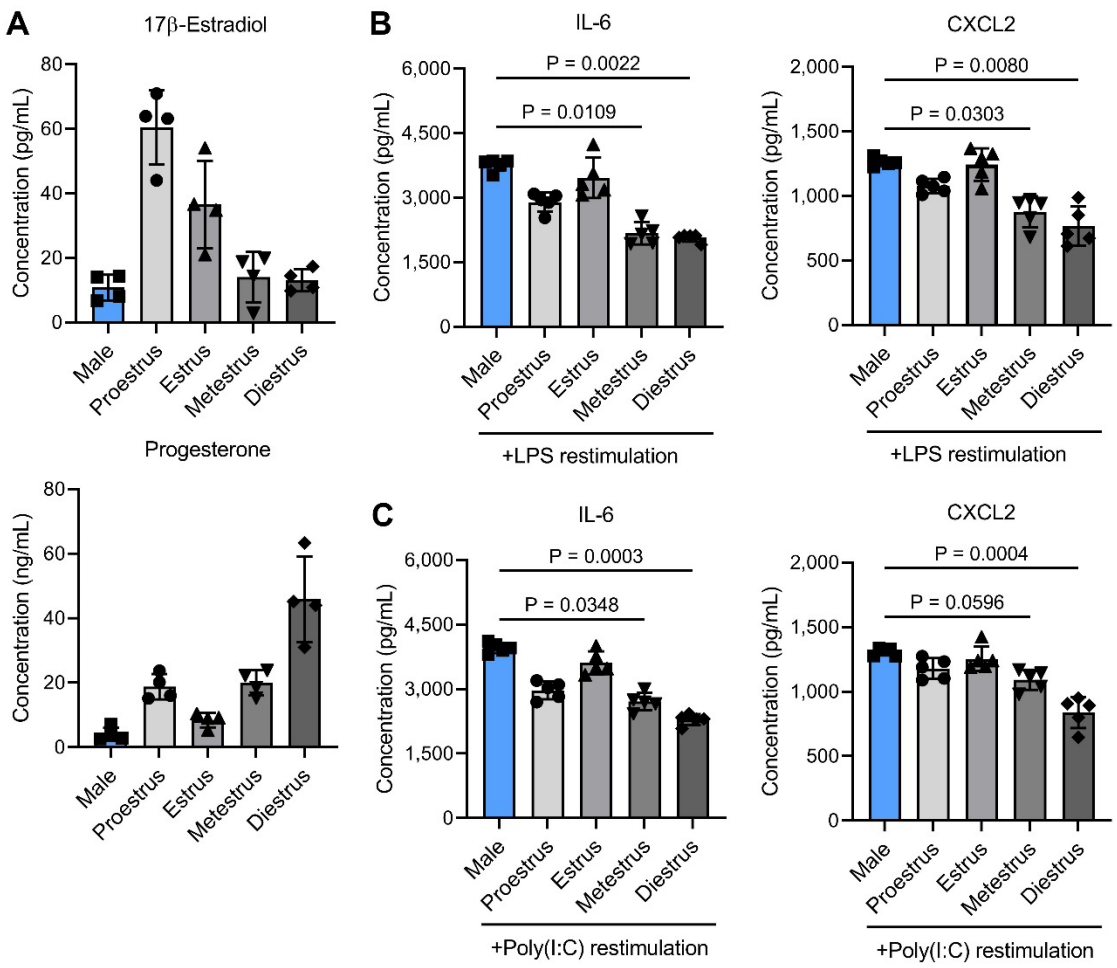

Figure 2.3. Serum from mouse estrous cycle stage influences attenuation of training of BM-MNCs.

(A) 17b-estradiol (top) and progesterone (bottom) concentrations in mouse serum measured from each stage of the estrous cycle, compared against male serum levels.

(B) Concentrations of IL-6 (left) and CXCL2 (right) released in supernatant from female *Rag1*$^{-/-}$ BM-MNCs after LPS restimulation, when cells had been b-glucan-trained in serum from each stage of the estrous cycle, compared to levels from male cells trained in male serum.

(C) IL-6 (left) and CXCL2 (right) concentrations in supernatant after poly(I:C) restimulation from cells trained as in (B).

Data were analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's multiple comparisons tests. Each dot plotted represents a mouse, *n* = 4 per group in panel (A), and *n* = 5 per each group (B-C) with mean ± SD shown. P-values < 0.05 are significant.

D. Progesterone receptor antagonism restores trained immune responses and survival

Because P4 primarily binds to the nuclear progesterone receptor (PR) to regulate the female reproductive system (Mulac-Jericevic et al., 2000; Halasz & Szekeres-Bartho 2013), we examined whether PR activity was involved in attenuating trained immunity. *In vitro*, female *Rag1*$^{-/-}$ BM-MNCs were pre-treated with either vehicle control, estrogen receptor (ER) antagonist ICI 182,780, PR antagonist PF-02413873, or both antagonists, and two hours later the training protocol was performed, with male cells exog-trained in male serum included in parallel for comparison. Following LPS or poly(I:C) restimulation, we found that female cells trained in the presence of ER antagonist yielded a moderate yet nonsignificant increase in IL-6 and CXCL2, while PR antagonism significantly restored trained secondary responses compared to male cells trained in male serum (Figure 2.4A,B). Adding both ER and PR antagonists did not enhance trained responses beyond PR antagonism (Figure 2.4A,B). We conclude that PR can contribute to attenuation of trained immunity in female mouse BM-MNCs.

To test possible effects of exogenous P4 on trained immunity, we performed experiments using csFBS as serum source so that controlled addition would provide the major source of steroid hormone. Addition of P4 to female and male BM-MNCs during exog-training significantly reduced cytokine output after restimulation by a mechanism inhibited by

the PR antagonist drug (Figure 2.4C,D). The PR antagonist alone had no effect on training in csFBS media (Figure 2.4C,D), nor on male cells exog-trained in male serum (Figure 2.4E,F), suggesting that male serum had P4 levels below that which could attenuate immune training. We conclude that P4 can attenuate trained immune responses in mouse BM-MNCs.

It is well-established that an induced switch to glycolytic metabolism underlies trained immunity (Cheng et al., 2014; Arts et al., 2016; Keating et a., 2020). Binding of dectin-1 by β-glucan engages the Akt-mTOR-HIF-1α axis to activate glycolysis leading to markedly increased levels of acetyl-CoA and histone modifications (i.e., acetylation) of pro-inflammatory and pro-glycolytic enhancer and promoter sites to enable increased activity after secondary stimulation (Cheng et al., 2014; Bekkering et al., 2018; Riksen & Netea 2021). Therefore, we examined whether P4 affected glycolysis upon β-glucan-induced training of female *Rag1*$^{-/-}$ BM-MNCs. Cells were plated with vehicle control or exogenous P4, followed by β-glucan-induced training for 24 hours. Six days later, we measured glycolytic activity in supernatant via extracellular acidification rate (ECAR). Upon injection of glucose to the cell culture, basal glycolysis was observed to be significantly increased in exog-trained cells compared with control-untrained cells, an effect that was inhibited in a PR-dependent manner if training occurred in the presence of exogenous P4 (Figure 2.5A). Following subsequent injection into culture of oligomycin, a potent ATP synthase (complex V) inhibitor which stops oxidative phosphorylation in

the mitochondria, maximum glycolytic capacity was assessed and was found to be unchanged from basal glycolysis in each condition (Figure 2.A). These patterns of glycolytic activity were also observed assessing exogenous P4 effects on male cells (Supplemental Figure 2.4). These results are consistent with a model where P4 attenuates training-induced glycolytic activity and trained secondary immune responses.

Finally, we assessed whether PR antagonism would increase survival of exog-trained female mice upon systemic infection. Sexually mature female *Rag1*$^{-/-}$ mice were intraperitoneally administered PBS control or PR antagonist PF-02413873 then again 48 hours later and immediately followed by intravenous infection with a lethal dose of *B. gladioli* on the seventh day. Survival was then monitored over 120 days. We observed that trained females receiving the PR antagonist during training survived longer than those given the vehicle control, with some PR antagonist female being the first in our dataset to survive to the end of the monitoring period (Figure 2.B). These data show that PR antagonism during immune training of female mice increased their overall survival. We conclude that PR antagonism can enhance trained immune responses and impact the fate of female survival from opportunistic infection.

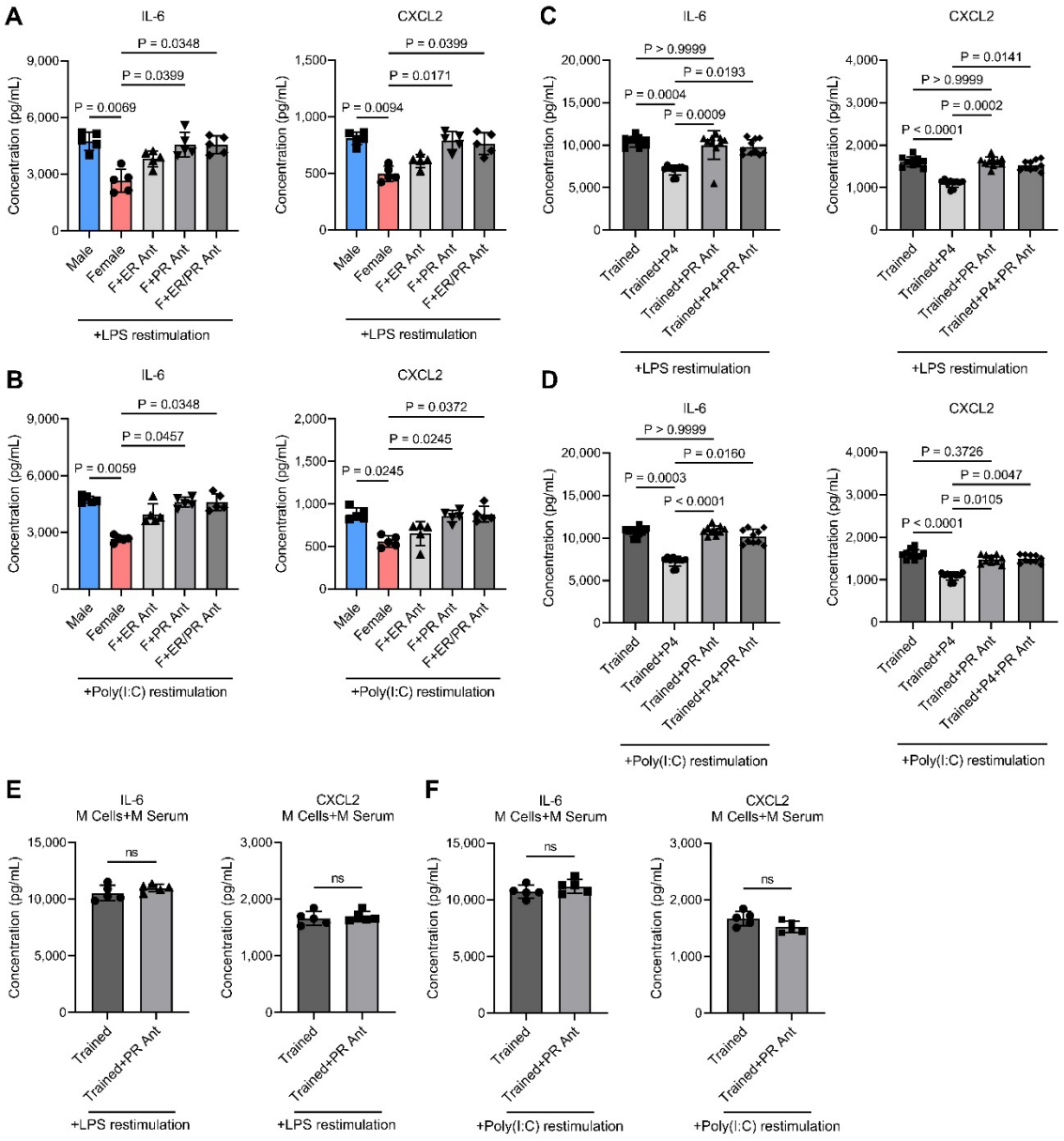

Figure 2.4. Attenuation of trained immunity is controlled by progesterone and the nuclear progesterone receptor, Part 1.

(A) After LPS restimulation, supernatant levels of IL-6 (left) and CXCL2 (right) from female *Rag1*$^{-/-}$ BM-MNCs that had been b-glucan-trained in female serum plus vehicle control (peach color) or estrogen receptor (ER) antagonist,

progesterone receptor (PR) antagonist, or both antagonists, compared with male cells trained in male serum (blue).

(B) Similar to (A) after poly(I:C) restimulation.

(C) After LPS restimulation, IL-6 (left) and CXCL2 (right) supernatant levels from female (*n* = 5) and male (*n* = 5) *Rag1*$^{-/-}$ BM-MNCs trained in csFBS media with vehicle or P4 alone, PR antagonist alone, or P4+PR antagonist combined.

(D) Similar to (C) after poly(I:C) restimulation.

(E) After LPS restimulation, IL-6 (left) and CXCL2 (right) supernatant levels from male BM-MNCs trained in male serum with vehicle or PR antagonist.

(F) Similar to (E) after poly(I:C) restimulation.

In vitro cytokine data were analyzed using Kruskal-Wallis one-way ANOVA with Dunn's tests *post hoc*. Each dot represents a mouse, *n* = 5 per sex per group with mean ± SD shown. P-values < 0.05 are significant.

See also Supplemental Figure 2.4.

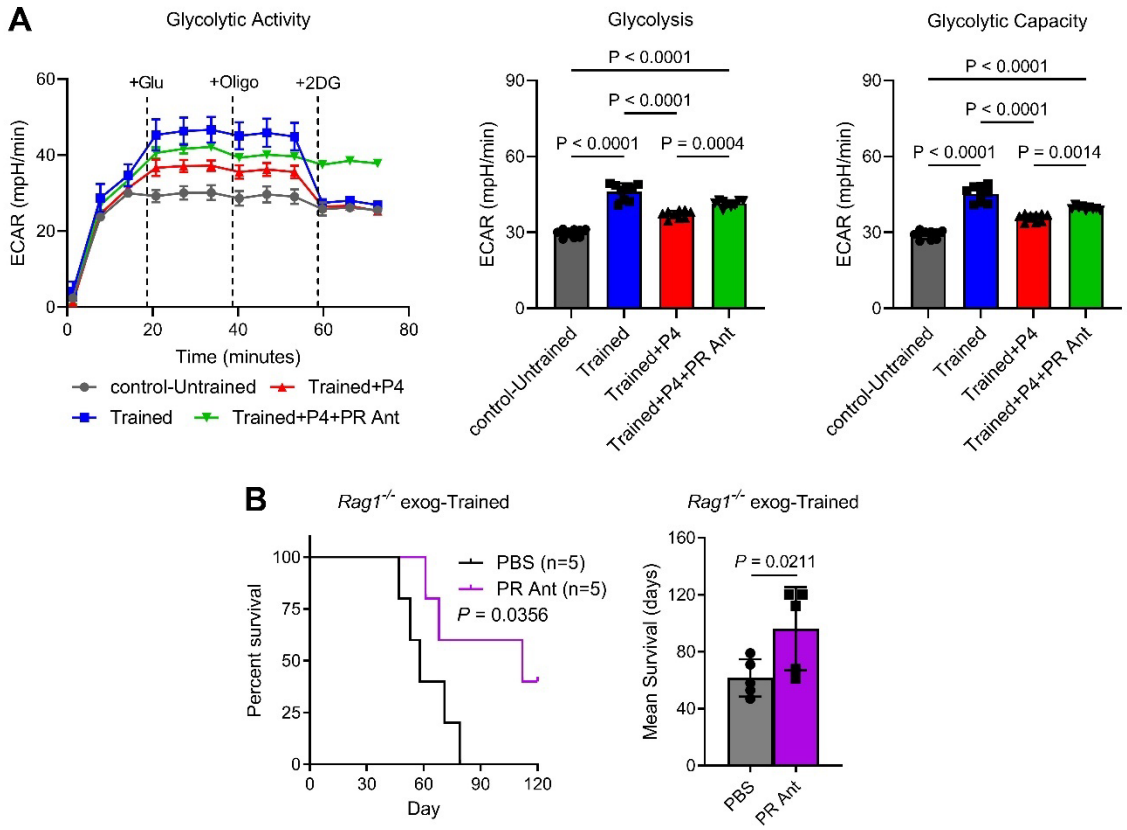

Figure 2.5. Attenuation of trained immunity is controlled by progesterone and the nuclear progesterone receptor, Part 2.

(G) Extracellular acidification rate (ECAR) of female *Rag1*$^{-/-}$ BM-MNCs that had been trained with b-glucan plus vehicle control, or plus progesterone (P4, 20 ng/ml) alone or combined with progesterone receptor antagonist (PR ant), compared against untrained cells (left), and analyzed for basal glycolysis (middle) and maximum glycolytic capacity (right).

(H) Survival curves (left) comparing *Rag1*$^{-/-}$ females exogenously trained in the presence of vehicle control versus PR antagonist prior to *B. gladioli* infection, and comparison of mean survival days (right).

Glycolysis was assessed using parametric one-way ANOVA. Survival curves were analyzed by Kaplan-Meier one-sided log rank statistics with P-values obtained as described in Methods. Log rank P-value 0.0356 has standard deviation of 2.18×10^{-4}. Mean survival (days) was compared using a one-sided Mann Whitney test of means. Each dot represents $n = 3$ mice per group with triplicate measurements at multiple time points with mean ± SD shown. P-values < 0.05 are significant.

See also Supplemental Figure 2.4.

IV. Discussion

The present findings reveal a sex bias in trained immunity, where females displayed decreased inflammatory responsiveness and survival from opportunistic infection compared to males, contributing to the growing evidence for sex hormone-based modulation of immune function. We demonstrated that trained female *Rag1*$^{-/-}$ mice lacking adaptive immunity carried an increased risk of death following systemic infection, and that exog-training of female mouse BM-MNCs in the presence of female mouse serum resulted in significantly decreased output of IL-6, CXCL2, and other cytokines. We speculate that one reason why previously published data may have observed no sex-based difference in immune training experiments *in vitro* might be due to the use of pooled serum rather than sex-controlled serum in tissue culture (de Bree et al., 2018), which would match our observations using commercial, pooled FBS (Supplemental Figure 2.3A). Training experiments utilizing female serum from all estrous cycle stages – each with differing levels of circulating sex hormones – and hormone receptor antagonism, implicated P4 and PR in attenuating female trained immune responses. We further showed that exogenous P4 decreased trained BM-MNC basal glycolysis, consistent with a model in which P4 inhibits the glycolytic switch known to accompany and program trained immunity, in line with the functional effects observed, here. Importantly, these principles may apply *in vivo* as PR antagonism during immune training significantly increased survival of trained female mice upon systemic infection with the opportunistic pathogen, *B. gladioli*. It is noteworthy that even without experimental exogenous application of

our immune training regimen, the control-untrained males exhibited increased survival compared with control-untrained females. Indeed, we found that heat-killed *B. gladioli* can induce trained immunity *in vitro*, and therefore we propose that all infected mice in our dataset likely experiences some positive degree of training intrinsic to the infection itself. If correct, this could mean that throughout the time course of infection training signals and their induced responses could have been occurring, such that we can neither prove nor rule out the possibility that immune training might be the basis for the entirety of increased survival we observed in males. Alternatively, it is possible that other sex-based and P4-based mechanisms of innate immune attenuation independent of training may have contributed to decreased female survival upon *B. gladioli* infection. Such alternate sex-based mechanisms will have responded to PR antagonist drug in combination with exogenous training to achieve the best female survival outcomes were observed, the only experiments to result in female survivors (Figure 2.4H). In addition, it is possible that other receptor and signaling pathways might influence the sex bias in immune training we identified. For example, we observed that ER antagonism produced an apparently moderate but statistically non-significant trend toward inhibiting immune training, *in vitro*. Since E2 signaling can induce PR expression (Kraus & Katzenellenbogen 1993; Couse et al., 1994), a marginal ER antagonism effect might indirectly reflect downstream PR signaling; but alternatively, E2, and possibly other sex hormones, might contribute to immune training by parallel signaling pathways separate from the progesterone-based one of focus in the present work. Further

studies will assess how signals downstream of PR and parallel to PR pathways may impact trained immunity, including signals and consequences of other sex hormones and reproductive conditions.

Limitations of the study

As discussed above, it may not be possible to completely eliminate immune training as a single variable *in vivo*, because animals with absolutely no immune training may not exist. Experimental mice are housed under specific pathogen-free conditions but live in the presence of microbes including unmonitored normal flora. Besides extrinsic microbes contacted through the environment, intrinsic products such as oxidized low-density lipoprotein and heme can induce immune training (Bekkering et al., 2014; Jentho et al., 2021). Therefore, it seems reasonable to speculate that some degree of natural immune training has occurred outside of controlled experimental conditions. As a result, here, we cannot know if or whether innate immune training is the basis for the entirety of the survival difference we observed between female and male mice. We can, however, conclude that immune training operates by a progesterone-dependent mechanism that results in sex bias, whose controlled manipulation increased female survival and cytokine readouts to reach magnitudes like those of males. A second limitation is regarding the female estrous cycle, where good *in vitro* data is not matched with similar *in vivo* protection data with exogenous training isolated to specific stages, for which best experimental design may require non-mouse models. We feel that separate mouse estrous stages endure too short a time to address experimentally using systemic PR inhibitor drugs. It is

conceivable that samples from human female patients +/- progesterone inhibitor drugs might provide experiments with insight into the question of how progesterone may affect immune training at specific stages of the estrous/menstrual cycles. Finally, aside from the nuclear PR there are also membrane receptors responsive to P4 (Meyer et al., 1998). If the PR inhibitor we used, PF-02413873, were to also affect those receptors, our conclusions would not be specific for nuclear PR alone.

V. Materials and Methods

Mice

B6.129S7-*Rag1*^{tm1Mom}/J (termed *Rag1*$^{-/-}$) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our facility. Mice were used in experiments between 8-16 weeks of age, when males and females were considered sexually mature. All mice were maintained in specific pathogen-free isolation facilities with a 12-hour light-dark cycle, an average temperature of 22-23 °C, and an average humidity of 68-72% at the University of Missouri School of Medicine mouse vivarium in Columbia, MO, USA. Mice were age- and sex-matched within each experiment. Estrous cycle stages were identified by gently flushing the vagina with PBS followed by microscopic examination of collected cells as previously described (Byers et al., 2012). Females were followed for at least 15 days (approximately 3 cycles). These experiments were approved by and performed according to the Institutional Animal Care and Use Committee at the University of Missouri.

In vivo *immune training of mice*

Female and male *Rag1*$^{-/-}$ mice were anesthetized with 3% isoflurane in a closed-box circulating system, and injected retro-orbitally with heat-killed *Candida albicans* (HKCA, 1x10^5 CFU per mouse) (Invivogen, San Diego, CA, USA) or PBS control. To examine the influence of the progesterone receptor (PR) on training, mice were injected intraperitoneally with PR antagonist PF-02413873 (1 mg/kg) (Sigma Aldrich, St. Louis, MO, USA) two days prior to training with HKCA or PBS control, again on the day of HKCA administration, and then two, four, and six days later.

Burkholderia gladioli *controlled systemic infection of mice*

Burkholderia gladioli obtained from prior infections of immune compromised mice was cultured in LB broth supplemented with gentamicin (10 U/ml) for 72 hours at 30 °C on an orbital shaker rotating at 200 rpm in biosafety level 2 conditions. Female and male mice were anesthetized as described above, and injected retro-orbitally with *B. gladioli* (3x10^4 CFU/mouse). For infections involving prior training with HKCA, mice were infected with *B. gladioli* 7 days after induction of training. Morbidity and sacrifice criteria were monitored for up to 120 days. The livers, lungs, and spleens from mice meeting end-point criteria were removed, pulverized in PBS with 5 ml syringe plungers, and plated on *Burkholderia cepacia* specific agar (BCSA) plates to assess growth of *B. gladioli*.

Isolation of mouse whole blood serum and bone marrow mononuclear cells (BM-MNCs)

Mice were anesthetized with 3% isoflurane in a closed-box circulating system, then transferred to a nose-cone system and maintained in anesthesia with 2% isoflurane. Cardiac exsanguination was performed with 21 G needle inserted slightly to the left of the sternum below the rib cage until puncture was confirmed and the blood slowly drawn out; approximately 0.8-1 ml blood was drawn from each mouse. Immediately following this, blood was allowed to clot and centrifuged at 600 x g at room temperature, separating the blood into the solid pellet and liquid serum components. The serum from specific experimental groups of female and male mice was pooled separately and immediately frozen at -80 °C until later use.

Following mouse sacrifice, femurs and tibias were removed from each mouse with the muscle tissue scraped away and bones cut at both ends to expose the marrow cavity. The bone marrow was then flushed with 10 ml cold RPMI using a 25 G short-tipped needle onto a 70 μm cell strainer attached to a 50 ml Falcon conical tube. This was followed by pushing the cells through the strainer with a 5 ml syringe plunger and washing with 5 ml cold RPMI. Cells were then centrifuged at 300 x g for 7 minutes at 4 °C. Following centrifugation, the cell pellet was resuspended in 10 ml cold RPMI and gently layered over 4 ml Ficoll-Paque solution for density gradient centrifugation at 400 x g for 35 minutes at 4 °C without braking. After this, the upper layer was removed exposing the BM-MNCs, which were transferred, resuspended in media, centrifuged again at

300 x g for 7 minutes at 4 °C, and finally resuspended in viability freezing medium (90% FBS, 10% DMSO) at a concentration of $5x10^6$ BM-MNCs/ml. Cells thus prepared were slowly frozen to -80 °C overnight and then preserved in liquid nitrogen until needed for later experiments.

Quantification of serum female sex hormones

Serum from female mice at each stage of the estrous cycle, and from males, was isolated as described above. Concentrations of E2 and P4 in males and females from all estrous cycle stages were determined using competitive ELISA plates according to the manufacturer's instructions (RayBiotech, Peachtree Corners, GA, USA). Plates were read on an EPOCH microplate reader (Agilent Technologies, Santa Clara, CA, USA) by measuring optical density at 450 nm wavelength.

In vitro *BM-MNC training experiments*

Cryopreserved BM-MNCs were removed from liquid nitrogen, thawed in a 37 °C water bath, then immediately moved to 15 ml Falcon conical tubes, and washed and centrifuged twice with RPMI at 300 x g for 7 minutes at room temperature. The cell pellets were then resuspended in RPMI at a concentration of $1x10^6$ BM-MNCs/ ml, plated at $1x10^5$ BM-MNCs/well of 96-well flat bottom tissue culture-treated plates, and incubated at 37 °C +5% CO2 for 30 minutes to promote cell adherence. Each well was then washed in warm PBS, followed by the addition of different warm experimental media and incubated at 37 °C +5% CO2 for 4 hours.

These media included RPMI +10% FBS; RPMI +10% charcoal-stripped FBS (csFBS); RPMI +10% female mouse serum; or RPMI +10% male mouse serum. Each media type was supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, and 10 U/ml penicillin/streptomycin (pen/strep). All media was added to groups of female and male BM-MNCs, separately. After incubation, the dectin-1 agonist beta-1,3-(D)-glucan (β-glucan) (Invivogen, San Diego, CA, USA) was added to experimental wells for immune training (10 μg/ ml), while a matched volume of PBS +10% DMSO was added as vehicle control to other wells. Plates were then incubated at 37 °C +5% CO2 for 24 hours, washed in warm PBS, rested in RPMI for 6 days, followed by secondary stimulation with RPMI negative control, LPS (10 ng/ml), or poly(I:C) (10 μg/ml) (Sigma Aldrich, St. Louis, MO, USA). After 24 hours, the supernatants from each well were collected and stored at -20 °C.

To examine the potential influence of estradiol (E2) and progesterone (P4) levels on BM-MNC immune training, female cells were thawed, and plated with RPMI media +10% pooled female serum from each estrous cycle stage (proestrus, estrus, metestrus, or diestrus), separately, then trained with β-glucan as described above. Cells were then washed, rested, and restimulated with LPS and poly(I:C) for 24 hours. For steroid receptor antagonism experiments, female cells were plated with RPMI alone, supplemented with 200 nM of either an estrogen receptor or progesterone receptor antagonist (ICI 182,780 or PF-02413873, respectively) (Sigma Aldrich, St. Louis, MO, USA) and incubated at 37 °C +5% CO_2 for 2 hours. Each well was washed, and RPMI +10% pooled female

serum media was added with 200 nM of either ICI 182,780 or PF-02413873 and again incubated at 37 °C +5% CO_2 for 2 hours, followed by training with β-glucan and subsequent secondary stimulation with LPS and poly(I:C). To examine the effects of PF-02413873 alone, male cells were trained in male serum media plus vehicle or antagonist. Some female and male cells were also trained in csFBS media with heat-killed *B. gladioli* ($5x10^4$) for 24 hours to evaluate secondary responses from LPS and poly(I:C) restimulation. All supernatants from these experiments were collected 24 hours later and stored at -20 °C. Supernatants were later thawed, diluted two-fold, then analyzed for concentrations of IL-6 and CXCL2 (or IL-1b and IL-18) using a Bio-Rad Luminex 100 multiplex system according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA; ThermoFisher Scientific Inc., Waltham, MA, USA).

Cell metabolism experiments

To examine the influence of P4 on training, BM-MNCs were plated at $1x10^5$ cells/well of an XFe96-well cell culture plate (Agilent Technologies, Santa Clara, CA, USA) and trained in csFBS media supplemented with negative control volume or 20 ng/mL P4 (Sigma Aldrich). After 6 days, cells were washed and left in glycolysis stress test buffer (Agilent XF DMEM media, supplemented with 2 mM glutamine, pH 7.4) at 37 °C with no CO_2 for at least 1 hour. Afterwards, glycolytic activity by means of extracellular acidification rate (mpH/min, or the accumulation of protons per minute) of the supernatant was analyzed with an Agilent Seahorse XFe96 analyzer. Measurement of three time points each of

stable non-glycolytic acidification, followed by the addition of 10 mM glucose to each well to measure basal glycolysis; addition of 2 μM oligomycin, an ATP synthase (complex V) inhibitor to induce maximum glycolytic capacity; and finally addition of 50 mM 2-deoxy-D-glucose to inhibit glycolysis was performed according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). These experiments were performed on both female and male cells.

Quantification and statistical analysis

To compare survival curves, we used the log rank statistic. Under the null hypothesis, we assumed that the distribution of the survival times in each of the two groups under consideration are the same. The alternative will either be one-sided or two-sided. The test statistic derives from the fact that conditional on the total number of subjects, the total number of failures, and the total number of subjects at risk at the time of failure, the number of subjects in one group at that time of failure is distributed according to a hypergeometric distribution. When the sample size is large, this quantity, suitably normalized by its mean and standard deviation, is approximately normally distributed. However, since the sample sizes are small in our experiments, this approximation is not valid. Instead, we simulated the distribution of the normalized quantity under the null hypothesis to obtain P-values for each of the experiments. Each simulation consisted of one million runs and we repeated the simulations for each of the experiments ten times. Here we report the mean and standard deviation of the simulated P-values of the ten sets of runs in each of the experiments.

For the estimated survival mean change (increase) in days provided by immune training, survival results for each sex assigned each mouse an ordered rank, 1-5, with 1 having least and 5 having most survival days. Then the difference in survival days for each rank between trained and untrained mice was calculated, plotted, with arithmetic mean and standard deviation used to compare females with males (Fig. 1D). For this comparison, and every other instance of comparing two groups (untrained-control vs. trained, female vs. male, etc.) a one- or two-sided Mann-Whitney test for equality of means was used. Holm-Šidák correction was applied for multiple hypothesis testing. Comparisons of three or more groups utilized Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post-hoc tests, or parametric one-way ANOVA as noted in figure legends. *P*-values less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 9.0 or MATLAB with C. Data are shown as individual mouse measurements with mean ± SD unless otherwise noted in figure legends. All data are included, and no outliers have been removed.

VI. Acknowledgments

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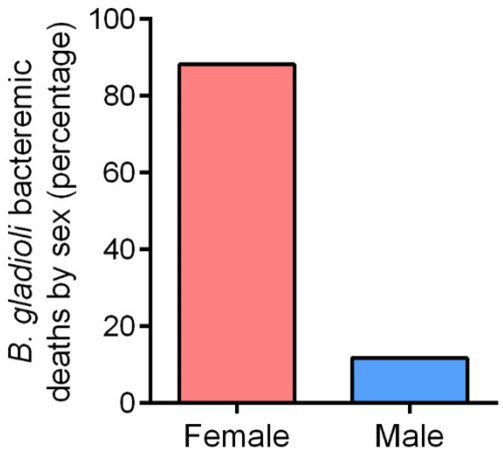
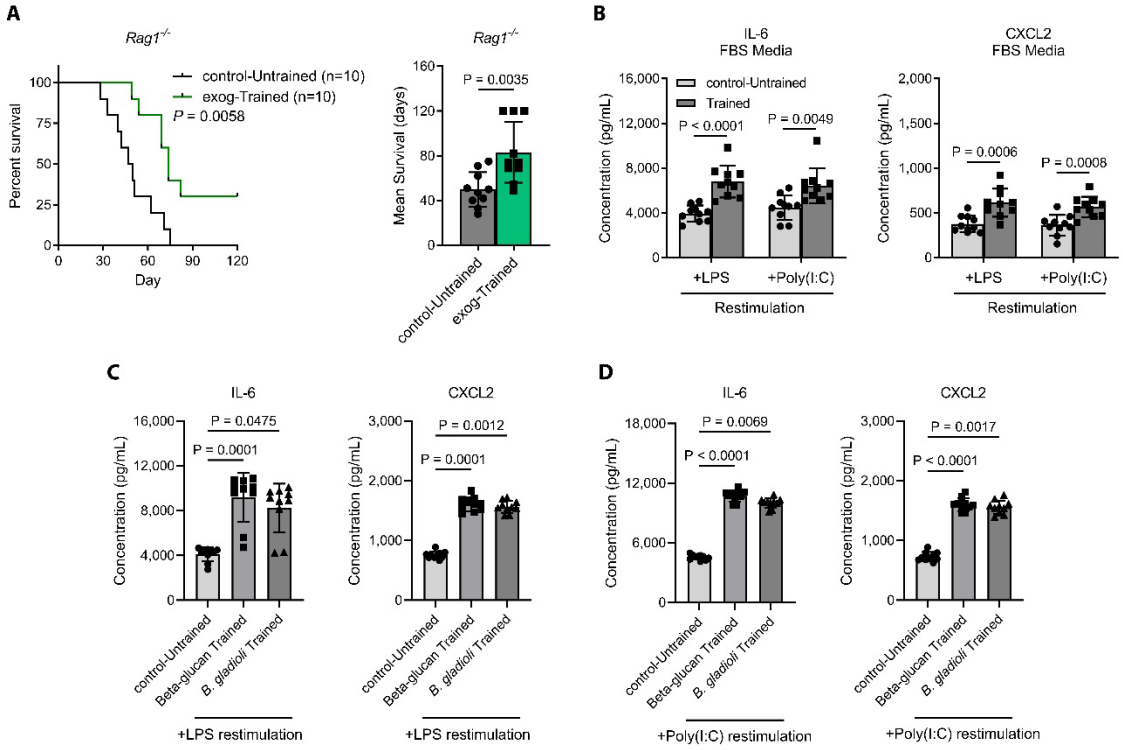


Supplemental Figure 2.1. Epidemiology of unintentional, confirmed *B. gladioli* bacteremic deaths in mouse colony, related to Figure 2.1.

Of unintentionally infected mice that died or were euthanized and confirmed to be *B. gladioli* bacteremic by proteomic analysis, PCR, and/or live bacterial culture, 17 were female and 2 were male (89.47% vs. 10.53%; many other mice died and appeared to follow the sex-biased pattern, but here we only include mice with *B. gladioli* sure confirmation). These mice included genotypes explained in a previous publication (Neier et al., 2019). The number of succumbed mice and their genotypes included: one *OT1.b2m*$^{-/-}$*.Rag2*$^{-/-}$, one *MHCII*$^{-/-}$*.b2m*$^{-/-}$, two *CD3d*$^{-/-}$, two *OT1.Rag2*$^{-/-}$, four *CD3d*$^{-/-}$*.MHCII*$^{-/-}$*.b2m*$^{-/-}$, six *CD3d*$^{-/-}$*.Rag2*$^{-/-}$, one C57BL6, and two mice of unknown genotype.



Supplemental Figure 2.2. Trained versus control-untrained mouse survival from *B. gladioli* infection, and effect of *B. gladioli* on training, related to Figures 2.1 and 2.2.

(A) Survival curves (left) and mean survival days (right) of intravenous heat-killed *Candida albicans* exogenously trained *Rag1*$^{-/-}$ mice of female (*n* = 5) and male (*n* = 5) mice, compared against equal sex numbers of control-untrained mice.

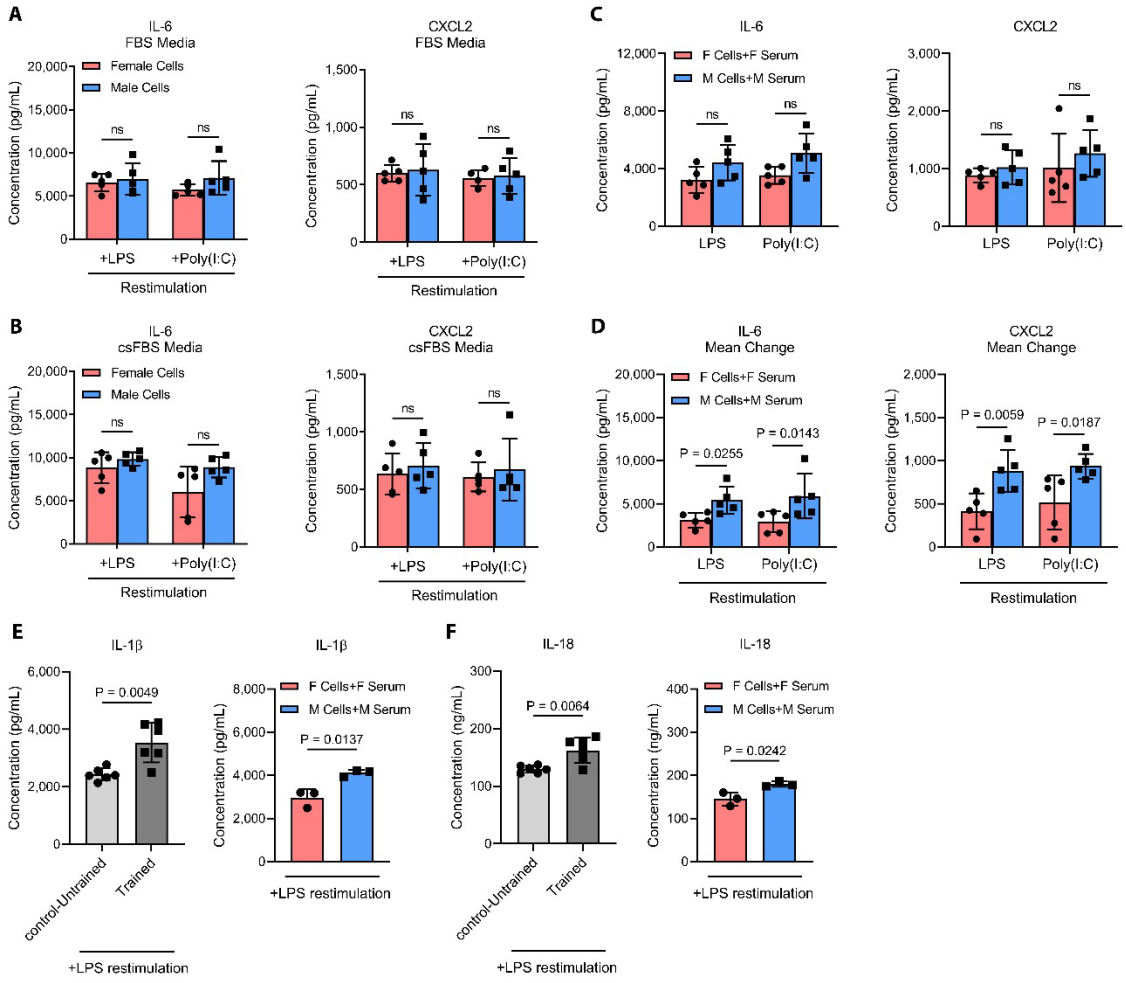
(B) β-glucan-trained *Rag1*$^{-/-}$ BM-MNCs including female (*n* = 5 per group) and male (*n* = 5 per group) cells produced significantly increased output of IL-6 (left) and CXCL2 (right) after LPS (10 ng/ml) or poly(I:C) (10 mg/ml) restimulation in FBS media compared against control-untrained BM-MNCs.

(C) After LPS restimulation, levels of IL-6 (left) and CXCL2 (right) from BM-MNCs including female (*n* = 5 per group) and male (*n* = 5 per group) control-untrained

cells in csFBS media compared against cells trained with β-glucan or heat-killed *B. gladioli* ($5x10^4$ cells).

(D) Similar to (C) after poly(I:C) (10 mg/ml) restimulation.

Survival curves were analyzed by Kaplan-Meier two-sided log rank statistics with *P*-values obtained from simulations of survival values with one million runs repeated ten times. Log rank p-value 0.0058 has standard deviation of $1.21x10^{-4}$. Mean survival days comparison between control-untrained versus exogenously trained mice utilized a two-sided Mann Whitney test of means. For both experimental groups, each dot plotted represents one mouse, $n = 10$. Concentrations of IL-6 and CXCL4 were analyzed using Mann-Whitney tests with Holm-Šidák correction for multiple hypotheses (B), or Kruskal-Wallis ANOVA test with Dunn's *post hoc* tests (C-D). For all groups (B-D), each dot represents one mouse, $n = 10$, with mean ± SD shown. P-values < 0.05 are significant.



Supplemental Figure 2.3. Immune training increases secondary responses of mouse BM-MNCs, *in vitro*, related to Figure 2.2.

(A) Supernatant levels of IL-6 (left) and CXCL2 (right) after restimulation of female or male BM-MNCs trained in fetal bovine serum (FBS) supplemented medium.

(B) Post-restimulation concentrations of IL-6 (left) and CXCL2 (right) of female or male BM-MNCs trained in charcoal-stripped FBS (csFBS) supplemented medium.

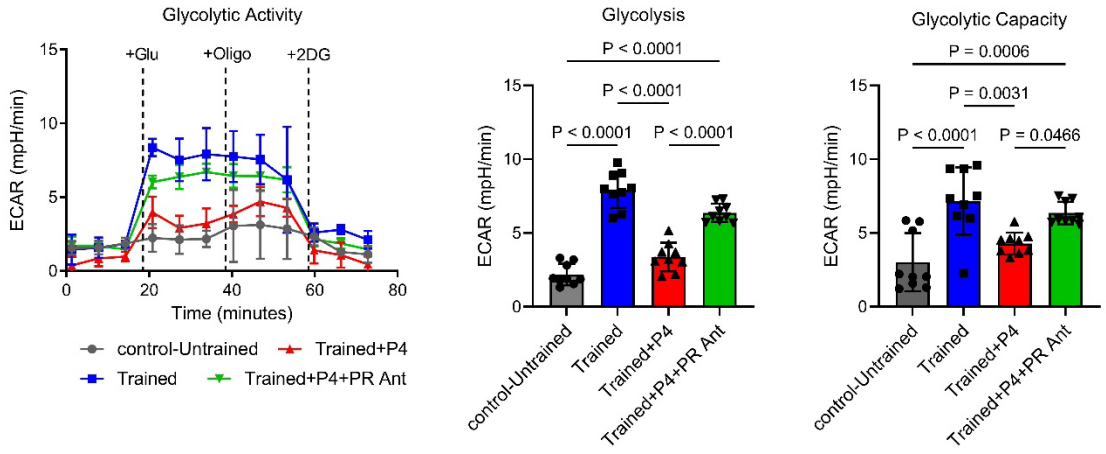
(C) After stimulation with LPS or poly(I:C), IL-6 (left) and CXCL2 (right) concentrations from control-untrained female cells in female serum, and male cells in male serum.

(D) Mean change in amounts of IL-6 (left) and CXCL2 (right) between β-glucan-trained and control-untrained female cells in female serum, and male cells in male serum.

(E) After restimulation with LPS, levels of IL-1β in supernatants from female (*n* = 3) and male (*n* = 3) BM-MNCs trained in their respective sex-matched serum and compared against control-untrained cells (left). Comparing female versus male trained response from sex-matched cells and serum samples (right).

(F) Similar to (E), assessing IL18.

For each panel, means were compared with two-sided Mann Whitney tests with Holm-Šidák correction for multiple hypotheses. Each dot plotted represents one mouse, *n* = 5 per sex for each experimental group in (A-D), and *n* = 3 per sex per group in panels (E,F), with mean ± SD shown. P-values < 0.05 are significant.



Supplemental Figure 2.4. Exogenous P4 can induce PR-dependent changes in immune training associated glycolytic activity in male BM-MNCs, related to Figure 2.4.

Extracellular acidification rate (ECAR) of male *Rag1*$^{-/-}$ BM-MNCs trained with β-glucan in the presence of vehicle control, progesterone (P4, 20 ng/ml) alone, or P4 combined with progesterone receptor antagonist (PR ant), compared to control-untrained BM-MNCs (left), and analyzed for basal glycolytic function (middle) and maximum glycolytic capacity (right).

Glycolysis was assessed using parametric one-way ANOVA with Tukey's *post hoc* tests. Each dot represents a mouse, n = 5 per sex per group except for glycolysis data, where n = 3 mice per group with triplicate measurements at multiple time points with mean ± SD shown. P-values < 0.05 are significant.

PART II:

Sex in COVID-19, and Disease Severity

Chapter 3

Introduction: Immune Activity and COVID-19

I. Adaptive Immune Cells

As discussed in Part I, Chapter 1, B and T lymphocytes of the adaptive immune system form customized genes of antigen-specific receptors at the single-cell level such that large populations of these cells present a repertoire to bind potentially millions of antigens. Upon antigenic stimulation with the aid of innate immune antigen-presenting cells (APC) such as DCs and macrophages, CD4+ T helper (Th) cells further differentiate into effector and memory cell subsets with various functions, including Th1, Th2, and Th17 cells. They assist, or “help” other cells including B cells, cytotoxic T lymphocytes (CTLs), and macrophages in their functions. The Th1 subtype predominantly induces pro-inflammatory activity in order to fight against bacteria and viruses, while Th2 cells are more anti-inflammatory but also provide protection against parasites and help induce allergic responses. Th17 cells are generally pro-inflammatory, typically helping in defense against pathogens in gut and other epithelial surfaces. Although there are other T cell types, such as CD8+ CTLs, this section focuses on Th cells that help B cells with antibody production, particularly in regard to the coronavirus disease 2019 (COVID-19) pandemic, and how sex of the patient may influence immune activity and disease outcomes. I will also examine innate immune responses in COVID-19 and roles played in pathogenesis and severity, including mortality, particularly due to PMN activity.

A. Basics of B cell development

B cell receptors

The development of B lymphocytes is a multifaceted process that begins within the bone marrow niche. Stromal cells secrete IL-7, which helps induce hematopoietic stem cells to become common lymphoid progenitors which further give rise to B, T, and other lymphocyte subsets (Kondo 2010; Corfe & Paige 2012). B cell differentiation starts with the formation of pre-pro B cells from these lymphoid progenitors, where IL-7 also aids in early proliferation (Corfe & Paige 2012). This stage is followed by immunoglobulin (Ig) gene heavy chain (HC) locus rearrangement, the beginning of B cell receptor (BCR) genesis in now pro-B cells (Pelanda & Torres 2012; Melchers, 2015). The nuclear regulatory proteins EBF-1 and E2A bind to the diversity-joining, or D-J, section of the heavy chain locus and allow for its accessibility by other factors (Hagman & Lukin 2006; Boller & Grosschedl 2014). At this point, the recombination-activating genes *Rag1* and *Rag2* are activated to splice and rearrange D-J, and variable (V) region, sections to generate diverse genetic sequences (Van Gent, Ramsden & Gellert 1996; Fugmann et al., 2000; Schatz & Swanson 2011). From here, EBF-1 also acts on the *Pax5* transcription factor site to promote the joining of the new D-J to V segments, the full process of which is called V(D)J recombination (Boller & Grosschedl 2014). This

produces unique HC segments, forming the basis of what will become expressed, below, as the pre-BCR, now at the transitional pre-B cell stage.

Expression of the pre-BCR multiprotein complex represents a first checkpoint step where antigen-independent selection occurs. Here, the pre-BCR is “selected” for functionality when an in-frame, properly folded HC protein can associate with Vpre-B and Vλ5 surrogate light chains (LCs) together with Igα and Igβ (CD79a and CD79b, respectively) to form the pre-BCR. CD79a and/or CD79b initiate intracellular processes favoring survival and proliferative processes via PI3K/Akt and subsequent MAPK activation (Mårtensson et al., 2010; Huse et al., 2022). If a the pre-BCR signal is not transduced, then the cells either undergo apoptosis or another round of recombination to form more functional pre-BCR candidate HCs (LeBien & Tedder 2008; Mårtensson et al., 2010). Upon Pre-BCR signaling, pre-B cells continue in their developmental progression. The Ig LC is formed via VJ recombination through *Rag1/2* activity, as this locus does not contain D segments. At this point, two HC:LC heterodimers physically associate and are stabilized by cysteine-based disulfide bonds, forming the full BCR, of IgM class, which is expressed on the now immature B cell surface.

Now, the final BCR-based developmental cell stage occurs. Different bone marrow cells expose these immature B cells to self-antigens to test for autoreactivity. If these cells bind to self-antigens and are thus autoreactive, three possible fates befall them: clonal deletion by

apoptosis; reactivation of *Rag1/2* to further rearrange new LCs to reenter this negative selection process; or anergy, an inactive and non-functional state with short half-life (LeBien & Tedder 2008). However, if these cells fail to bind self-antigens, then they exit the bone marrow niche and egress to the secondary lymphoid organs, primarily the spleen, and complete their maturation into naïve peripheral B cells (Pelanda & Torres 2012). This process is referred to as B cell central tolerance.

Later B cell differentiation

Early transitional, or T1, B cells first go to the splenic T cell zone, where they undergo an additional round of negative selection (Loder et al., 1999; Chung, Silverman & Monroe 2003). T1 B cells that strongly bind to antigens die by apoptosis, while cells that fail to bind self-antigens survive and upregulate production of the anti-apoptotic protein Bcl-XL. These cells then move into the follicular zone, begin to express another Ig-receptor HC class, IgD, and are known as T2 B cells (Chung, Silverman & Monroe 2003). Here, another round of positive selection occurs where those that bind antigens survive and become B-2, or follicular, cells capable of memory and production of many variable antibodies (Chung, Silverman & Monroe 2003; Cerutti, Cols & Puga 2013).

Two other B cell subsets, which will not be discussed much further, are B-1 and marginal zone B cells. B-1 cells are produced in the fetus move into the circulatory periphery instead of the spleen, where they are then readily responsive to carbohydrate moieties from foreign microbes,

becoming short-lived IgM antibody-producing plasma cells to help quickly fight against invading pathogens (Haas et al., 2005; Ghosn et al., 2008; Verbinnen et al., 2012; Cunningham et al., 2014; Baumgarth 2016). Importantly, they do not typically have “memory” like other B cell types. The other, marginal zone B-2 type cells, have broadly cross-reactive surface IgMs that respond to microbial carbohydrate and protein antigens with restricted diversity, and are specialized for blood-borne pathogenic insults as the blood largely circulates through the marginal zone (Ghosn et al., 2008; Baumgarth 2016). These two types generally do not produce many different antibodies or antibody classes after activation.

B. Antibody generation

B cell activation

When naïve B-2 cells are activated by follicular dendritic cells (FDCs) expressing recently encountered foreign antigens, they uptake the antigen via their surface Ig, degrade it and present peptide fragments on class II MHC receptors for presentation to Th cells (Pieper, Grimbacher & Eibel 2013). The B cells then move to the interfollicular zones of lymphoid organs in response to secreted chemokines like CXCL13 binding to the receptor CXCR5 (Ansel et al., 2000). Here, they face two primary types of further activation: the first is T cell-dependent, in which B-2 cells expressing MHC-II/antigen bind to TCRs and obtain T cell help via CD40L binding to B cell surface-expressed CD40, and also with aid from the

cytokines IL-4 and IL-21 (Pieper, Grimbacher & Eibel 2013). The second activation type is T cell-independent, whereby B-2 cells are exposed to foreign polysaccharides or unmethylated DNA; this also requires costimulation, typically in the form of TLRs.

Somatic hypermutation, class switching, and antibody production

Such stimulation, usually T cell-dependent, begins the formation of the B cell germinal center (GC), composed of two distinct zones. In the dark zone, after stimulation, B-2 cells rapidly proliferate and further undergo a process wholly unique to B lymphocytes: somatic hypermutation (SHM). During SHM, the now active enzyme cytidine deaminase randomly mutates the variable chain gene loci of the BCR in the recently proliferated cells (Chi, Li & Qiu 2020). Following this, they express these new BCRs, move into the GC light zone, and undergo a round of positive selection, where low affinity binding to antigens presented by FDCs leads to apoptosis while those bound with high affinity, now called GC B cells, again internalize antigens and can repeat the cycle when they migrate further into the light zone (Chi, Li & Qiu 2020). The GC B cells further present antigens on class II MHC receptors to T follicular helper (Tfh) cells, where those with the highest affinity end the maturation process and develop into antibody-producing plasma cells with the help of NF-κB signaling and IRF4 expression (Nutt et al., 2015). Those with lower affinity typically upregulate the *Hhex* transcription factor and develop into memory B cells (Laidlaw et al., 2020; Good-Jacobson & Groom 2020).

From here, plasma cells move into the bone marrow, while memory B cells circulate to the periphery such as in lymphoid organs.

While in the GC, mature B cells undergo another unique phenomenon called class switching, whereby they rearrange Ig constant domain genes to produce new antibody classes (IgG1, 2, 3, 4, IgA, and IgE) and excise the upstream constant domain genes (Markey & Papavasiliou 2003; Chi, Li & Qiu 2020). This process occurs due to certain cytokines produced by Tfh cells: IFN-γ, IL-4, 5, 10, and TGF-β all help induce production of the different antibody classes (Xu et al., 2012; Chi, Li & Qiu 2020). These mature B cells now produce different classes of antibodies recognizing the same antigens, although with different structures and production in different parts of the body (Xu et al., 2012; Chi, Li & Qiu 2020). Plasma cells in the bone marrow constantly produce high amounts of antibodies that enter circulation and bind recognized antigens they encounter, while memory B cells, when bound by antigens, proliferate into antibody-producing cells to fight pathogens (McHeyzer-Williams et al., 2012; Kurosaki, Kometani & Ise 2015; Nutt et al., 2015; Lightman, Utley & Lee 2019; Robinson et al., 2022). Of interest to this dissertation, females typically have higher basal levels of circulating antibodies concomitantly with increased B cell counts compared to males, and greater antibody responses against viral infections (Teixeira et al., 2011).

II. The Coronavirus Disease 2019 (COVID-19) Pandemic

In November 2019, an unusual spike of pneumonia cases occurred in the Chinese city of Wuhan, and, by the end of December, the cause was traced to a novel coronavirus spreading via human-to-human transmission. On the evening of December 31, Chinese authorities alerted the World Health Organization (WHO) to the situation, setting off an international response to understand and contain the disease and its causative agent. By February, this virus, then termed 2019-nCoV, and its associated disease were renamed by the WHO to severe acute respiratory distress syndrome coronavirus-2 (SARS-CoV-2) and coronavirus disease 2019 (COVID-19), respectively. Since then, the virus spread globally, having been officially declared a pandemic on March 11, 2020 by the WHO. As of September, 2023, there have been approximately 770 million confirmed cases worldwide, with many repeat infections, and nearly 7 million official deaths, although this number may actually be as high as 20 million or more attributable deaths (Adam 2022; Wang et al., 2022). These numbers indicate a case fatality rate (CFR), or the proportion of diagnosed people who end up dying from it, of at least 1%. Early enactment of social containment measures, including mask-wearing, school shutdowns, lockdowns, and mass vaccination resulting from perhaps the most rapid development of a vaccine in history, have almost certainly kept these numbers lower than they would be without them.

A. SARS-CoV-2 and pathogenesis

The SARS-CoV-2 virus

The causative agent of COVID-19, SARS-CoV-2, is a sarbecovirus in the coronavirus family. Coronaviruses are comprised of positive-sense, single stranded RNA genomes, meaning the genome itself acts as mRNA and can be directly translated into proteins by infected host ribosomes. SARS-CoV-2 is closely related to the sarbecovirus responsible for the outbreak of SARS in 2003-2004, SARS-CoV-1. Though many genetic lineage studies have examined SARS-CoV-2 throughout the course of the pandemic, its precise ancestral origins remain unclear. It appears to be closely genetically related to several bat coronaviruses known to circulate in southern China, particularly RaTG13 and BANAL-2, with 96.1% and 96.8% similarity, respectively (Zhou et al., 2020; Temmam et al., 2022). Early speculation based on genetic relatedness suggested this virus may have also arisen, in part, from pangolin coronaviruses (Xiao et al., 2020). Interestingly, there is no known animal reservoir for SARS-CoV-2, and it has been suggested that perhaps a bat virus mutated in an infected human into the initial strain (Holmes et al., 2021).

The SARS-CoV-2 genome consists of 6 open reading frames (ORFs), spans of genetic material with transcriptional start and stop codons, producing four structural proteins which hold the virion together and aid in its pathogenesis (Wu et al., 2020). The nucleocapsid (N) protein holds the RNA genome in place within the viral membrane, which is itself

held together by the envelope (E), membrane (M), and spike (S) proteins (Masters 2006; Tseng et al., 2010; Chang et al., 2014; Schoeman & Fielding 2019). The E protein is the least well-understood, although it is believed to play a nonessential role in viral assembly within host cells, while the M protein is necessary for assembly (Tseng et al., 2010; Schoeman & Fielding 2019). However, the most well-studied and pathogenetically important of these is the S protein, which plays an essential role in viral entry into host cells (Li 2016; Zhu et al., 2021). One gene, *ORF3d*, is nearly identical to one found in pangolin coronaviruses, implicating them in the SARS-CoV-2 lineage; its resultant protein is highly immunogenic and likely plays a role in disease severity (Hachim et al., 2020; Nelson et al., 2020).

SARS-CoV-2 pathogenesis and immune responses

The SARS-CoV-2 virus was initially believed to only spread through respiratory droplets, and not through airborne transmission, although later studies showed that it is capable of both (Mittal, Ni & Seo 2020). Upon exposure, SARS-CoV-2 infects ciliated epithelial cells within the nasal cavities, pharynx, and upper airway via S protein binding to host cell surface ACE2 receptors (Hofmann et al., 2020; Zhao et al., 2020). Uniquely among coronaviruses, the S protein contains a furin host enzyme cleavage site which helps to facilitate entry into cells, while the host surface protein TMPRSS2 also aids in this process, although neither are absolutely required for infection (Verdecchia et al., 2020; Hu et al., 2021;

Hossain et al., 2022). Upon entry, different classes of PRRs, including TLR7, bind the viral RNA and initiate downstream signaling, including those of NF-κB and IRFs, leading to the production of pro-inflammatory and antiviral mediators such as CXCL1, CXCL2, CXCL3, IL-1β, IL-6, IL-8, TNFa, and type I and III IFNs, respectively (DeDiego et al., 2014; Soy et al., 2020; Wong & Perlman 2022). However, upon translation of the viral genome, the viral N protein appears to interfere with the host nuclear functions of STAT1, leading to decreased IFN production, and thus lessened antiviral activity (Mu et al., 2020). At this point, the virus readily replicates.

Upon escape from host upper respiratory cells, viral particles can be inhaled into the lower respiratory tract, infecting type II pneumocytes within the gas-exchanging alveolar spaces in the lungs, and resident macrophages, inducing further production of the pro-inflammatory cytokines and chemokines described above, while also downregulating the same IFNs (Mu et al., 2020; Chakravarty, Panchagnula & Patankar 2023). In cases of severe disease, this sets off a chain reaction whereby monocytes, macrophages, and PMNs infiltrate the alveoli in great numbers, while T cell migration into the lungs is reduced. At this point, infiltrating innate immune cells greatly exacerbate inflammation, while PMNs also produce NETs, which thicken mucus to clog alveoli, participating in hypoxia (Middleton et al., 2020; Jing et al., 2021). This persistent pro-inflammatory activity can lead to hypercytokinemia, not just

within the lungs but also in circulation, resulting in systemic host damage and eventual multiple organ compromise, potentially ending in death (Soy et al., 2020; Zhao et al., 2022). SARS-CoV-2 also enters the bloodstream, and here also induces immune functions, sometimes leading to exacerbated innate cell activity, especially from circulating monocytes and PMNs, contributing to the development of potentially fatal blood clots seen in many severe COVID-19 patients, due in large part to PMN NETosis (Middleton et al., 2020; Jing et al., 2021).

While T cells play a significant role in eliminating viral infections through both helper and direct cytotoxic cell activities, their functions can be dampened in COVID-19, especially severe disease, as the virus downregulates production of the IFNs which induce them (Mu et al., 2020). However, B cells appear to have a much greater direct influence against SARS-CoV-2. Circulating anti-SARS-CoV-2 S-protein IgM antibody (anti-S) levels increase quickly after infection (Figueiredo-Campos et al., 2020). High IgM indicates an early response to infection. As infection continues, levels of circulating IgG, which have greater affinity, increase to fight the specific pathogenic insult. Anti-S IgAs also greatly increase over time in COVID-19, helping to fight the virus at the mucosal sites of infection (Figueiredo-Campos et al., 2020). Antibodies bound to antigen are then readily recognized and engulfed by innate cells like classical monocytes, macrophages, and PMNs in order to facilitate pathogen destruction. However, not all antibodies are specific to the S

protein, as most cases of infection develop broad antibodies against multiple SARS-CoV-2 proteins, including M, N, and S (Hashem et al., 2020; Post et al., 2020; Jeannet et al., 2022).

Interestingly, men are more likely than women to develop severe COVID-19 and are thus more susceptible to fatal outcomes (Klein et al., 2020; Penna et al., 2020; Takahashi et al., 2020). Indeed, the odds of severe disease, hypoxemia, sepsis, and death were all significantly higher in men throughout the pandemic (Klein et al., 2020; Penna et al., 2020; Takahashi et al., 2020). Of note, men and women appeared to have similar characteristics at the beginning of infection, with similar white blood cell counts and levels of anti-S IgM (Huang et al., 2021; Scully et al., 2020). However, as disease progressed, especially in severe patients, women had increased T and B cell counts, and higher anti-S IgG1; while men showed decreased lymphocyte and antibody levels but higher monocyte, macrophage, and PMN counts alongside increased pro-inflammatory cytokines, on average (Huang et al., 2021; Scully et al., 2020).

However, it remains unclear as to why men were more predisposed to this increase in morbidity and mortality. Many deaths from COVID-19 may, at least in part, be attributable to hypercytokinemia induced by the innate immune system (Soy et al., 2020; Hu, Huang & Yin 2021). As the female sex hormones E2 and P4, in a broad sense, might tend to dampen innate immune responses, it is conceivable their higher levels in women

provided some protection against severe disease. Other factors may include expression patterns of the ACE2 and TMPRSS2 receptors. Although many studies have found no sex differences in the overall expression of ACE2, men do tend to have higher numbers of ACE2+ type II pneumocytes in the lungs (Song et al., 2020). While the androgen receptor acts on the *TMPRSS2* gene promoter region, there is no clear evidence that men have increased expression of this gene (Lin et al., 1999).

B. Consideration of dornase alfa for the treatment of severe COVID-19 ARDS

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Earhart et al., 2020, *New Microbes and New Infections*, 35, 100689.

Abstract

We propose a likely contribution to severe COVID-19 morbidity by extracellular DNA in neutrophil extracellular traps (NETs). Dornase alfa degrades extracellular DNA to reduce mucus rigidity and accumulation, and was associated with respiratory improvement in a first patient. Dornase also should be considered for clinical trials in treatment of severe COVID-19.

Letter to the Editor

Dornase alfa (Pulmozyme; Genentech, South San Francisco, CA, USA) is recombinant human deoxyribonuclease I, the only drug in its class that acts as a mucolytic by cleaving extracellular chromosomal DNA from neutrophil extracellular traps (NET) and other cell-free DNA. The drug's on-label clinical use is to reduce the viscosity and quantity of airway mucus in individuals with cystic fibrosis, thereby improving mucociliary clearance (Yang & Montgomery 2018). Dornase alfa is commonly used in individuals with cystic fibrosis, including those with severe complications requiring mechanical ventilation in intensive care units, and is compatible with co-administration of other routine drugs including antibiotics. Off-label use of dornase alfa includes reports treating acute respiratory distress syndrome (ARDS), where the drug can lead to mucus plug clearance and accelerated recovery in humans and mice (Morris & Mullin 2004; Riethmueller et al., 2006). A controlled clinical trial for treating ARDS with dornase alfa is currently underway (Pottecher et al., 2020). In the Critical Care setting, rare and minor adverse effects associated with dornase alfa include voice alteration and rash (Yang & Montgomery 2018).

The cellular and molecular mechanisms proposed for dornase alfa activity in severely distressed lungs of individuals with cystic fibrosis and many ARDS patients are as follows. Inflammation results in neutrophilia and neutrophil infiltration in the lungs, where these cells produce NETs, largely comprised of sticky, large chromosomal DNA that physically

reinforces airway mucus viscosity and accumulation (Cheng & Palaniyar 2013; Matinez-Aleman et al., 2017). Thick mucus that clears poorly can lead to airway obstruction, bronchiectasis, lung injury, hypoxia and respiratory failure. Dornase alfa facilitates airway clearance by breaking up reinforcement of mucus by NETs, by far the greatest source of extracellular DNA in inflamed lungs (Cheng & Palaniyar 2013; Matinez-Aleman et al., 2017).

Severe cases of coronavirus disease 2019 (COVID-19) have shown an inflammatory neutrophil and mucus-mediated airway exclusion pathway with striking similarities to that described above (Figure 3.1). Unlike mild COVID-19, which is often associated with fever and upper-airway symptoms, individuals with severe COVID-19 often progress to an ARDS condition: hypoxemic respiratory failure associated with neutrophilia and neutrophil infiltration in the lungs, thick mucus in bronchi, and bronchiectasis (WHO 2020; Ye et al., 2020; Zhang et al., 2020; Barnes et al., 2020). Because lung neutrophilia in ARDS is generally known to involve high NET production (Cheng & Palaniyar 2013), we feel it is rational to hypothesize that NETs contribute to severe pathology in COVID-19. Indeed, lung neutrophilia and NET production have been shown to contribute to the development of ARDS in other severe viral respiratory infections, including H1N1 influenza (Narasaraju et al., 2011).

We postulate that nebulized dornase alfa may effectively treat a deleterious effect of NETs in the airways and so promote recovery in

individuals with COVID-19-related ARDS (Fig. 3.1). Dornase alfa can be easily administered to mechanically ventilated patients and is well tolerated in intensive care unit settings. Anecdotally, an individual with COVID-19 who had been intubated for 5 days was given 3 days of nebulized dornase alfa (2.5 mg twice daily) with continued standard intensive care unit care. Improvements in oxygenation and lung compliance were observed comparing before with after the 3-day period (changes: arterial oxygen partial pressure (PaO2)/fractional inspired oxygen (FiO2) = P/F, 212 to 305; FiO2, 65% to 40%; positive end-expiratory pressure (PEEP), 20 to 14). Four additional days were followed by extubation and six more days in hospital before the patient was considered recovered and discharged home. At this juncture of a rapidly evolving pandemic associated with high mortality in severely ill individuals and the concepts discussed above, we suggest the consideration of including inhaled dornase alfa in clinical trials for severe COVID-19 associated with ARDS.

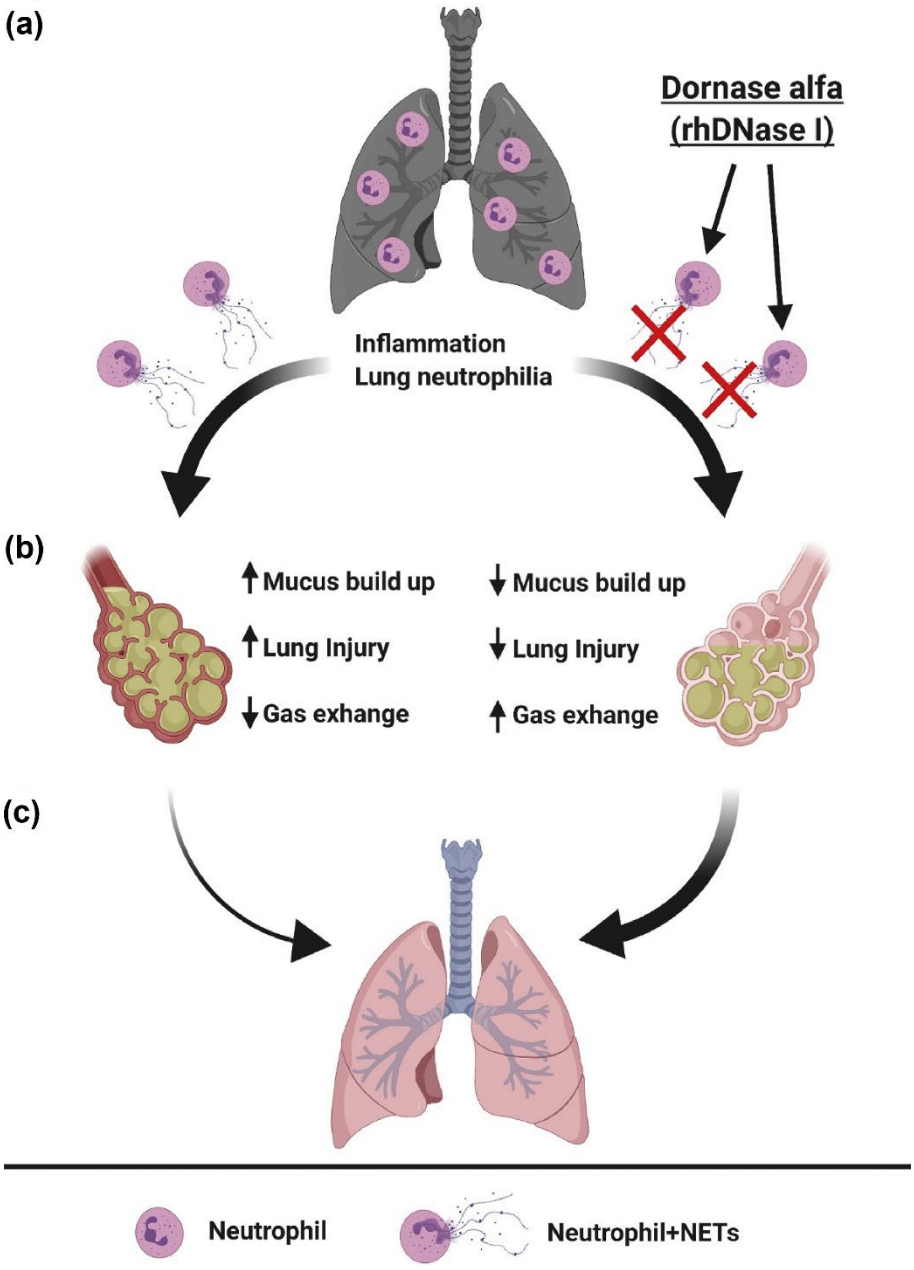

Figure 3.1: Model of how dornase alfa-sensitive neutrophil extracellular traps (NETs) from neutrophils may reinforce mucus accumulation, rigidity and airway occlusion in severe coronavirus disease 2019 (COVID-19). (a) Viral infection causes inflammation and respiratory distress (darkened lungs). Lung-infiltrating neutrophils produce NETs whose large quantities of

chromosomal, extracellular DNA are susceptible to degradation by dornase alfa (recombinant human deoxyribonuclease I, right). **(b)** Close up view of alveoli. Without dornase alfa treatment (left), NETs reinforce the accumulation and rigidity of mucus that can increase lung injury and reduce oxygenation. Dornase alfa treatment (right) reduces NET-mediated reinforcement of mucus, making it less rigid (lighter yellow) and facilitating mucus clearance, so reducing lung injury and increasing gas exchange. **(c)** The rate at which recovery from severe COVID-19 occurs naturally (left, thin arrow) might be increased by dornase alfa treatment (right, thicker arrow). Figure was created with BioRender.

Chapter 4

SARS-CoV-2 antibody positivity and the persistence of protective antibody responses: evidence from the University of Missouri community in the fall semester of 2020

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I: Abstract

Tests of SARS-CoV-2 seroprevalence within defined populations can inform local public health decisions about the level of exposure in the population and, by proxy, estimate spread of disease, independent of the frequency of positive Polymerase Chain Reaction test results. These data can be used as a robust measure of community-level disease exposure. Our goal was to determine the percentage of the University of Missouri-Columbia (MU) community that had antibodies indicative of exposure to SARS-CoV-2 and the factors that contributed to antibody levels and apparent persistence. Through stratified random sampling and self-enrollment, 2,202 individuals, including faculty, staff members, and students took part in the cross-sectional MU Study of Seropositivity and Risk for SARS-CoV-2 and COVID-19, over the course of the Fall 2020 semester. The study included a blood draw and serology analysis for SARS-CoV-2 anti receptor binding domain (RBD) antibodies. Among 2,202 participants, seropositivity was 10.9%. The peaks for the highest percentages of participants testing positive for SARS-CoV-2 antibodies occurred roughly two weeks after Halloween (October 31st) and Thanksgiving (November 26th). Seropositivity was higher in young people. Older male participants had higher antibody levels. There were 114 participants who declared they had previously been tested for SARS-CoV-2 by PCR, and, among these individuals, 10.5% were seronegative. Additionally, the antibody levels were much lower in males than females the greater the time since a positive PCR test. Seroprevalence serves as a more definitive measure of disease exposure within a community. There was increased seropositivity near

seasonal holidays noted in this study, demonstrating the importance of risk mitigation strategies at such times in the face of a disease pandemic. Future longitudinal studies should also consider gender differences in the immune responses to SARS-CoV-2 and our findings should be considered when analyzing potential herd immunity scenarios, including responses to vaccination.

II: Introduction

SARS-CoV-2 is responsible for the Coronavirus Disease of 2019 (COVID-19) pandemic. Even with the world-wide deployment of multiple vaccines and continued vaccine research, there is still much to be learned about the immune response and long-term protection that results from SARS-CoV2 infection. Current models suggest that a well-balanced immune response to the virus, including antibody production and T cell activation, is protective and antibodies against the receptor-binding domain (RBD) have the ability to neutralize infection (Sette & Crotty 2021). COVID-19 severity is largely dependent on innate immune system-based lung pathology brought on by high viral loads and deficient T cell responses (Sette & Crotty 2021; Magleby et al., 2020; Moderbacher et al., 2020). Importantly, given the probability of symptom-free transmission among younger individuals (DeBiasi & Delaney 2021) and the variable duration of antibody responses in infected individuals (Chia et al., 2021), tests of seroprevalence within defined populations can inform local public health decisions about the level of exposure and, by proxy, an estimate of disease spread, independent or in parallel with the frequency of positive Polymerase Chain Reaction (PCR) test

results. Taken together with an assessment of the duration of protective immunity, these can provide a measure of the state of “herd immunity” within a community.

With the practical need to re-open colleges for the Fall semester of 2020, there was great concern for how the opening of college campuses might increase the incidence of COVID-19 on campus and in surrounding areas (Paltiel, Zheng & Walensky 2020). Local communities where the colleges are located were expected to be impacted by both the universities’ implementation of public health measures and the prevalence of active infection and health measures at distant “home” communities where students travel to and from during the semester.

With the emergence of more readily transmissible mutant SARS-CoV-2 strains, there is an increased need to track disease prevalence. At the University of Missouri-Columbia (MU), we undertook a semester-long campus community-based study that quantified the proportion of students, faculty and staff participants that were positive for SARS-CoV-2 antibodies, including a subset of participants that had previously received PCR tests. The seropositivity rate was used to advise MU Incident Command about ongoing exposure in the campus community, informing the need to revise disease mitigation plans. The presence or absence of RBD specific antibodies among participants who had reported a positive COVID-19 test provided preliminary evidence of the persistence of an immune response by comparing time of positive PCR test date and subsequent antibody levels measured in this study.

III: Results

A. Antibody seroprevalence increased throughout the semester

In Table 4.1, we identify key characteristics of our sample by university status, age, and gender. Fig. 4.1 depicts the weekly results of SARS-CoV-2 antibody testing among faculty, staff, and students. The peaks for the highest percentages of participants testing positive for SARS-CoV-2 antibodies occurred the weeks of November 9 at 12.8% and December 7 at about 15.5%, these peaks occur roughly two weeks after major Fall events—Halloween (October 31) and Thanksgiving (November 26), respectively. These results reflect the self-reported positive PCR test rate amongst our sample in the weeks leading up to these peaks. After mid-October, the sample positivity for SARS-CoV-2 antibodies increased from 5.4% to 10.9% by December 11—the blood collection end date.

	Frequency (%)
University Status	
Faculty/Postdoc/Resident	331 (15.0)
Staff	870 (39.5)
Graduate	555 (25.2)
Undergraduate	292 (13.3)
Missing	154 (7.0)
Age, years	
17-24	757 (34.4)
25-29	290 (13.2)
30-34	235 (10.7)
35-39	192 (8.7)
40-44	152 (6.9)
45-49	153 (7.0)
50-54	137 (6.2)
55-59	121 (5.5)
60-64	98 (4.5)
65+	46 (2.1)
Missing	21 (1.0)
Gender	
Male	628 (28.5)
Female	1410 (64.0)
Identified Outside of Gender Binary	17 (0.8)
Missing	147 (6.7)
Note: Missing values are due to item non-response or select cases where there were too few comparable cases, such as the 143 who simply did not answer the gender question, or the 17 participants who identified with a gender other than male or female (counted with “missing” group, here).	

Table 4.1: Analytic sample, individual characteristics (n = 2,202)

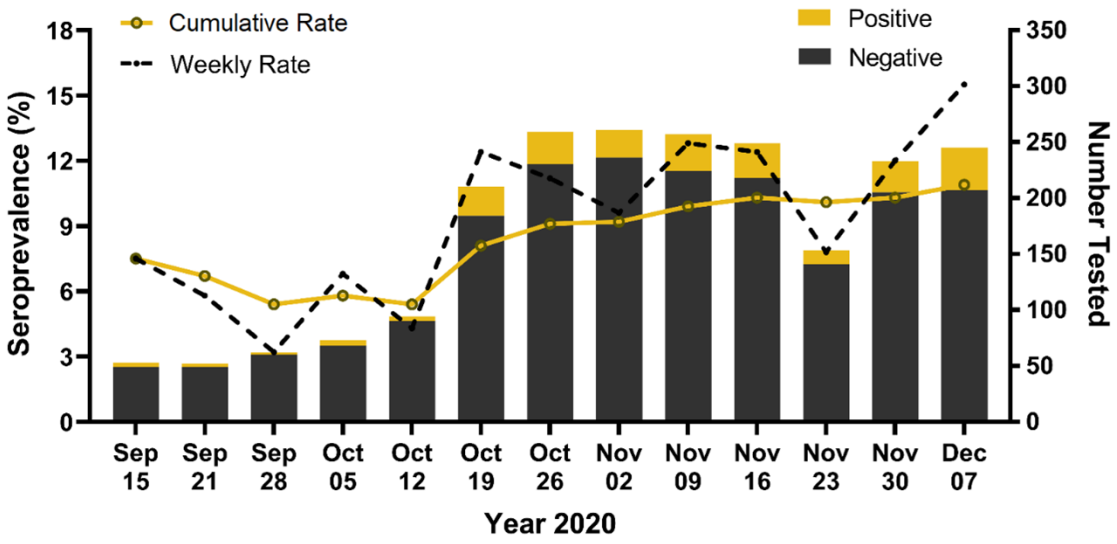

Figure 4.1: Overall SARS-CoV2 seropositivity at the University of Missouri, Columbia during testing periods from September 15 to December 11, 2020. The Y-axis on the right side corresponds to the bars, showing the frequencies of participants who tested negative (grey) and positive (gold) for SARS-CoV-2 antibodies; The Y-axis on the left side corresponds to weekly percentages of seropositivity (dotted black) and the study's cumulative seropositivity (solid gold).

B. Undergraduate students had higher antibody titers

Undergraduate students had nearly double the seropositivity (21%) compared to faculty, staff, and graduate students at any point in the latter two months of the study— October 14 to November 12, and November 13 to December 11 (Fig. 4.2). Those who were 17 to 24 years old had the highest seropositivity over the course of the entire study (20%), with 45- to 49-year-olds the second highest (11.1%). Lastly, despite differential participation rates in the study, there was only a 1.5% difference in seropositivity between female and male participants.

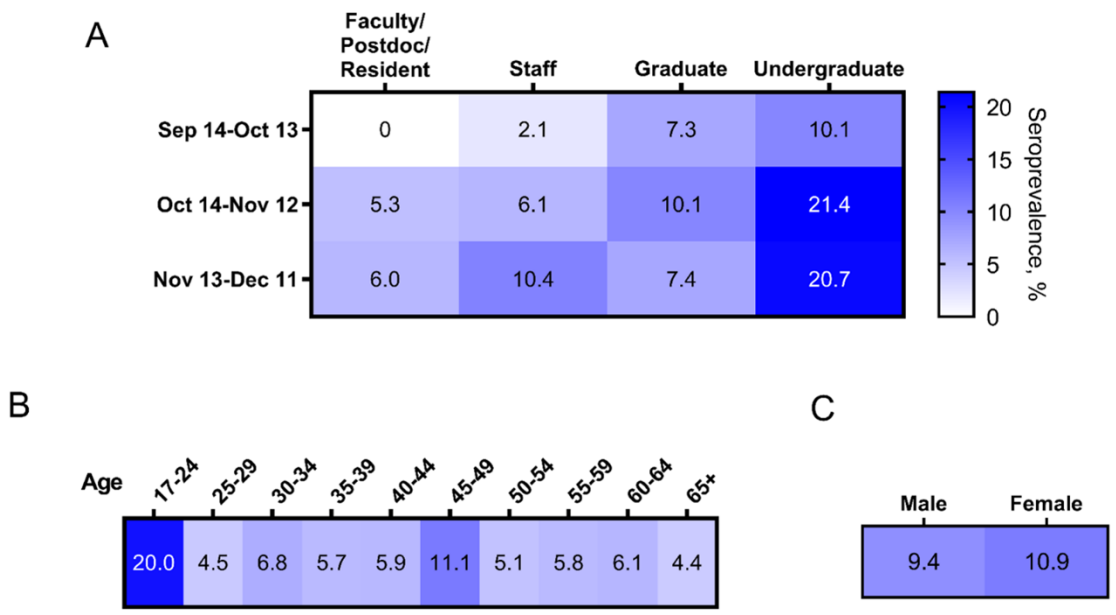

Figure 4.2: University status, age and gender-stratified SARS-CoV2 prevalence. (A) University status-stratified seroprevalence is shown in the indicated testing period; numbers indicate the positive rate in each group. **(B-C)** Age and gender stratified seroprevalence in the whole testing period.

C. Antibody levels were higher in older individuals, males

The SARS-Cov-2 Total Ig and IgG assays provide a numerical index value of antibody responses against the RBD of the S1 spike protein that quantitates antibody levels. A total of 241 individuals tested positive for either total Ig or IgG or both assays. We observed considerable heterogeneity in these responses across our study. Our cross-sectional analysis between antibodies and age revealed that older ages were associated with higher anti-RBD total Ig and IgG levels (Fig. 4.3A,B). Notably, overall, males had greater anti-RBD levels than females and this difference increases with age (Fig. 4.3C,D). To rule out the possibility that the difference in antibody levels between groups was related to participants' hematology characteristics, we routinely performed whole blood cell counts, including 5-part differentials for every blood sample collected. There were no significant differences in various blood parameters between antibody positive and antibody negative groups (table S4.1) except for eosinophil whole blood counts and percentages being higher in Ig-positive samples, suggesting the role of eosinophils in maintaining antigen secreting cells.

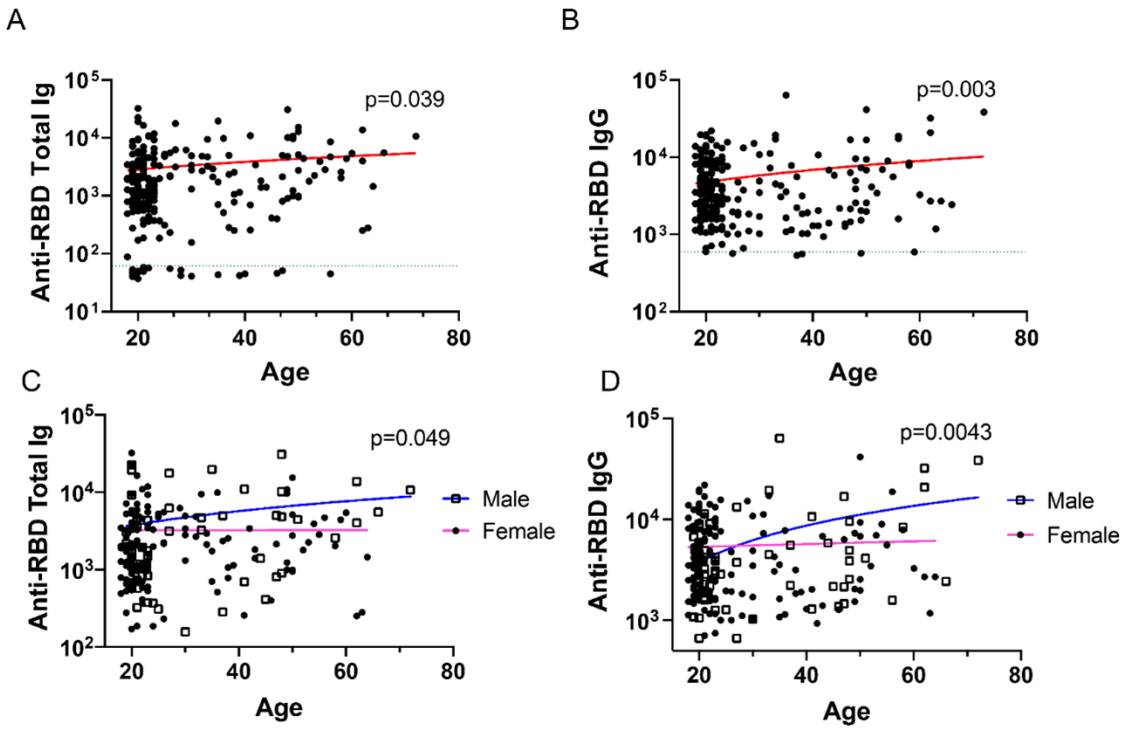

Figure 4.3: Age and gender are associated with anti-RBD antibody levels. **(A-B)** Total Ig **(A)** and IgG **(B)** antibodies against RBD from the 239 individuals who assayed positive from either test are analyzed by age. Linear regression models for the best fit curve (age information was missing for 2 subjects). P values in each graph represent the significance of the parameter. The green dotted line indicates negative control background level. **(C-D)** Association of gender and age with Total IgG **(C)** and IgG **(D)** response. Linear regression curves are shown. For total Ig **(C)**, 139 female subjects and 51 male subjects were plotted. Test of homogeneity of regressions F=3.9, p=0.049. For IgG **(D)** 148 female subjects and 58 male subjects were plotted. Test of homogeneity of regressions F=8.3, p=0.0043. (Gender information was missing for 28 subjects, and the ones with antibody level below background were excluded).

D. Antibodies decreased over time in those with previous PCR-positive infections

A total of 122 participants reported a positive SARS-CoV-2 PCR test prior to the start of our study. Most participants reported no symptoms to mild/moderate symptoms, 8 reported severe symptoms, and none were hospitalized for COVID-19. Those with self-reported severe symptoms had significantly higher total Ig and IgG than the rest among the participants who previously tested positive by PCR (Fig. 4.4A,B). Because antibodies take 4 to 14 days to be detectable after infection (Sethuraman, Jeremiah & Ryo 2020), we analyzed the antibody results among the subjects whose blood were collected 2 weeks after their positive PCR tests (n = 114). Remarkably, 10.5% of these subjects tested seronegative at the time of blood collection for this study (Fig. 4.4C). The frequency of seronegative results increased from 0% in participants who donated on days 15-30 to 13.5%, in those who had waited for more than 30 days after their positive PCR tests (Fig. 4.4C). In addition, for those who donated blood more than 100 days post-positive PCR test, the people older than 40 years were less likely to remain seropositive compared to those who were less than 40 years old (Fig. 4.4D). Further analysis supported a moderate negative correlation between age and antibody persistence (r = -0.31).

We also assessed antibody levels by days after positive PCR tests and gender. Fig. 4.4E,F shows that females had positive levels of both anti-RBD total Ig and IgG from 15-170 days. By contrast, males with a

self-reported positive PCR test had significantly higher total Ig and IgG than females in the first 30 days, consistent with the analysis in Fig. 5.2 and other studies (Klein et al., 2020). However, the levels of antibodies were significantly lower in males over time from self-reported positive PCR test results. This suggests that the endurance of anti-RBD antibody response depends on gender, with levels of anti-RBD antibody levels in males likely declining over time.

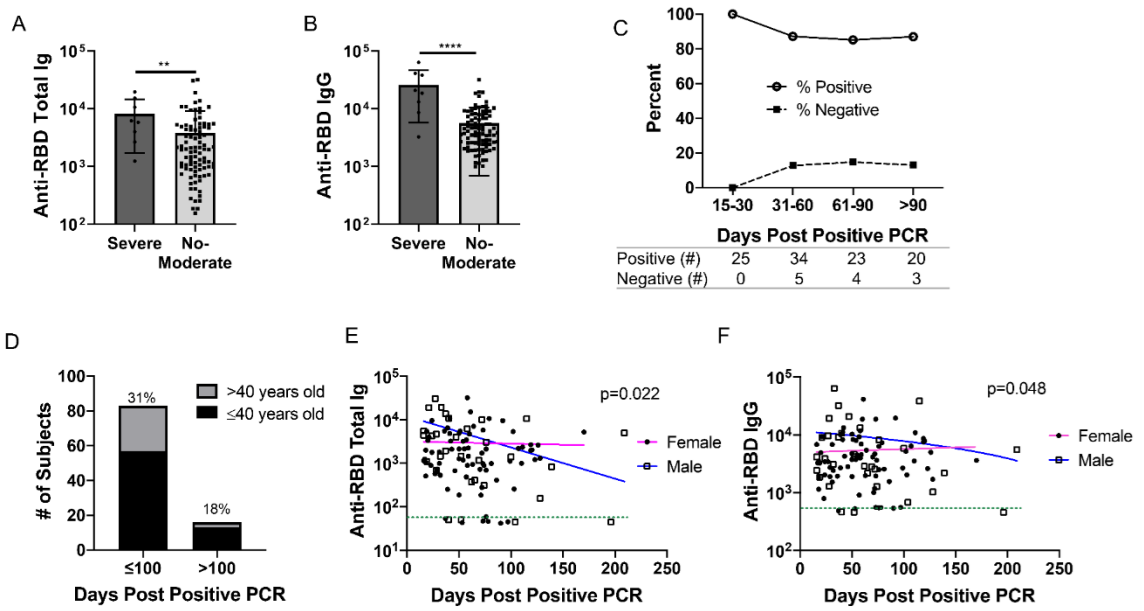

Figure 4.4: Anti-RBD antibody levels in subjects with positive COVID-19 PCR results. (A) Total Ig and **(B)** IgG level in the group reported severe symptoms (n=8) and the ones with no symptoms or mild to moderate symptoms (n=91). **(C)** Antibody negativity is observed 30 days post positive PCR tests. The time from the positive PCR was divided into four periods and the frequency of subjects present and absent of anti-RBD antibody are plotted. The number of subjects is listed in each period. **(D)** Association between antibody duration and older age. The number indicates the percentage of seropositive donors aged 40 or older in the indicated groups. **(E-F)** Relationship between gender (77 females and 31 males) and anti-RBD antibody level by days after positive COVID-19 PCR Subjects. **(E)** Total Ig are analyzed by the days post positive PCR tests. Exponential growth equation curves are shown. Test of homogeneity of regressions F=4.0. **(F)** IgG are analyzed by the days post positive PCR tests. Linear regression curves are shown. Test of homogeneity of regressions F=4.0.

The green dotted line indicates negative control background level. **P < 0.01, ****P < 0.0001.

IV: Discussion

By testing for the presence of SARS-CoV-2 antibodies in faculty, staff members, and students at MU, we were able to estimate seropositivity in the general university population. Increases in seropositivity aligned with major seasonal social events such as Halloween and Thanksgiving—the latter of which the Centers for Disease Control and Prevention (CDC) specifically warned college students about the risks posed (Quintana 2020). Further, through self-reports of prior PCR test dates and results, we documented antibody seropositivity ranging between 15 and 207 days following self-reported PCR positivity.

The virus RBD is the immunodominant epitope in both T and B cell responses to the virus. Importantly, antibodies against this domain can neutralize SARS-CoV-2 infection (Rogers et al., 2020; Suthar et al., 2020). Therefore, quantitative detection of antibodies to the RBD domain we provide here, are an indication of potential protection against the virus. It is important to note, while the index values as shown, have a strong relationship with neutralizing antibody titers (Siemens Healthineers 2020), PRNT-50 studies to equate antibody levels with neutralizing antibody titers are ongoing. Regardless, these assays allow us to assess and compare participants' antibody levels across different populations.

From the total of 241 blood donor participants who tested positive on total Ig or IgG or both assays, antibody levels were variable, spanning a roughly 200-fold range. This heterogeneity is plausible when considering the participants wide span in age (19-75 years old) and the fact that seropositivity was assessed from

2-weeks to up to 9-months after possible infection (the first case of COVID-19 disease at MU was reported in March 2020). Additionally, variation between individuals can be contributed to wide age range and often unknown interval between infection onset, PCR test and blood collection, especially in cases of asymptomatic infection (Li et al., 2020).

Biologically, IgM is produced first during viral infection and its level decreases after symptom resolution, while the production of IgG and IgA begin a few days after IgM and persists over time. Along these lines, several individuals had a much higher total Ig (IgM, IgA, IgG) when tested, suggesting they were early in their response to the virus. This result (Total Ig>IgG) was confirmed early in the immune response in several participants from a parallel ongoing longitudinal COVID response study at MU (to be reported elsewhere).

The magnitude of the antibody response against SARS-CoV-2 spike protein has been linked to the severity of the disease (Li et al., 2020). It has been reported that age is correlated with COVID-19 disease severity (Moderbacher et al., 2020) and that antibody levels significantly increased among those with severe symptoms and/or those who were hospitalized (Li et al., 2020; Prevost et al., 2020). Our results support this as both older age and reported severity was associated with higher antibody titer. Furthermore, the analysis of gender revealed that, within donors tested early in the infection (less than 30 days post-positive PCR test), older males produced greater antibody responses than age-matched females. This gender-related difference in male bias in COVID-19 severity and mortality has been reported elsewhere (Scully et al., 2020;

Takahashi et al., 2020), however the mechanism behind this phenomenon is still poorly understood.

There were 122 participants who self-reported a prior positive SARS-CoV-2 PCR test result. Among them, 8 subjects donated blood between 6-10 days post their PCR tests. 75% of them were seropositive and developed higher total Ig titer than IgG, parallel with early stage of infection (data not shown). Among the rest, 114 subjects who gave blood more than 2 weeks post-PCR testing, 10.5% of people did not have detectable anti-RBD antibodies, even several weeks after a positive PCR test. Our observation is in line with recent reports describing patients that do not seroconvert (rare) or show a decline in neutralizing antibodies after resolution of the infection, especially in cases of asymptotic and mild SARS-CoV-2 infections (Prevost et al., 2020; Long et al., 2020; Zhou et al., 2021). Again, we see similar frequencies of patients whose antibody levels drop to negative levels in our ongoing longitudinal study. Therefore, lower antibody levels suggest these individuals had a weaker immune response to SARS-CoV-2 infection, and therefore may be more susceptible to re-infection. Additional longitudinal studies and characterization of T and B cell memory are ongoing to identify causative factors responsible for these differences.

We observed that the lack of SARS-CoV-2 antibodies is associated with older age. Notably, our data showed that although males mounted stronger antibody responses shortly after infection, their antibody levels were lower, over time, than females. This finding is striking and raises concerns that males may be

more vulnerable to reinfection to SARS-CoV-2. A recent study reported that older individuals often exhibit uncoordinated adaptive immune responses which linked to disease severity (Moderbacher et al., 2020). The fact that, poor T cell responses negatively correlate with patients' age and has been linked to worse disease outcomes in male patients, but not in female patients suggests that B cell responses and severity of disease may not maintain any causal relation and the link to disease is rather related to the efficiency of T cell responses at the early phase of disease onset (Takahashi et al., 2020). Future longitudinal studies should consider gender differences in the immune responses to SARS-CoV-2 and our findings should be considered when analyzing potential herd immunity scenarios, including responses to vaccination.

Despite the large sample size, participation rates for middle-aged and older individuals were lower than those at younger ages. In addition, half of the seropositive group did not report a prior PCR test or date. Therefore, important data on the persistence of antibody responses in these individuals was not available for our analyses. Further, the cross-sectional study collected an individual's information only at one point in time even though we tracked MU seropositive rate for the campus community over time. Longitudinal data for individuals is required to better understand the kinetics and duration of antibodies. Lastly, the study only focused on anti-RBD antibodies in plasma. Additional characterization of the sample with antibodies against other SARS-CoV-2 antigens as well as T and B cell responses will facilitate a more complete evaluation of the relationship between antibody responses and other factors.

While the majority of participants, in the Fall 2020 semester, did not have detectable SARS-Cov-2 antibodies, seroprevalence still varied widely by age. Even though seropositivity was higher among younger participants—parallel to the rest of the world—data revealed that various sectors of the campus community had been exposed at an institution where numerous safeguards, plans, and communications were designed to mitigate the spread of COVID-19. Based on the increased seropositivity near seasonal holidays noted in this study, it seems prudent to expand education and messaging on risk mitigation strategies at such times in the face of a disease pandemic.

The fact that 10.5% of participants were seronegative despite reporting testing positive for the SARS-CoV-2 infection, with some being seronegative as early as two months post-positive PCR test, strongly suggests that prior positive PCR test results should not be considered alone as a strong indication of protective immunity. These data also portend the importance of analyzing T and B cell memory in these patients in a longitudinal fashion and highlight the need to analyze these parameters in vaccinated individuals.

V: Materials and Methods

Context

MU held in-person classes and had students living on-campus at the start of the Fall 2020 semester (mid-August). A number of restrictions were put into place over the course of the semester such as, limiting class sizes, adjusting room capacity to accommodate social distancing, and mandatory use of face

coverings or masks. MU moved most classes online after Thanksgiving break (the week of November 23) to reduce the number of students on campus, and, particularly, to decrease the number of students who would travel to and from campus. Students who needed to stay in on-campus housing were allowed to do so, and a number of health profession and other lab-intensive programs continued in-person while utilizing virus transmission mitigation strategies. Other than a program for MU's inter-collegiate student-athletes, there was no mandate for an on-campus COVID-19 testing program in Fall 2020.

Study design and sampling

We began enrolling members of the MU Community—faculty, staff members, and students—in mid-September 2020 into the MU Study of Seropositivity and Risk for SARS-CoV-2 and COVID-19. The study included a blood draw and serological analysis, as well as an online behavioral risk and perceptions survey (data reported elsewhere), for eligible individuals. The study was performed under a protocol (#2028427) approved by the Institutional Review Board of MU. Originally, study enrollment was by e-mail invitation through stratified random sampling: each batch of invitations to participate included a random sample of 950 undergraduates, 250 graduate/professional students, and 400 faculty/staff, for a total of 1,600 individuals. The first three sets of invitations were sent on September 15, September 25, and October 5, but due to low response rates, 2 sets of 1,600 invitations were sent weekly (1,200 students and 4,00 faculty/staff) starting the week of October 12. Due to significant interest

among the MU community, in mid-October, we received ethical approvals to allow individuals to self-enroll in the study. All data were collected, and stored, in REDCap. Collection of blood samples ended on December 11, 2020 (the last day of classes for the Fall semester), the survey remained open through the end of December.

For our analyses, we draw upon 2,202 individuals who volunteered their blood for SARS-CoV-2 antibody testing and received a positive or negative antibody test report. Self-enrolled participants had different demographics from the randomly selected participants in these categories, but, most importantly, did not differ in terms of antibody results.

Blood sample processing

A total of 10-20ml of blood was collected from each participant. The whole blood was run on a single-tube Advia 560 Hematology System (Siemens) for complete blood cell counts, including: white blood cell counts and percentages, red blood cell counts and characteristics, and hemoglobin levels and characteristics. The plasma was then separated from the blood cells by centrifugation and subsequently stored overnight at 4°C, before testing for anti-SARS-CoV-2 S1 RBD antibodies.

Detection of SARS-CoV2 antibodies

All serological tests were performed on Siemens Dimension@ EXL™ integrated chemistry system. Siemens Healthineers SARS-CoV-2 Total Antibody

(CV2T) and SARS-CoV-2 IgG (CV2G) assays, that target the S1 receptor binding domain (RBD) of the spike protein, were used to measure antibodies in each sample. CV2T is a LOCI immunoassay for semiquantitative detection of total antibodies (including IgG, IgA and IgM) against SARS-CoV-2 in human serum/plasma with 100% sensitivity and 99.8% specificity after 14 days from symptom onset. A specimen was considered positive at a cutoff analyte value in units of 1000 or greater without serum dilution. The unit numbers were reported for each specimen and the negative antibody Quality Control result was consistently below 70 units. CV2G assay is also a chemiluminescent immunoassay for semiquantitative detection of IgG antibodies to SARS-CoV-2 with 100% sensitivity and 100% specificity after 14 days from symptom onset. The assay cutoff analyte value is 1000 Ind Units and is used to distinguish between positive and negative. The assay also reports a numeric value in Ind units, and the negative antibody Quality Control result was below 600 Ind units. A specimen was considered positive when either or both assays had a cutoff value of 1000 or greater. All assays were granted Emergency Use Authorization by the US FDA and used according to the manufacturer's instructions.

Statistical analyses

For cross-sectional analyses and descriptive purposes only, simple linear regression, one-phase decay, and exponential growth equations were performed, for unpaired comparisons, Mann-Whitney tests were performed (via GraphPad Prism 9.0). Best curve fit was defined by comparing R^2 values and an extra F-

test, selecting the simpler model unless $p<0.05$. For gender curves, slopes and intercepts were compared for linear regression, and comparison of one curve fit all data set was done with non-linear curves. Details and significance are noted in figures, where appropriate.

VI: Acknowledgments

YG, TM, AE, MD, ES, EM, SW, MT, LAA, AG, JH, JM, MM, ASMM, and ID conceptualized and designed the study. YG, TM, AE, ID, MD, ES, and JM acquired, analyzed, and interpreted the data. YG, TM, AE, MD, and ES drafted the manuscript. YG, TM, AE, MD, ES, ET, LAA, AS, JM, JS, and SW reviewed and revised the manuscript. YG, TM, and AE statistically analyzed the data. JM, MM, ASMM, MP, JS, MT, PB, ID, SE, KL, MBS, SNW, and SW provided administrative, technical, and/or material support. This work was supported by the University of Missouri CARES funding and Siemens Healthcare Diagnostics Inc, which also supplied sample analysis platforms and antibody detection reagents. We could not have successfully collected these data without the support of the University of Missouri Student Information Services and Human Resources to supply randomly selected email lists (Chase Hickman, Allen Johanning, Jeremy Wiebold), IT staff who helped with appointment and website development (Lei Jiang), the team of consenters (Brady Chung, Emma Foust, Emily Perisho, Kamilla Sarvestani, Peyton Kusgen, Megan Polniak, Sarah Simmons, Tess Willemse), the clinical staff (Allison Thomas, Collin Welch, Ella Bochenski, Madison Enright, Madison Hassler, Tess Goldenberger), our Student

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	Antibody (Ab) Status			
Parameter (Units)	Reference Range	Ab Positive (±SD)	Ab Negative (±SD)	*P* value
WBC Characteristics				
WBC Count (x10^3/µL)	5.00 - 10.00	6.55 (1.76)	6.58 (1.89)	0.8613
Neu Count (x10^3/µL)	2.00 - 7.50	3.99 (1.45)	3.97 (1.48)	0.5889
Lym Count (x10^3/µL)	1.30 - 4.00	1.98 (0.57)	1.99 (0.62)	0.3662
Mon Count (x10^3/µL)	0.15 - 0.70	0.35 (0.12)	0.36 (0.14)	0.2905
Eos Count (x10^3/µL)	0.00 - 0.50	0.16 (0.12)	0.18 (0.15)	**0.0183**
Bas Count (x10^3/µL)	0.00 - 0.15	0.07 (0.03)	0.07 (0.04)	0.1446
Neu %	40.0 - 75.0	59.97 (8.37)	59.17 (9.10)	0.0606
Lym %	21.0 - 40.0	30.96 (7.36)	31.05 (7.57)	0.4277
Mon %	3.0 - 7.0	5.53 (1.88)	5.66 (2.11)	0.3406
Eos %	0.0 - 5.0	2.46 (1.86)	2.76 (1.99)	**0.0186**
Bas %	0.0 - 1.5	1.07 (0.51)	1.10 (0.54)	0.2218
RBC/HGB Characteristics				
RBC Count (x10^6/µL)	4.00 - 5.50	4.92 (0.51)	4.87 (0.52)	0.1209
HGB (g/dL)	12.0 - 17.4	14.5 (1.36)	14.32 (1.64)	0.3064
HCT (% total blood)	36.0 - 52.0	43.27 (4.62)	42.95 (4.88)	0.3338
MCV (fL)	76.0 - 96.0	88.63 (4.67)	88.54 (6.15)	0.7850
MCH (pg)	27.0 - 32.0	29.52 (2.03)	29.37 (2.64)	0.8805
MCHC (g/dL)	30.0 - 35.0	33.15 (2.33)	32.98 (2.87)	0.8359
RDW SD (fL)	46.0 - 59.0	37.39 (3.74)	37.29 (4.86)	0.8277
RDW CV (%)	0.0 - 16.0	14.21 (1.27)	14.08 (1.41)	0.2692

Table S4.1: Hematologic characteristics of first donation antibody (Ab) positive and negative persons at the University of Missouri

Chapter 5

Non-randomized trial of dornase alfa for acute respiratory distress syndrome secondary to COVID-19

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I: Abstract

The most severe cases of Coronavirus-Disease-2019 (COVID-19) develop into acute respiratory distress syndrome (ARDS). It has been proposed that oxygenation may be inhibited by extracellular deoxyribonucleic acid (DNA) in the form of neutrophil extracellular traps (NETs). Dornase alfa (Pulmozyme, Genentech) is recombinant human deoxyribonuclease I that acts as a mucolytic by cleaving and degrading extracellular DNA. We performed a pilot study to evaluate the effects of dornase alfa in patients with ARDS secondary to COVID-19. We performed a pilot, non-randomized, case-controlled clinical trial of inhaled dornase for patients who developed ARDS secondary to COVID-19 pneumonia. Improvement in arterial oxygen saturation to inhaled fraction of oxygen ratio (PaO2/FiO2) was noted in the treatment group compared to control at day 2 (95% CI, 2.96 to 95.66, P-value = 0.038), as well as in static lung compliance at days 3 through 5 (95% CI, 4.8 to 19.1 mL/cmH2O, 2.7 to 16.5 mL/cmH2O, and 5.3 to 19.2 mL/cmH2O, respectively). These effects were not sustained at 14 days. A reduction in bronchoalveolar lavage fluid (BALF) myeloperoxidase-DNA (DNA : MPO) complexes (95% CI, -14.7 to -1.32, P-value = 0.01) was observed after therapy with dornase alfa. Treatment with dornase alfa was associated with improved oxygenation and decreased DNA : MPO complexes in BALF. The positive effects, however, were limited to the time of drug delivery. These data suggest that degradation of extracellular DNA associated with NETs or other structures by inhaled dornase alfa can be beneficial. We propose a more extensive clinical trial is warranted.

II: Introduction

Health care systems across the world are being inundated with patients who are critically ill due to infection with Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) causing Coronavirus Disease 2019 (COVID-19). Around 10% of those infected will develop the most severe manifestation of the disease requiring admission to an intensive care unit (Emanuel et al., 2020). Early reports suggested neutrophil extracellular traps (NETs) as being a potential contributor to the severity of disease in some patients with COVID-19 (Earhart et al., 2020). As originally described, NETs were proposed to ensnare and potentially kill invading microbes, but it is now clear that these extracellular DNA-protein complexes are almost always deleterious and contribute to tissue destruction and pathogenesis in many diseases (Brinkmann et al., 2004; DeLeo & Allen 2020). In severe cases of COVID-19, NETs appear to cause significant morbidity in the lungs with associated microthrombi formation, endothelial damage, capillaritis, neutrophilic mucositis and mucus accumulation (Earhart et al., 2020; Barnes et al., 2020; Schönrich & Raftery 2016; Kambas et al., 2014). Plasma levels of NETs are increased in patients requiring intubation and are inversely correlated with arterial blood oxygen content to fraction of inspired oxygen ratio (PaO2/FiO2) (Middleton et al., 2020). Dornase alfa (recombinant human DNase I) is currently used in patients with Cystic Fibrosis and reduces mucus viscosity by degrading extracellular DNA in the airways (Shak et al., 1990; Ramsey et al., 1993). We proposed a trial using inhaled dornase alfa as a therapeutic target to reduce extracellular DNA and NETs in patients with acute

respiratory distress syndrome (ARDS) secondary to COVID-19 pneumonia, with outcome aims including improved lung compliance and gas exchange (Wang, Doerschuk & Mizgerd 2004).

III: Results

A. Dornase alfa initially enhances lung oxygenation and compliance in COVID-19 patients with ARDS

A total of 10 dornase alfa-treated patients were included in the pilot trial and all 10 were included in the final analysis. As the trial was not established as a randomized trial, a total of 20 case control patients were also included in the final analysis (Fig. 5.1). Recruitment for the study was performed from June 19, 2020 to December 1, 2020. A significant improvement in change of PaO2/FiO2 from baseline was noted in the treatment group compared to change of PaO2/FiO2 from baseline in case-control subjects at day 2 (Fig. 5.2A; Difference between means 95% CI, 2.96 to 95.66, P-value = 0.038). The significant difference in measured PaO2/FiO2 was not maintained after drug treatment completion with measurements obtained out to 14 days on mechanical ventilation. Improvement was also noted in static lung compliance at days 3 through 5, which again was not sustained durably beyond the drug treatment period out to 14 days (Fig. 5.2B; Difference between means 95% CI, 4.8 to 19.1, 2.7 to 16.5, and 5.3 to 19.2 mL/cmH2O respectively). Secondary outcomes including adverse events did not show statistically significant

differences between treatment and case-control groups assessed up to 90 days (Table 5.1).

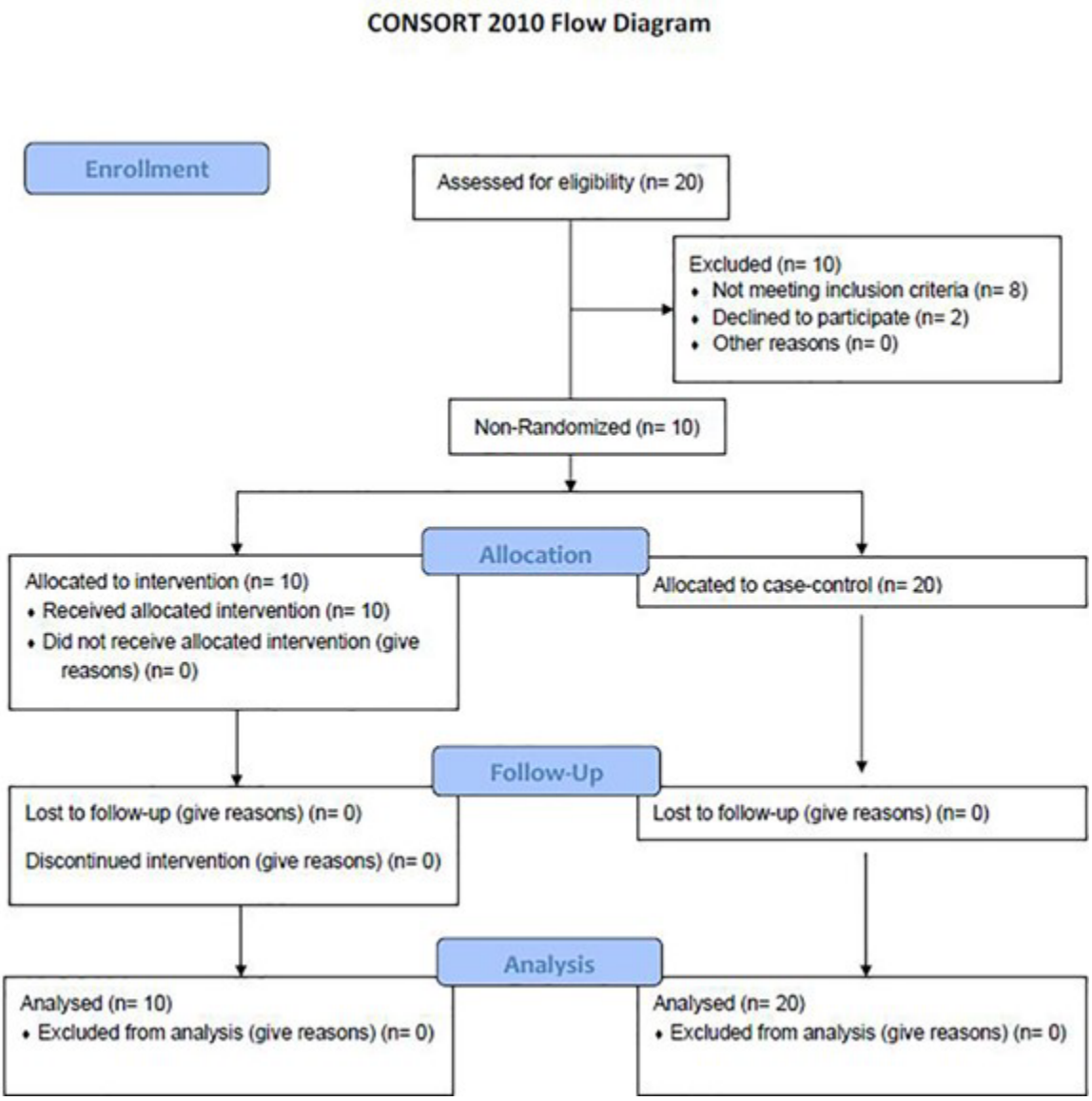

Figure 5.1: Consort flow diagram. A total of 20 patients were evaluated for the study with 8 patients not meeting inclusion criteria and 2 declining to participate. 10 patients were included in the treatment arm with no patients lost to follow up. 20 patients were randomly selected to be a part of the case-control arm (Schulz, Altman & Moher 2010).

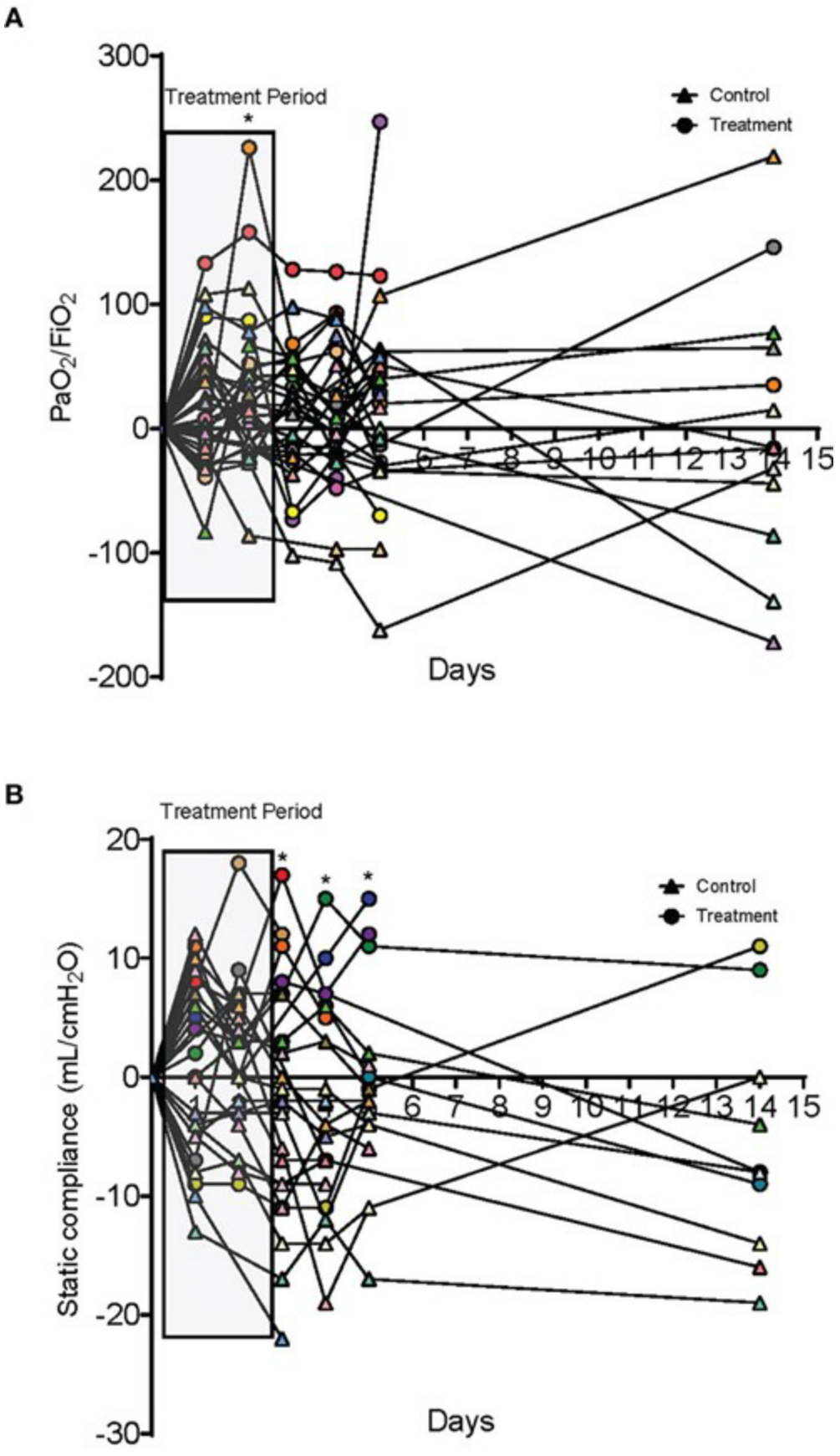
A
300
200
100
0
-100
-200
Treatment Period
*
Control
Treatment
PaO2/FiO2
6 7 8 9 10 11 12 13 14 15
Days
B
Treatment Period
20
10
0
-10
-20
-30
*
*
*
Control
Treatment
Static compliance (mL/cmH2O)
1
6 7 8 9 10 11 12 13 14 15
Days

Figure 5.2: Oxygenation and compliance responses to dornase alfa treatment. (A) Change in PaO_2/FiO_2 compared to day 0. Each colored circle symbol represents a unique patient in the treatment group and each colored triangle symbol represents a unique patient in the control group. A significant increase is noted in the treatment group at day 2 of treatment with a non-statistically significant increase for the additional 14 days. **(B)** Change in static lung compliance (mL/cmH_2O) compared to day 0. Each colored circle symbol represents a unique patient in the treatment group and each colored triangle symbol represents a unique patient in the control group. Starting on day 3 there was a significant improvement in lung compliance in the treatment group compared to the case control group that was sustained to day 5. No statistically significant difference was noted at 14 days.

Primary and Secondary Outcomes

	Control (N = 20)	Dornase alfa (N = 10)	P-Value
Primary Outcomes			
$\Delta PaO_2/FiO_2$ from Day 0 (95% CI)			
Day 1	-0.8 to 43.6	-29.7 to 51.1	0.59
Day 2	-9.6 to 33.1	5.2 to 117	**0.04**
Day 3	-9.7 to 34.7	-21 to 68	0.59
Day 4	-24.8 to 31.7	-21.8 to 70.1	0.38
Day 5	-28.8 to 38.8	-35.9 to 111	0.32
Day 14	-92.6 to 70.2	-150.8 to 260.8	0.38
Secondary Outcomes			
Δ Lung compliance from Day 0 (95% CI)			
Day 1 (mL/cmH_2O)	-4.2 to 3.9	-1.7 to 7.9	0.28
Day 2	-3.1 to 2.3	-1 to 9	0.08
Day 3	-9.8 to -1.6	-0.8 to 13.3	**<0.01**
Day 4	-9.4 to -1.8	-3.2 to 11.3	**<0.01**
Day 5	-8.4 to -1.3	-1.8 to 16.6	**<0.01**
Day 14	-17.9 to -2.4	-16.3 to 17.8	0.09
Δ PEEP from Day 0 (95% CI)			
Day 1 (cmH_2O)	-0.7 to 1.7	-0.8 to 1.2	0.74
Day 2	-0.8 to 1.7	-1.7 to 0.9	0.34
Day 3	-1.1 to 2.4	-3.1 to 1.3	0.27
Day 4	-1.6 to 2.2	-4.5 to 1.6	0.28
Day 5	-1.4 to 3.1	-5.5 to 2	0.19
Days on Mechanical Ventilation*	18.2 (8-38)	15.2 (5-29)	0.47
Days in ICU*	22.1 (11-47)	16.5 (7-30)	0.23
Days of Hospitalization*	28.7 (15-60)	22.5 (8-52)	0.61
Secondary Pulmonary Infections**	5 (25%)	3 (30%)	0.78
Mortality, 28-day**	9 (45%)	4 (40%)	0.8
Mortality, 90-day**	11 (55%)	4 (40%)	0.46

*(mean, min to max) **(total and % of patient population). ICU, Intensive Care Unit. Significant P-values are in bold.*

Table 5.1: Primary and Secondary Outcomes of patients in case-control group (N=20) and Dornase alfa treatment group (N=10).

B. Lung neutrophil counts are unchanged, but lung NETs are decreased after dornase alfa treatment

BALF and blood samples obtained in the treatment group before and after therapy were analyzed for cell differential and accumulation of MPO-DNA complexes. Eleven patients in the control group also underwent diagnostic bronchoscopy and samples in the treatment group were also compared to this control group. There was no difference in neutrophil counts or percentages between the before treatment samples and the control group (Fig. 5.3). Despite the non-significant increase in neutrophil percentage and absolute log10 neutrophil count (Fig. 5.3), there was a significant reduction in BALF NETs measured as MPO-DNA complexes (Difference between means 95% CI, -14.7 to -1.32, P-value = 0.01) (Fig. 5.4A) after therapy with inhaled dornase alfa. There was no difference in the serum MPO-DNA activity before or after therapy, nor in NETs that were experimentally induced from neutrophils isolated from patient blood that were treated with PMA ex vivo (Fig. 5.4B,C).

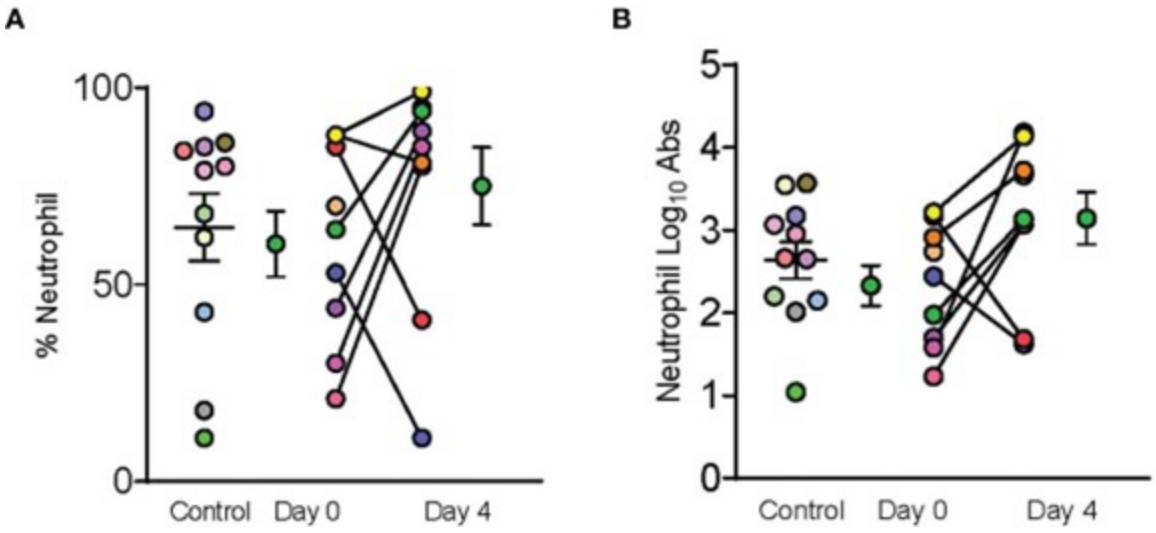

Figure 5.3: Dornase alfa treatment did not reduce BALF neutrophils.

Control, day 0 and day 4 symbols are displayed as mean ± SEM of 11 subjects in the control group and 9 subjects in the treatment group. Each individual color-coded point with connecting line between day 0 and 4 represents a unique patient in the treatment group (N=9). Two patients only had samples from a single time point due to sample degradation. Only 11 subjects in the control group underwent BALF sampling during hospitalization and are represented by individual colored coded data points. No statistically significant difference was noted in **(A)** % Neutrophils, or **(B)** log transformed absolute neutrophil counts in the bronchoalveolar fluid between case control patients and patients prior to receiving dornase alfa.

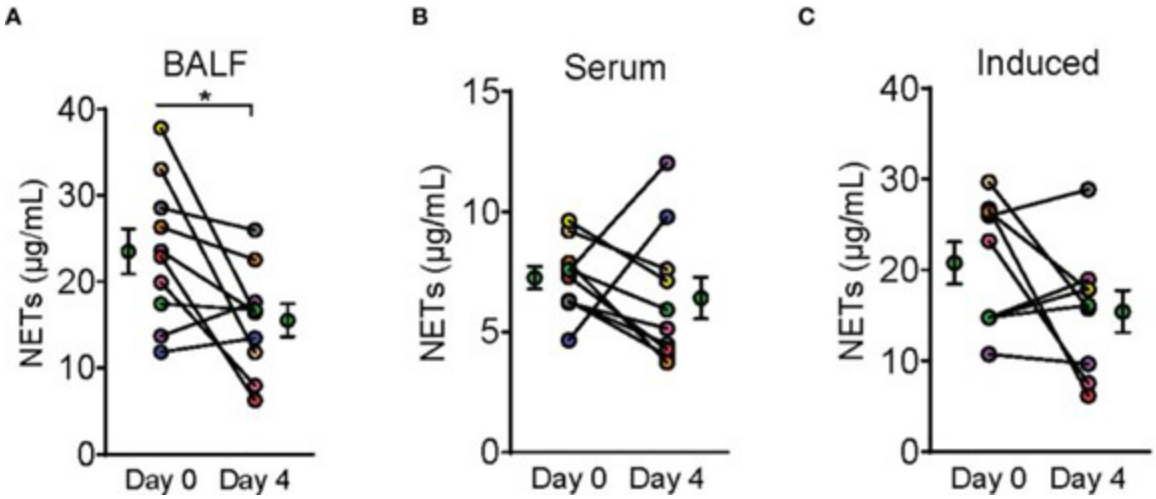

Figure 5.4: Dornase alfa treatment reduced BALF NETs. Symbols on Day 0 and Day 4 are represented as mean ± SEM of 10 subjects in the bronchoalveolar fluid (BALF) analysis and serum neutrophil extracellular traps (NETs) analysis and 9 subjects in the induced NETs analysis. Due to not having the appropriate material prior to sample collection from the first enrolled patient, we were only able to perform induced NET analysis on 9 subjects **(C)**. Each connecting line with points at Day 0 and 4 represents a unique patient sample and is patient colored coded to previous graphs (N=10). NETs defined by NET MPO : DNA complex measurement was significantly reduced in the BALF after 3 days of therapy with dornase alfa **(A)**. There was no significant change in serum NETs or NETs induced *in vitro* from patient neutrophils harvested after dornase alfa therapy **(B,C)** respectively.

IV: Discussion

Neutrophils play an important role in the innate immune response in the lungs and have been shown to be higher in BALF in patients with COVID-19, particularly those with increased disease severity (Wang, Doerschuk & Mizgerd 2004; Zhou et al., 2020). An associated increase in neutrophil chemoattractants have also been observed (Middleton et al., 2020). Neutrophils can release complexes of chromosomal DNA, histones and granule enzymes, such as MPO, as NETs trapping microorganisms, but also causing collateral damage in the form of thrombosis and lung injury (Zhou et al., 2020; Frantzeskaki, Armaganidis & Orfanos 2017). Earlier studies noted an increase in circulating markers for NETs in patients with COVID-19 compared to healthy controls, as well as autopsy findings have confirmed the presence of NETs in the lungs (Barnes et al., 2020; Zuo et al., 2020). The concept of NET-driven lung injury is not unique to COVID-19. Targeting NETs in ARDS has been applied to other causes of pneumonia as the level of NETs has been associated with disease severity (Ebrahimi et al., 2018; Bendib et al., 2019).

Our group began by applying dornase alfa to one severe COVID-19 patient who subsequently showed improvement in oxygenation after treatment (Earhart et al., 2020). Therefore, we designed this initial clinical trial with PaO2/FiO2 improvement as the primary outcome objective. We predicted that the inhaled drug would decrease extracellular DNA and NETs in the lung and improve oxygenation. Currently, the data appear to support both predictions. There was a decrease in MPO-DNA complexes, which suggests a decrease in

NETs (Figs. 5.2–5.4). This result makes sense given the accumulation of neutrophils in the lungs in response to infection, with lytic death and DNA release having deleterious effects. At the same time, the ability of dornase alfa to degrade DNA and improve oxygenation wanes rapidly upon drug withdrawal. A more durable effects of decreased magnitude may be possible to determine upon increasing patient N in a more extensive trial. One concern was the potential negative effect of degrading NETs whose function is in part to fight off invading pathogens. There was no observed significant increase in secondary pulmonary infections. We conclude that the premise of applying dornase alfa to degrade DNA in NETs and improve oxygenation in severe COVID-19 induced ARDS appears sound and should be further explored in a more extensive clinical trial. We also propose that the time of drug administration be extended, predicting that a greater duration of beneficial effects may thereby be induced, with the goal of decreasing morbidity and mortality rates in these patients. There is currently a phase 2 open-label, randomized, Best-Available-Care (BAC) and historic-controlled trial ongoing at University College London Hospital looking into the effects of inhaled dornase alfa over 7 days on the outcome of patients admitted to the hospital who are at risk of ventilatory failure (COVASE 2020).

Our study is limited by being a single center study and a small sample size which does not allow for power to determine many of the secondary outcomes. If plausible, future studies should aim to include multi-center patient data sets and a larger patient population. Our population was also predominantly white, non-Hispanic and may underrepresent the effect on other ethnic groups. Standard of

care for severe COVID-19 patients includes treatment with systemic corticosteroids, which has been reported to reduce systemic NET activity (Ebrahimi et al., 2018). The limited serological change in MPO-DNA activity may have been affected by this therapy. Another limitation is that MPO-DNA complexes in patient samples were discussed as known NET products, but the mechanism of their generation was not determined.

Severe COVID-19 pneumonia leading to ARDS is associated with increased extracellular MPO-DNA complexes, potentially as NETs, in the alveolar space. Inhaled dornase alfa, via degradation of alveolar DNA, significantly improved oxygenation and lung compliance. Due to a persistent inflammatory state of the lungs in COVID-19 and ARDS, the effect is short lived after drug removal. Results of our pilot study warrant further consideration of a larger randomized trial in patients with ARDS secondary to COVID-19, as well as other causes of ARDS.

V: Materials and Methods

Study design

A single center, non-randomized, controlled before-and-after clinical study was designed to examine the effects of inhaled dornase alfa in patients with ARDS secondary to COVID-19 pneumonia. The Institutional Review Board at the University of Missouri approved the clinical trial including collection of clinical data and clinical samples from participating patients (trial #2022206) and case control clinical data from patients with COVID-19 infection with exemption of

informed consent from each patient due to the case control portion of the study involving only information collection and analysis (#2025101). A nonrandomized model was used to determine if an effect could be noticed to justify a larger randomized controlled study. Patients were recruited from the medical intensive care unit at the University of Missouri, a 250-bed academic tertiary care medical center. Recruitment was considered after confirmation of SARS-CoV-2 infection induced ARDS and progression of care requiring mechanical ventilation. Inclusion criteria included age ≥ 18 years, hospitalized, and mechanically ventilated for illness related to SARS-CoV-2 infection, with individual or surrogate ability to sign informed consent, and negative urine-based pregnancy test in female patients. Exclusion criteria included contraindication or intolerance to dornase alfa, length of mechanical ventilation expected to be less than 48 hours, life expectancy less than 24 hours based upon judgment of treatment physician, pregnancy, or inability to obtain informed consent. Patients in the treatment group received nebulized dornase alfa (2.5mL) via vibrating mesh through the ventilator circuit twice daily for three days after enrollment. Control group patients received standard of care for severe COVID-19 pneumonia and ARDS. No placebo group was included in this pilot study. The primary outcome measure was change in arterial oxygen saturation to inhaled fraction of oxygen (PaO2/FiO2) compared to the day of enrollment. Secondary outcomes included change in static lung compliance (Tidal Volume (mL) Plateau Pressure−PEEP) compared to the day of enrollment, duration of mechanical ventilation, length of ICU stay, length of hospitalization, secondary bacterial infections, and mortality. PaO2/FiO2 and

static lung compliance measurements were all obtained when patients were supine for at least 2 hours prior to measurement. Sample size was determined based upon ability to calculate significance of the primary outcome. Interim analysis was performed on every 3 patients to evaluate for trends in outcomes and consideration of trial continuation.

Data collection

Demographic and clinical data of the patients was obtained from electronic medical records at enrollment. Clinical study information collected included age, sex, co-morbidities, therapies received, serological testing, ventilator data, bacterial and viral culture data, days of hospitalization, days in the intensive care unit, days of mechanical ventilation, and mortality.

Sample acquisition and processing

An optional part of the trial consisted of collecting patient samples including blood and bronchoalveolar lavage fluid (BALF) before and after receiving therapy with dornase alfa to compare frequency of neutrophils and NET MPO-DNA complex concentrations. BALF was either obtained from any unused fluid collected as part of routine clinical care, or if approved by the patient, additional research samples were obtained. BALF cell count and differential were all performed by the clinical laboratory with frequency of neutrophils being expressed by percent of total white blood cells per mcL and absolute cell counts per mcL. BALF used for detection of NET MPO-DNA complex concentrations were processed as follows. Mucus in

BALF samples was manually broken up by gentle pipetting, followed by straining through a cell strainer, then split into 1 mL aliquots and immediately frozen at -80°C. Blood to be discarded after clinical laboratory analysis was also obtained. Approximately 1 mL blood was centrifuged at 150 g for 10 minutes at room temperature, followed by collection and freezing of the plasma at -80°C until further use. Meanwhile, neutrophils from 1 mL blood were isolated using the EasySep Human Neutrophil Enrichment Kit (Stemcell Technologies) per manufacturer instructions and diluted to 1x106 cells/mL in RPMI +5% FBS. Isolated neutrophils from the blood were then seeded at 5x105 cells/ well of a 12 well plate (Corning), allowed to adhere for 30 minutes, followed by NET induction via stimulation with 10 µg/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 4 hours at 37°C. NETs were then detached from adherent cells by gentle pipetting, centrifuged at 150 g for 20 minutes at room temperature to separate NETs from cells and debris, and the supernatant was immediately frozen at -80°C. Control NETs from a healthy volunteer were prepared and stored in this same manner.

NET MPO-DNA complex detection

To detect the presence of NET MPO-DNA complexes, 96-well plates (Corning) were coated with 4 µg/mL of anti-MPO monoclonal antibody (clone 4A4, Bio-Rad) in a sodium carbonate/bicarbonate buffer (pH 9.6), incubated at 4°C overnight, washed with PBS, and subsequently blocked with 1% bovine serum albumin in PBS for 30 minutes at room temperature. Thawed BALF, serum, and PMA-neutrophil-induced NET samples were immediately added after washing,

followed by incubation for 90 minutes at room temperature. After washing, a 1:100 dilution of anti-DNA-POD antibody (clone MCA-33, Roche) was added to each well and incubated again for 90 minutes at room temperature. The amount of single or double-stranded DNA bound to MPO was then detected by adding 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate], or ABTS substrate (Roche) and incubated for 15 minutes at room temperature on a plate shaker at 250 rpm. Plates were then read on an EPOCH microplate reader (Bio-Tek) by measuring optical density at 405 nm wavelength. MPO-DNA complexes were quantified by comparing against a standard curve of known DNA concentrations (in µg/mL) as determined by spectrophotometry from healthy volunteer control NETs prepared as described above and reported as micrograms/milliliter.

Statistical analyses

Statistical tests and graphs were made using GraphPad Prism software. Data are presented as difference between means 95% confidence intervals. Statistical difference was assessed by two-tailed, paired or unpaired, t-test. Statistical significance for the study was defined as P-value ≤ 0.05.

Patient demographics

Baseline demographics and clinical characteristics of the treatment group and case control group are depicted in Table 5.2. There was no significant difference noted between the two groups regarding co-morbidities and common therapies received for COVID-19.

Demographics	Case-Control (N=20)	Dornase alfa (N=10)	P-Value
Age (years)*	60 (24-84)	63 (47-79)	0.65
Gender**			
Male	13 (65%)	6 (60%)	0.8
Female	7 (35%)	4 (40%)	0.8
Ethnicity**			
White, non-hispanic	16 (80%)	8 (80%)	>0.999
African American	1 (5%)	0 (0%)	0.49
Hispanic/Latino	3 (15%)	2 (20%)	0.74
Co-Morbidities**			
Diabetes	11 (55%)	7 (70%)	0.45
Hypertension	14 (70%)	9 (90%)	0.24
Coronary artery Disease	7 (35%)	5 (50%)	0.45
Chronic lung disease (COPD, asthma, ILD)	7 (35%)	3 (30%)	0.79
Obesity	17 (85%)	8 (80%)	0.79
Therapies Received**			
Remdesivir	20 (100%)	9 (90%)	0.16
Corticosteroids	20 (100%)	9 (90%)	0.16
Antibiotics	19 (95%)	10 (100%)	0.49
Convalescent Plasma	11 (55%)	9 (90%)	0.06
Anticoagulation	10 (50%)	4 (40%)	0.62
Paralytics	19 (95%)	8 (80%)	0.21
Prone positioning	16 (80%)	8 (80%)	>0.999

*(mean, min to max) **(total and % of patient population). COPD, Chronic Obstructive Pulmonary Disease; ILD, Interstitial Lung Disease.*

Table 5.2: Demographics and concurrent medical therapies of patients in the case-control group (N=20) and Dornase alfa treatment group (N=10).

VI: Acknowledgments

ZH, AE, and AS conceptualized the study. ZH, AK, AS, AE, and MA collected and analyzed the data. ZH, AE, and AS wrote the manuscript. AS, AE, AK, L-AA, and MA reviewed/edited the manuscript. All authors contributed to the article and approved the submitted version. Funding was provided by the University of Missouri Division of Pulmonary and Critical Care Research Fund.

Chapter 6

Discussion

I. Conclusions

In these works, we have observed that sex exerts clear differential effects on innate immune memory, or trained immunity, by way of the female sex hormone progesterone (P4) acting on its cognate nuclear receptor (PR); and the production of anti-SARS-CoV-2 antibodies after natural infection with the earliest circulating COVID-19 viral variants. We also proposed, and found, together with other groups, that PMNs, the most numerous of immune cells, play a prominent role in the immunopathology of COVID-19 disease. These observations are in concordance with other findings showing that P4 attenuates trained immunity; men create more against the SARS-CoV-2 virus; and that increased neutrophil counts and activity negatively impact survival of COVID-19. It is important to note that COVID-19 outcomes during the pandemic showed a sex bias in that men fatally succumbed to disease more than women.

Such findings suggest that sex directly influences the severity of disease, albeit in potentially different ways through similar mechanisms. Progesterone appears to attenuate trained immunity of both circulating human and bone marrow-derived mouse innate cells and, as such, can weaken protection against systemic bacterial opportunistic infection. Interestingly, this is seemingly in contrast to the disease severity and mortality seen in COVID-19, as men generally exhibited stronger innate immune responses sometimes leading to

severe pneumonia and hypercytokinemia resulting mostly in respiratory distress and failure, and also multiple organ failure in certain cases, leading to death. In contrast, as men in our studies produced more anti-SARS-CoV-2 antibodies yet worldwide were more susceptible to death from the virus, it appears that something about this antibody production may not have been sufficient to protect the host. These differences highlight the effects sex exerts on immune function, responses to bacterial and viral infections, and also between innate and adaptive immunity.

A. Progesterone and trained immunity

An unintentional bacterial infection spread amongst adaptive immune-deficient mice in our lab, with the causative agent later identified as the near-ubiquitous soil bacterium *Bukrholderia gladioli*. This bacterial species is known to be an emergent pathogen causing life-threatening infections in cystic fibrosis and immune-deficient individuals. Interestingly, approximately 90% of fatalities were females from our infected mouse population. Because these mice were adaptive immune-deficient, this led us to investigate whether training of innate cells exhibited a sex bias in subsequent responses and during an opportunistic infection. We utilized a unique approach to address these questions, supplementing our cell media with female or male mouse serum with sex-matched or sex-switched bone marrow cells. Importantly, bone marrow-resident precursors readily express all three primary sex hormone nuclear receptors. As such,

we found significantly increased output of the pro-inflammatory mediators CXCL2 and IL-6 in both female and male bone marrow cells trained using β-glucan in male serum media, while female serum supplemented media showed relatively decreased responsiveness, suggesting that some soluble factor in female serum attenuates training. We further showed that female serum from each estrous cycle stage influenced training, especially from the metestrus and diestrus phases which reduced responsiveness and correlated with higher progesterone levels. Serum from each of these stages had variable levels of E2 and P4, with the highest P4 concentration in the diestrus stage correlating with the lowest output of CXCL2 and IL-6. These findings were strongly suggestive that P4 directly attenuates the trained immune response; exposure of bone marrow cells to exogenous P4 recapitulated these findings. This also significantly reduced glycolytic activity, indicating this training attenuation effect correlates with an effect on underlying metabolic functions. Meanwhile, antagonism of the nuclear PR restored these responses and glycolytic activity, while also rescuing trained female mice from *B. gladioli* infection, yielding similar survival to that of males.

Given these findings, we conclude that P4 attenuates trained immunity responses via engagement of its cognate nuclear hormone receptor. Though we did not examine the epigenetic modifications known to underlie trained secondary responses, we can further conclude that P4, acting on the nuclear PR, negatively augments the metabolic switch to

glycolysis. It is likely that this is at least partly responsible for the attenuation in trained activity, as this switch plays a vital role in chromosomal remodeling and previous studies have shown that blocking glycolysis prevents the induction of training. Since antagonizing the PR during training in female mice significantly enhanced survival, we may also conclude that this P4/PR-dependent training effect remains intact in living subjects.

So far, few studies have focused on the role of sex in trained immunity, while those that have, found some contrasting results. One study focused on how P4 affects training. There, the authors Groh et al. found that P4 attenuated trained responses by way of IL-6 and TNFa output after secondary stimulation in vitro, although they targeted this effect to P4 acting on the glucocorticoid receptor (GR) and not the nuclear PR. Yet, their focus was on adult human circulating monocytes, which may not express the PR. Human and mouse bone marrow cells, however, do indeed express this receptor and are affected by changes in circulating P4 levels. Our focus here on mouse bone marrow cells is likely to explain why we were able to target the nuclear PR to ameliorate the attenuation of training induced by P4, yet Groh et al. were not but did find the GR attenuated training via P4 in adult circulating monocytes. Interestingly, both the GR and nuclear PR act against pro-inflammatory responses via similar mechanisms. Engagement of the GR both induces production of IκBα, which inhibits NF-κB translocation into the nucleus, while also

directly complexing with NF-κb to prevent it binding to pro-inflammatory genes. Binding of the GR to DNA also induces transcription of the anti-inflammatory *Il10* gene. Meanwhile, activation of the nuclear PR also directly interacts with NF-κB to prevent its downstream functions, while also leading to IL-10 production.

B. Anti-SARS-CoV-2 antibodies

While the COVID-19 pandemic shut down universities worldwide in Spring 2020, in-person classes were set to resume in the Fall semester. Due to the ever-changing nature of the pandemic and continued spread of a newly emergent pathogen, our group worked with university officials and Siemens Healthcare to track the spread of the SARS-CoV-2 virus among the University of Missouri population during the Fall 2020 semester via anti-S protein antibody seroprevalence. We initially sent emails at random to students, staff, and faculty inviting them to participate in this study, later inviting everyone in the university community. In all, 2,202 members participated by donating blood and providing information such as days post-PCR positive results for COVID-19 infection and demographics, including university status, sex, and age. As expected, we found that antibody seroprevalence increased weekly from mid-September until late-October, and then plateaued through December. A plurality of those were younger undergraduate students, as opposed to older students, staff, and faculty. Interestingly, total Ig and IgG anti-S protein antibody levels

increased with age, with older men having the highest concentrations. For those reporting a positive PCR test at any point, antibody levels among men, although higher than women at first, decreased much more rapidly over time. Antibodies levels in women, however, remained more constant, on average.

From these data, we can conclude that younger individuals are more susceptible to infection due to greater seroprevalence among the 17-24 years-of-age and undergraduate populations, with the speculation that this demographic group typically engages in less risk-averse behaviors, on average, such as attending more social activities. Meanwhile, our findings also suggest older men with COVID-19 infections have higher antibody levels compared to women. We can also conclude that men had higher anti-S protein antibody levels shortly after positive PCR tests, which decreased over time compared to women. Interestingly, in studies from other groups, the magnitude of antibody levels has been linked to COVID-19 disease severity, as higher circulating concentrations correlate with morbidity and hospitalization (Amjadi et al., 2021: Chen et al., 2020). Since men are more likely to develop greater morbidity and require hospitalization, our findings here showing men initially had increased antibody titers are in concordance with other studies.

C. Neutrophil extracellular traps in COVID-19 patients

Early case reports of severe COVID-19 patients suggested it was a respiratory disease with a presentation that included some striking similarities to late-stage cystic fibrosis (CF), characterized by hypoxemia with excessive PMN infiltration into the lungs, thick mucus-like buildup, and bronchiectasis – thickening of the airways (Barnes et al., 2020; Ye et al., 2020; Zhang et al., 2020). Early studies showed that PMN to lymphocyte ratios were significantly increased in these severe patients over those with mild disease, indicating an aberrantly strong innate immune response (Zhang et al., 2020). Such findings are characteristic of acute respiratory distress syndrome (ARDS). As such, cases with high PMN infiltration could be predicted to result in production of NETs, which in CF thickens the mucus found in the lungs leading to airway blockages (Konstan & Ratjen 2012). We therefore hypothesized that drug dornase alfa, a recombinant human DNase I used to help clear the airways of cystic fibrosis patients by breaking apart the DNA component in NETs that leads to mucus thickening, would improve outcomes for mechanically ventilated COVID-19 patients. An initial patient treated with this in the Intensive Care Unit showed apparently positive results, and this strategy was therefore applied to a small Phase III clinical trial directed by Dr. Zach Holliday, MD. During the course of treatment, the PaO2/FiO2 ratio, an important clinical indicator of hypoxemia, significantly improved, although this did not continue past the treatment period. Meanwhile, static lung

compliance, the ability of the lungs to stretch and expand, also significantly improved, albeit again only during the treatment period. These results suggest that this period of dornase alfa administration via nebulized inhalation should be expanded to help maintain improved clinical parameters. Although not significantly different, those patients that received dornase alfa treatment spent fewer days mechanically ventilated, in the ICU, and hospitalized altogether; death rates were also decreased, although not statistically significantly. Treatment did not reduce BALF PMN counts but did lead to a significant decline in BALF NETs marked by MPO-bound DNA. This effect was specific for BALF samples since it was observed neither in peripheral blood samples nor in NETs experimentally induced ex vivo from isolated PMNs.

Given these results, we can conclude that nebulized, inhaled dornase alfa treatment of mechanically ventilated COVID-19 patients improved clinical lung parameters, although only during the period of treatment and not enduring thereafter. Similarly, we can also say that dornase alfa was able to reduce the amount of airway blocking NETs in the lungs. Though our treatment sample was small (only 10 patients), we find it is reasonable to hypothesize that a greater sample size may result in significant differences for the other trending outcomes mentioned previously, the most attractive parameter being survival. Notably, because our sample size was small, we were not able to design this clinical trial for evaluation of outcomes based on sex.

II. Recommendations for Future Work

In further examining the role of sex in trained immunity in the future, several routes of study should be considered. Firstly, changes in training induced epigenetic modifications due to PR activity should be fully elucidated to gain better insight into the mechanistic underpinnings of P4-based training attenuation. This is a critically important aspect of trained immunity that we did not explore. Aside from the nuclear PR, most cells also express membrane bound P4 receptors; these are not nuclear factors, but instead regulate cellular activity within the cytosol. No membrane P4-specific antagonists or inhibitors have yet to be reported; we must consider it possible that the antagonist we used here, PF-02413873, might act on both the nuclear PR and membrane P4 receptors. Future work should try to isolate these factors, possibly using PR or membrane P4 receptor knockout mice to elucidate whether this attenuation effect is dependent on one or the other, or perhaps both. Another area we did not explore is regarding important reproductive variables, factors, and states such as pregnancy; as circulating levels of both E2 and P4 increase throughout gestation until immediately before parturition, it is conceivable to hypothesize this may affect training of bone marrow, circulating, and/or even uterine immune cells. This may further add to our understanding of immune suppression during pregnancy.

As the COVID-19 pandemic has ended, we wonder when a future viral respiratory pandemic will occur. Retrospective studies may help to better understand antibody development after natural pandemic viral infections. This would also be useful in evaluating sex-based differences not only in antibody

production, but also the strength of antibody responses. Experimental infections of female and male mice expressing human ACE2 receptors will also be useful. This may also help to better understand exactly how SARS-CoV-2 reduces T cell counts and dampens activity, which may further aid in developing more effective treatments for viral infections that exhibit these effects including any future emergent coronaviruses.

A small but significant proportion of recovered COVID-19 patients develop long-lasting symptoms, termed post-acute sequelae of SARS-CoV-2, or PASC (also known as long-COVID). Approximately 50% of these patients have circulating autoantibodies, including antinuclear and antineutrophil cytoplasmic antibodies (Chang et al., 2021; Lee et al., 2022; Seeßle et al., 2022). These autoantibodies are commonly found in those with autoimmune conditions such as systemic lupus erythematous, autoimmune hepatitis, and Still's disease, and are strongly associated with pathogenic PMN activity including NETosis. Several other small dornase alfa clinical trials have also been conducted, finding improved clinical parameters after treatment in mechanically ventilated COVID-19 patients, including increased oxygen saturation, improvement in FiO2/PaO2 ratios after treatment (which we did not report, though our data trended in this direction during the treatment period), lung compliance, and even in radiological findings (which we did not examine here) (Okur et al., 2020; Toma et al., 2021; Weber et al., 2020). Extracellular DNA was also significantly decreased in the lungs (Okur et al., 2020). Interestingly, circulating levels of damage-associated, inflammatory, and thrombotic markers such as C-reactive protein, ferritin, lactate

dehydrogenase, and d-dimer were also reduced (Weber et al., 2020). Although this does not necessarily mean that dornase alfa treatment improved parameters of these circulating markers, its use in cystic fibrosis patients has been reported to decrease systemic inflammatory markers (Konstan & Ratjen 2012). Several larger trials, including COVIDORNASE in Europe, and several other international trials were initiated and patients were recruited, although their findings are yet to be published while some (such as COVIDORNASE) were terminated. Follow up studies of surviving patients should be performed evaluating the link between these autoantibodies and the development of PASC.

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VITA

Alexander Parker Earhart was born on April 28, 1991, in Belleville, Illinois to Donna Parker Earhart and Tony Randall Earhart. After seeing the movie *Jurassic Park* at an alarmingly early age, Alex was fixated on becoming a scientist – first to be a paleontologist, then geneticist. He spent his early years and education in Granite City and Collinsville, Illinois. Alexander graduated *summa cum laude* from Collinsville High School in 2009, and then attended Murray State University to study molecular biology/biomedical sciences with a minor in biochemistry. During his time in undergraduate school, he researched insect genetic relatedness and changes due to climatic events, and mathematically modeled species distribution of termites and their colonies in the US Virgin Islands, doing field work on the island of St. John, and enjoying every moment living in a jungle camp. He finished his BS degree *cum laude* in May 2013, and later found a job at Washington University Medical Center in St. Louis as a research technician in the Thoracic Immunobiology Lab of Andrew Gelman, Daniel Kreisel, and Sasha Krupnick researching the role of neutrophils in respiratory system injuries – thanks to the recommendation of his dear high school friend Kelsey Toth.

After nearly four years as a research tech, Alex wanted to continue his education and career with a PhD focusing on innate immunology, accepting an offer from the University of Missouri in the Molecular Pathogenesis and Therapeutics program in 2018. Here he began work on and developed a

completely *de novo* project on sex differences in trained immunity, the primary focus of his dissertation work, in the lab of Adam Schrum. Although the COVID-19 pandemic put his primary work on hold, he immediately proposed a mechanism of the disease by way of pathologic neutrophil activity in the lungs and a means to treat it, leading to a clinical study utilizing the cystic fibrosis drug dornase alfa to break apart damaging neutrophil extracellular traps in ICU COVID-19 patients. During this time, he also oversaw the laboratory work involved in examining anti-SARS-CoV-2 antibody positivity in the University of Missouri community during the Fall 2020 semester. Following the completion of his PhD, Alex will be starting a postdoctoral research position at Washington University School of Medicine in St. Louis in the lab of Dr. Hrishikesh Kulkarni studying the influence of complement factors in lung pathology and on the induction of trained immunity.