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Trained Immunity Enhances the Immune Response and Maintains Microbiome Diversity in Aging and Sepsis

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by

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ABSTRACT

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The global population is rapidly aging. It is estimated that over the next thirty years, the number of individuals ≥60 years of age will increase by over a billion, and the number of individuals over age 80 may increase by 300 million. As humans age, our immune system becomes progressively weaker through a process called immune senescence. This age-related decrease in immune function increases susceptibility to infection and chronic diseases. Sepsis is a leading cause of death worldwide. Over the past two decades, there has been an increased incidence of sepsis which is due, in part, to our aging population and immune senescence. The gut microbiome, which plays an essential role in health and disease, is altered in aging and sepsis. Specifically, the commensal microorganisms of the gut microbiota are replaced with potentially pathogenic bacteria. This contributes to immune dysfunction and worsened outcomes in critical illness. The innate immune system can be "trained" to respond more effectively to pathogens. We examined trained immunity as an approach to modulating immunosenescence and microbiome diversity in aging. We investigated the effect of trained immunity on: i) immune cells from healthy aging subjects and sepsis patients and ii) the diversity of the microbiome in aging and sepsis. Our results indicate that trained immunity is effective in combatting age-related immunosenescence. We found that β-glucan induced trained immunity enhances monocyte metabolism, increases functionality as well as alters the transcriptome and epigenome in aging

individuals and sepsis patients. We also found that trained immunity induced the expansion of a unique population of myeloid cells in sepsis. These cells are defined as FSC^{hi}, $CD11b^{+}$, $GR\text{-}1^{hi}$ and express high levels of immunosuppressive PD-L1. In addition, we found that trained immunity reversed age-related changes to the microbiome and prevented alterations to the microbiome in septic mice. We found that the Firmicutes/Bacteroidetes ratio increased in aging; however, trained immunity reversed this increase and increased *Clostridia* in aged mice. In sepsis, trained immunity prevented expansion of Proteobacteria observed in control mice. Thus, our results indicate that trained immunity may be effective in modulating immunosenescence and the microbiome in aging and sepsis.

DEDICATION

This dissertation is dedicated to my loving fiancé, Abby. You have been so patient and supportive while I worked towards this goal, and we have finally made it. Through the good times and the bad, you have been there for me. I do not think I can thank you enough. I am so grateful and fortunate to have you in my life. I am looking forward to whatever is next for us.

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CHAPTER 1. INTRODUCTION

Trained Immunity

Overview and Definition

The immune system has classically been categorized into two separate arms; the innate immune response, which occurs quickly and nonspecifically upon exposure to a pathogen, and the adaptive response, which is slower and more specific, forming long-lasting immune memory (Netea et al. 2016). However, plants and invertebrates, which lack adaptive immune systems, still display forms of immune memory (Netea et al. 2020). There is also evidence for the existence of innate immune memory in mice and humans. Immunization of mice and humans with the Bacillus Calmette Guerin (BCG) tuberculosis vaccine confers the host with nonspecific protection from non-target infections (van 't Wout et al. 1992; Biering-Sørensen et al. 2017). Furthermore, the enhanced nonspecific protection from infection provided by BCG vaccination also occurs in SCID mice that lack functional adaptive immune systems (Kleinnijenhuis et al. 2012), thus implicating the role of innate immune memory for the non-target effects of BCG vaccination. Similar, non-target effects have been discovered with other vaccines (Benn et al. 2013).

Several recent publications have found that exposure to specific pathogens or microbial components can train the innate immune system to respond to subsequent infections more effectively [7-9]. This concept is referred to by different names, most commonly it is called “trained immunity,” “innate immune memory,” or “immune training” (Netea et al. 2020). Trained immunity can be induced by exposure to microbes, such as *Candida albicans* (Cheng et

al. 2014a); pathogen-associated molecular patterns (PAMPs), such as β-glucans (Cheng et al. 2014a; Ifrim et al. 2014); synthetic products, such as Monophosphoryl lipid A (MPLA) (Fensterheim et al. 2018); and more complex preparations, such as vaccines (Arts et al. 2016). The trained immune phenotype is characterized by epigenetic and metabolic reprogramming and subsequent immunologic changes and protection from infection upon exposure to an immune training agent (Netea et al. 2016; Netea et al. 2020).

Epigenetic Reprogramming

The molecular mechanism responsible for trained immunity is epigenetic reprogramming (Netea et al. 2020). In inactive innate immune cells, most pro-inflammatory genes are epigenetically suppressed (Smale et al. 2014). Upon exposure to a pathogen or PAMPs, genes are quickly activated by increased chromatin accessibility, RNA polymerase II recruitment, and specific histone acetylation (Netea et al. 2016). These changes, along with the recruitment of transcription factors, lead to markedly increased transcription of pro-inflammatory genes (Smale et al. 2014; Netea et al. 2016). This stimulation response can leave a "scar" at the epigenetic level, thereby increasing the cells' capacity to respond quickly to future insults (Netea et al. 2020). Two specific epigenetic markers distinguish the trained immunity phenotype, *i.e.,* histone 3 lysine 27 acetylation (H3K27ac) and histone 3 lysine 4 methylation (H3K4me) (Cheng et al. 2014a; Netea et al. 2020). These changes can persist for months after the induction of trained immunity (Kleinnijenhuis et al. 2012). This suggests that the epigenetic changes in trained immunity occur at the progenitor cell level (Netea et al. 2016).

In the well-described phenomenon of LPS tolerance, myeloid cells that were previously exposed to LPS undergo immunoparalysis and have decreased pro-inflammatory cytokine

responses upon secondary stimulation (Saeed et al. 2014). Like trained immunity, LPS tolerance induces specific epigenetic changes in myeloid cells. Tolerized cells lose H3K27ac that is typically present upon immune activation (Saeed et al. 2014). Additionally, tolerized cells display epigenetic suppression of IRF and STAT, which are transcription factors critical to the pro-inflammatory response (Novakovic et al. 2016). Novakovic et al. found that epigenetic changes associated with LPS tolerance are overridden by β-glucan-induced immune training and that previously tolerized monocytes that are treated with β-glucan have their cytokine production capacity restored (Novakovic et al. 2016). This indicates that trained immunity may be beneficial in circumstances where tolerance contributes to immunosuppression, such as in sepsis (Hotchkiss et al. 2013b).

Metabolic Reprogramming

One critical hallmark of trained immunity is increased glycolytic metabolism (Cheng et al. 2014a; Netea et al. 2020; Riksen and Netea 2021). While glycolysis is less energy efficient than its metabolic counterpart, oxidative phosphorylation, it is favored by activated immune cells (O'Neill et al. 2016). Glycolysis may be preferential for immune activation for several reasons. Firstly, glycolytic enzymes can be induced and activated more quickly when compared to the mitochondrial biogenesis that must occur to upregulate oxidative phosphorylation (O'Neill et al. 2016; Riksen and Netea 2021). Secondly, glycolysis involves the conversion of NAD^+ to NADH, which is used as a cofactor by many enzymes (O'Neill et al. 2016). Lastly, glycolysis intermediates may be used in key biosynthetic pathways, allowing the host to produce necessary amino acids and proteins to aid in the immune response (O'Neill et al. 2016; Riksen and Netea 2021).

Enhanced glycolysis is essential in the induction of the trained immunity phenotype. Immune-trained monocytes have enhanced glycolic rates (Cheng et al. 2014a; Arts et al. 2016). However, direct inhibition of glycolysis using 2-deoxy-D-glucose (2-DG) abolishes the enhanced cytokine production after immune training with β-glucan (Cheng et al. 2014a; Keating et al. 2020b). Additionally, activation of the AKT-mTOR pathway is a requirement for trained immunity. AKT phosphorylation increases in monocytes upon treatment with β-glucan (Cheng et al. 2014a). Inhibition of this pathway using Wortmannin, rapamycin, Torin, and AICAR prevent the metabolic and immunologic changes associated with trained immunity (Cheng et al. 2014a; Arts et al. 2016). Furthermore, lactate, a major product of glycolysis, inhibits histone deacetylase function, allowing for increased transcription and upregulation of genes (Latham et al. 2012; Hajishengallis et al. 2019).

The tricarboxylic acid (TCA) cycle and oxidative phosphorylation are also involved in trained immunity. TCA cycle metabolites are upregulated in trained immunity. One such TCA cycle metabolite, fumarate, can induce the trained immunity phenotype (Arts et al. 2016). Itaconate, another TCA cycle metabolite, possesses antimicrobial properties and is essential in resolving inflammation (Hooftman and O'Neill 2019). Administration of exogenous itaconate prevents the formation of the trained immunity phenotype. Additionally, itaconate is increased in tolerized monocytes; however, β-glucan administration reverses tolerance by inhibiting itaconate signaling (Riksen and Netea 2021).

The role of oxidative phosphorylation in trained immunity is less well understood. Several studies have found that immune training with a low dose of β-glucan (1μg/mL) leads to increased oxygen consumption rates (Arts et al. 2016; Keating et al. 2020a; Keating et al. 2020b;

van der Heijden et al. 2020). Conversely, Cheng et al. found a reduction in oxidative phosphorylation using a high concentration (10 μg/mL) of β-glucan (Cheng et al. 2014a). However, studies in our lab have found increased basal and maximum oxygen consumption rates in cells treated with the higher dose (manuscripts submitted). Administration of oligomycin, an ATP synthase inhibitor, during LPS-restimulation of β-glucan-immune trained monocytes prevented the increased cytokine production of trained immunity (Keating et al. 2020a); however, oligomycin did not inhibit the trained immunity phenotype in BCG-induced trained immunity (Arts et al. 2016). Thus, the role of oxidative phosphorylation in immune training requires additional investigation.

Immunologic Changes

Trained immunity has been most studied in monocytes/macrophages, dendritic cells, and NK cells; however, trained immunity is likely, not unique to these cell populations (Netea et al. 2016). There is also preliminary evidence for trained immunity in neutrophils (Moorlag et al. 2020a). It has also become clear that trained immunity occurs in progenitor cells, allowing the duration of immune training effects to far exceed the lifespan of monocytes and dendritic cells (Netea et al. 2020). Additionally, trained immunity may induce the expansion of myeloid hematopoietic stem cells and other myeloid progenitors (Hajishengallis et al. 2019). Foremost among the immune changes associated with trained immunity is the increased production of pro-inflammatory cytokines, specifically TNF-α and IL-6 (Cheng et al. 2014a; Ifrim et al. 2014). Additionally, others have found the anti-inflammatory cytokine IL-10 increased in immune training (Bekkering et al. 2016a; Garcia-Valtanen et al. 2017; Jeljeli et al. 2019). Immune-trained monocytes also display patterns of cell surface markers that are different from tolerized cells.

Immune training with β-glucan induces a broad range of monocyte sizes and granularity, and most trained monocytes express CD14, CD68, and CD11b (Ifrim et al. 2014). Natural killer cells exposed to mouse cytomegalovirus or IL-12 and IL-18 display enhanced cytokine secretion for weeks following exposure (Netea et al. 2020). Autophagy also plays an important role in trained immunity. When autophagy is inhibited pharmacologically or by a genetic mutation, trained immunity induced by BCG vaccination is hindered (Buffen et al. 2014).

β-Glucan

C. albicans-derived β-glucan is one of the most potent and well-studied inducers of the trained immunity phenotype (Cheng et al. 2014a; Ifrim et al. 2014; Saeed et al. 2014; Netea et al. 2020). β-glucans are an abundant component of yeast, fungi, and some bacterial cell walls. Among the β-glucans, β-1,3-(D)-glucans with β-1,6 branching side chains have the most significant effects on immunity (Geller and Yan 2020). *C. albicans*-derived β-glucan induces the trained immunity phenotype through a Dectin-1 dependent mechanism (Cheng et al. 2014a). Exposure to β-glucan induces H3K4me and K3K27ac, increased glycolysis, and upregulated pro-inflammatory cytokine production that are all hallmarks of the immune training response (Cheng et al. 2014a; Ifrim et al. 2014; Saeed et al. 2014). β-glucan also induces expansion of myeloid progenitors and increased production of IL-1β and GM-CSF, the latter of which enhances the trained immunity phenotype (Mitroulis et al. 2018). mTOR, AKT, and HIF-1α are important signaling intermediates in β-glucan-induced trained immunity. Their signaling is required for the enhanced cytokine secretion and protection against infection associated with innate immune training (Cheng et al. 2014a). Immune training with β-glucan affords enhanced protection against a wide range of pathogens. Among these, administration of β-glucan confers enhanced protection

against *Staphylococcus aureus* (Di Luzio and Williams 1978; Cheng et al. 2014a), *C. albicans* (Saeed et al. 2014), *Mycobacterium tuberculosis* (Moorlag et al. 2020b), and *influenza* (Jesenak et al. 2013; Muramatsu et al. 2012). While the duration of immune training effects exceeds the typical lifespan of myeloid cells, the enhanced cytokine production associated with immune training with β-glucans is diminished by three weeks (Garcia-Valtanen et al. 2017).

Trained Immunity in Disease

While trained immunity is beneficial in improving the immune response and preventing infection, there may be some cases in which trained immunity is maladaptive. Firstly, the pro-inflammatory trained immunity phenotype may contribute to atherosclerosis (Netea et al. 2020). Christ et al. found that the western diet, a high caloric diet known to increase the risk of diabetes, obesity, and heart disease, induces the trained immunity phenotype in myeloid cells of *Ldlr*$^{-/-}$ mice. After being fed a western diet for four weeks, mice in this study experienced an enhanced inflammatory response to immune stimuli. Trained immunity in western diet-fed *Ldlr*$^{-/-}$ mice remained for four weeks after switching to a standard chow diet (Christ et al. 2018). A separate study found that monocytes from human patients with symptomatic atherosclerosis also display the trained immunity phenotype (Bekkering et al. 2016b).

Trained immunity may also contribute to neurodegenerative disease. Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are associated with chronic inflammation (Netea et al. 2020). Wendeln et al. found that exposure to LPS in a mouse model of Alzheimer's disease led to immune training of microglia, accompanied by enhanced epigenetic activation of HIF1α and correlated with worsened amyloid-β plaques (Wendeln et al. 2018). Trained immunity may also contribute to dementia (Netea et al. 2020). Monocytes from patients

with small vessel disease, a significant cause of dementia, also display some trained immunity characteristics, such as increased production of pro-inflammatory cytokines upon immune stimulation. This inflammatory response is associated with the progression of the disease (Noz et al. 2018). Because trained immunity may potentially be utilized to combat immunosenescence in vulnerable populations (Novakovic et al. 2016), the potential effect of immune training on neurodegenerative disease must be considered.

Age-related Immunosenescence and Inflamm-aging

Overview

The world's population is rapidly aging. By 2050 the number of individuals over 60 years old will increase by approximately 110%, and the number of individuals over 80 years old will increase by over 300% (Sadighi Akha 2018). The increase in the aging population does not correspond with an increase in "healthspan," suggesting that chronic diseases and infection will be an increasing burden (Aiello et al. 2019). Aging individuals often experience a combination of immunosenescence and chronic low-level inflammation, known as inflamm-aging, which contribute to many age-related diseases and disorders. These processes contribute to cancer (Jackaman et al. 2017), neurodegeneration (Liang et al. 2017), and decreased wound repair (Sadighi Akha 2018). They also increase the risk and severity of infection and sepsis (Martin et al. 2017; Fulop et al. 2018). Both innate and adaptive immunity are affected by aging, but immunosenescence is primarily attributed to changes within the adaptive immune system in the aging population (Fulop et al. 2018). Due to the pronounced impact that immunosenescence will have on the aging population, the World Health Organization has placed a priority on increasing

understanding of immunosenescence and counteracting its effects using immunization (Thomas-Crusells et al. 2012)

Immunosenescence and Changes to Adaptive Immunity in Aging

Immunosenescence is the age-related loss of the cellular and humoral immune responses (Aiello et al. 2019). While it is acknowledged that the innate immune system also changes with aging, changes to the adaptive immune system are currently thought to explain the bulk of the decline in immunity in aging (Fulop et al. 2018). One of the significant contributors to immunosenescence is the decline in naïve T-cells (Bektas et al. 2017). A decline in thymus size and thymopoiesis partially explains the reduced capacity to acquire immunity to previously unencountered pathogens (Palmer 2013; Bektas et al. 2017). Furthermore, naïve T-cells from aging individuals have reduced differentiation capabilities (Ventura et al. 2017b). As naïve T-cells are not being generated, T-cell receptor (TCR) diversity is significantly blunted. This partially explains why immune repertoire is greatly diminished, although overall memory and effector CD4+ and CD8+ T-cell numbers increase with age (Bektas et al. 2017). Additionally, latent virus exposure, such as that from CMV, increases corresponding memory $CD8^+$ T-cell clones, further reducing T-cell repertoire (Hadrup et al. 2006; Sadighi Akha 2018). Cytokine production may also play a role. In aging, $CD4^+$ T-cells predominantly switch from a Th1 phenotype to a Th2 phenotype and secrete primarily IL-4 and IL-10. This skewing of the T helper cell phenotype limits the range and effectiveness of immune responses (Ponnappan and Ponnappan 2011; Bektas et al. 2017). Additionally, this shift corresponds to increased reactive oxygen species (ROS) exposure that is known to occur in aging (Ponnappan and Ponnappan 2011).

Immunosuppressive regulatory T-cells (Tregs) also increase in age (Bektas et al. 2017; Fulop et al. 2018). Tregs are classified as T-cells expressing high levels of CD25 and Foxp3 and are a part of the anti-parasitic immune response and thought to be anti-inflammatory (Ponnappan and Ponnappan 2011; Bektas et al. 2017). While some studies suggest that Tregs increase in aging individuals, the function of Tregs declines, as they no longer effectively limit inflammatory conditions (Bektas et al. 2017). This is accompanied by an increase of pro-inflammatory Th17 T-cells in aging (van der Geest et al. 2014). Th17 cells arise from the same precursor as Tregs and are associated with protection from bacterial infection and autoimmunity (Bektas et al. 2017). The Th17 population increases more rapidly than Tregs in aging; thus, the Th17/Treg ratio increases, accompanied by decreased Treg function, leaving aging individuals increasingly susceptible to inflammatory conditions and chronic infection (Schmitt et al. 2013; Ventura et al. 2017b). One possible mechanism to explain these changes is increased Il-6 expression in aging (Ventura et al. 2017b), as Th17 and Treg differentiation are promoted by IL-6 (Ponnappan and Ponnappan 2011; Bektas et al. 2017).

B cells are also impacted by aging. B cell response and antibody production are significantly diminished in aging individuals (Fuentes et al. 2017). The decline in B cell function may be partially attributed to some of the previously mentioned changes in T cells, which are critical in inducing B cell responses (Ventura et al. 2017b). However, B cells themselves undergo cell-specific changes that impact their function (Sadighi Akha 2018). Firstly, B cell precursors decrease with age (Mckenna et al. 2001). Like T-cells, naïve B cells decrease in aging and are replaced with antigen-experienced cells (Sadighi Akha 2018). Interestingly, depletion of existing B-cells in aged mice improves their immune response and promotes B cell precursor expansion. Indicating that some age-related changes to B cells may not be irreversible (Keren et

al. 2011). B cell repertoire also decreases as a function of general health, and expansion of circulating plasma cells increases with age (Sadighi Akha 2018). However, plasma cells decrease in the bone marrow in aging, resulting in lower affinity antibodies (Fuentes et al. 2017). Furthermore, a recently discovered subset of B cells, referred to as age-associated B cells (ABCs) or atypical memory B cells, accumulate with age in mice and humans (Hao et al. 2011; Rubtsov et al. 2011; Karnell et al. 2017). In humans, these cells are associated with chronic inflammation, autoimmunity, and some infections (Sadighi Akha 2018). These cells are incompletely understood but are associated with some increases in autoimmunity observed in aging individuals (Rubtsov et al. 2011). Antibody production capabilities of B cells are also decreased in response to vaccination in aging humans (Frasca et al. 2011). Aging is associated with reduced levels of IgM and IgD and increased levels of IgG1, IgG2, IgG3, IgA1, and IgA2 (Ventura et al. 2017b). Decreases in IgM and IgD are associated with the development of naïve T-cells after exposure to an antigen (Ventura et al. 2017b). Increased IgG and IgA production are thought to be stimulated by increased circulating IL-6 and IL-4 in aging (Weksler and Szabo 2000).

Inflammaging and Innate Immune Changes in Aging

While changes to the adaptive immune system are primarily responsible for immunosenescence, age-related changes to the innate immune system also contribute to immune dysfunction in aging. As humans age, their natural barriers to infection, such as skin, begin to break down. This places an increased burden on the innate immune system, as it is exposed to increasing numbers of pathogens, leading to persistent immune activation (Ponnappan and Ponnappan 2011; Chambers et al. 2021; Kasler and Verdin 2021). This is one of the underlying

bases for inflammaging, chronic, low-grade, sterile inflammation that increases host susceptibility to infection and increases the risk of chronic inflammatory diseases (Franceschi et al. 2007; Fulop et al. 2018; Sadighi Akha 2018). This constant immune activation leads to cellular damage that produces inflammatory damage-associated molecular patterns (DAMPs), further stimulating the immune system (Sadighi Akha 2018). Inflammaging is closely associated with many age-related conditions such as atherosclerosis, diabetes, Alzheimer's disease, and frailty (Sadighi Akha 2018). The two currently identified major hallmarks of inflammaging are upregulated C-reactive protein and pro-inflammatory cytokines, especially IL-6. (Sadighi Akha 2018). In addition to persistent exposure to inflammatory stimuli, cells of the innate immune system experience a multitude of age-related changes that contribute to immune dysfunction.

Monocytes are an important cell linking the innate and adaptive immune arms, and they have many crucial immune functions. Firstly, monocytes express a multitude of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), that recognize a broad range of microorganisms (Yarbro and Pence 2018). Using PRRs, monocytes recognize and phagocytize pathogens, breaking them down to present critical information to the adaptive immune system in the form of antigens (Yarbro and Pence 2018). Monocytes also secrete cytokines and chemokines (De Maeyer and Chambers 2021) and express high levels of co-stimulatory molecules on their surfaces that stimulate and signal with other immune cells (Metcalf et al. 2015). Upon activation by immune stimuli, monocytes migrate to the site of infection or host damage and differentiated into macrophages (Saeed et al. 2014). In aging, monocyte/macrophage function is significantly altered. Macrophage migration, phagocytosis, cytokine production, and pathogen recognition are all decreased in aging (Martin et al. 2017). Additionally, macrophage precursors are decreased in the elderly. MHC II

expression on macrophages is also decreased (Martin et al. 2017). Macrophages in aging individuals also secrete elevated levels of IL-10, further contributing to immunosenescence (Jackaman et al. 2017). While circulatory IL-6 is increased in aging individuals in the absence of infection, macrophages' ability to secrete pro-inflammatory cytokines in response to immune stimuli is decreased (Jackaman et al. 2017).

Neutrophils are the first responders to invading microorganisms and are critical to infection clearance (Jackaman et al. 2017). Neutrophils quickly migrate to sites of infection, phagocytize and kill pathogens, generate neutrophil extracellular traps (NETs), and generate ROS (Fuentes et al. 2017). Comparatively, less is known about the effect of aging on neutrophils. Butcher et al. found that the cell surface molecule CD16 and phagocytic capacity is decreased in elderly individuals (Butcher et al. 2001). Neutrophil migration may also be impaired in aging (Chen et al. 2014). Moreover, neutrophils are less sensitive to activation in aging individuals, contributing to immunosenescence (Fuentes et al. 2017). Impaired neutrophil function in aging likely contributes to increased incidence of cancer and infection in the elderly (Jackaman et al. 2017; Martin et al. 2017).

Trained Immunity and Immunosenescence

The effect of trained immunity on age-related immunosenescence has been speculated about but has not been directly addressed. (Fulop et al. 2018; Bulut et al. 2020; Netea et al. 2020). In their review of the subject, Bulut et al. suggest that trained immunity could be used to combat immunosenescence in older adults (Bulut et al. 2020). There is preliminary evidence for this idea in the literature. Firstly, β-glucan-induced trained immunity can override immunological tolerance, a form of immune suppression (Novakovic et al. 2016). Additionally, a clinical study that administered the BCG vaccine to individuals between 60 and 75 years old

once a month for three months found that BCG vaccination prevented respiratory infections and improved cytokine production (Wardhana et al. 2011). Bulut et al. note that trained immunity may be detrimental in individuals experiencing inflammation from sterile causes, as it may contribute to atherosclerosis and other inflammatory syndromes (Bulut et al. 2020). Furthermore, Fulop et al. suggest that trained immunity, itself, may lead to persistent immune activation, causing some of the immune dysfunction in aging individuals (Fulop et al. 2018). Further studies are necessary to increase understanding of the effect of trained immunity on age-related immunosenescence.

Sepsis

The Sepsis-3 conference defines sepsis as "life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al. 2016b)." Changes to the immune system that occur in aging result in immune dysfunction which increases the risk and severity of infection in the elderly. This makes sepsis a condition that is disproportionally experienced by aging individuals (Martin et al. 2017). The progressive aging of the global population and better clinical recognition of sepsis are drastically increasing sepsis incidence (Dellinger et al. 2013; Singer et al. 2016a). Worldwide there were approximately 48.9 million sepsis cases and 11 million sepsis mortalities in 2017 (Rudd et al. 2020). In the US alone, sepsis costs the healthcare system nearly $40 billion, which makes up 9% of all hospital costs (Liang et al. 2006). Despite decades of attempts and research, there is still no FDA-approved drug for sepsis, and improvements that have been made in sepsis mortality can be credited to quicker recognition of sepsis and improved standards of care (Marshall 2014; Cavaillon et al. 2020).

The immune response to sepsis is complex and dysfunctional (D'Elia et al. 2013; Hotchkiss et al. 2013b; Venet and Monneret 2018; Huang et al. 2019). The immune response to sepsis is comprised of two phases that were classically considered to be distinct; however, modern sepsis research has identified that the initial hyper-inflammatory response and immunosuppressive responses overlap (Delano and Ward 2016b). The hyperinflammatory state induced early in sepsis is initiated by the interaction of pathogens and PAMPs with host PRRs (Raymond et al. 2017). In the case of sepsis, systemic immune activation results from these interactions, followed by a release of pro-inflammatory cytokines known as the "cytokine storm (D'Elia et al. 2013)." Elderly patients are at an increased risk for failing to resolve this inflammation, leading to worse outcomes (Goodwin et al. 2015). Although this hyper-inflammatory response can damage the host, efforts to target and suppress the cytokine response in sepsis have proved to be ineffective and sometimes harmful (Cohen et al. 2012; Hotchkiss et al. 2013a). Patients who survive the initial hyperinflammatory response often experience a protracted immunosuppressive state that makes them susceptible to recurring infections (Boomer et al. 2011). Contributing to this problem, sepsis survivors have long-term changes to immune function including reduced cytokine secretion (Arens et al. 2016), leukocyte depletion (Hotchkiss et al. 2013b), and an expansion of myeloid-derived suppressor cells (MDSCs) (Delano et al. 2007).

Myeloid-Derived Suppressor Cells

MDSCs represent a heterogeneous population of myeloid-derived cells that have immunosuppressive effects (Goh et al. 2013; Gabrilovich 2017; Schrijver et al. 2019). MDSCs were first discovered in the context of cancer; however, MDSCs also play a role in infection and

sepsis (Gabrilovich 2017; Schrijver et al. 2019). MDSCs modulate the immune system by secreting anti-inflammatory cytokines, such as IL-10, and interfacing with other immune cells using surface proteins such as PD-L1 (Wang et al. 2015b; Kulkarni et al. 2018). In cancer, MDSCs are seen mainly as harmful to the patient, as they suppress the patient's immune system, permitting tumor growth (Law et al. 2020). However, the role of MDSCs is less straightforward in infection and sepsis. MDSCs may be beneficial in limiting the hyper-inflammatory stage that is predominant early in sepsis (Schrijver et al. 2019). However, MDSCs may also limit the host's immune system's ability to effectively respond to infection (du Plessis et al. 2013; Goh et al. 2013) and contribute to the prolonged immunosuppression that occurs in many sepsis patients (Hotchkiss et al. 2013b; Venet and Monneret 2018). The stage of sepsis also may play an essential role in determining MDSC function. In a mouse model of sepsis, MDSCs ($Gr\text{-}1^+$, $CD11b^+$ cells) stimulate the immune response in the early stages of sepsis and transition to a more immunosuppressive phenotype in the later stages (Brudecki et al. 2012). There is also reason to believe that trained immunity may stimulate MDSC expansion. Rieber et al. found that *C. albicans* infection induces MDSC expansion through dectin-1 signaling (Rieber et al. 2015). The resulting MDSC population is protective against *C. albicans* infection but detrimental to the host in *Aspergillus fumigatus* infection (Rieber et al. 2015). Thus, the effect of trained immunity on MDSCs requires further investigation.

Microbiome

Overview

The gut microbiome plays a critically important role in host health and response to disease (Lloyd-Price et al. 2016; Buford 2017). Bacteria are the majority microorganism of the

microbiota and the total number of microorganisms in the human body total between 10^{13} and 10^{14} (Kim and Jazwinski 2018). Ninety percent of gut microbiome bacteria belong to 2 bacterial phyla: Firmicutes and Bacteroidetes (Qin et al. 2010). The microbiome plays a critical role in host health and well-being at every stage of life (Salazar et al. 2020). The microbiome is attained at birth and remains relatively stable throughout life but can be altered by host health, environment, and diet (Kim and Jazwinski 2018). However, major disruptions of diversity to microbiota communities are linked to a wide range of diseases, including cancer, metabolic diseases, and worse outcomes in critical illness (Goulet 2015; Lloyd-Price et al. 2016; Steves et al. 2016).

Gut microbiota contribute to host health in several ways. First, symbiotic microorganisms colonize the intestines and outcompete potentially pathogenic bacteria and fungi (Nagpal et al. 2018). Second, the microbiome has a significant impact on host metabolism (Steves et al. 2016; Buford 2017). Microorganisms serve many metabolic functions that are important to human hosts, including the production of short-chain fatty acids (SCFAs) and the breakdown of otherwise un-digestible carbohydrates (Lloyd-Price et al. 2016). Furthermore, the microbiome is heavily involved in host immunity (Buford 2017). Many studies have found that the immune system and microbiome communicate and influence one another. Germ-free (GF) mice have deficient cytokine production and a reduced spectrum of T-cell function (Ivanov et al. 2008a; Maslowski et al. 2009; Molloy et al. 2012). However, when GF mice are colonized with microbiota, Th1, Th17, and regulatory T-cell functions were restored (Gaboriau-Routhiau et al. 2009). The microbiome also impacts host physiology by influencing tight junctions and preventing intestinal leakage that could cause systemic inflammation if microorganisms or

inflammatory stimuli reach the bloodstream (Nagpal et al. 2018). The microbiome even influences brain physiology and function through gut-brain communication (Nagpal et al. 2018).

Age-Associated Changes to the Microbiome

Interest in the critical role of the microbiome in aging has increased dramatically over the last two decades (**Figure 1**), making the microbiome the target of anti-aging research (Vaiserman et al. 2017). Aging in humans is a complex process that involves progressive declines in many biologic systems (Maffei et al. 2017; Vaiserman et al. 2017; Nagpal et al. 2018). While life expectancy is increasing and the elderly population is rapidly growing (Sadighi Akha 2018), advances in human lifespan have not been accompanied by increased healthspan, *i.e.* the period of life in which a person experiences generally good health (Vaiserman et al. 2017). Recent studies suggest that the microbiome plays a significant role in human lifespan and overall health (Biagi et al. 2016; Vaiserman et al. 2017). Researchers have also found that crucial microbial communities are dissipated, and microbial diversity of the microbiome decreases with advanced age (Jackson et al. 2016; Buford 2017). Loss of vital microbial populations and diversity in aging has been associated with frailty, dementia, hypertension, diabetes, arthritis, and many other age-related conditions (Buford 2017; Nagpal et al. 2018).

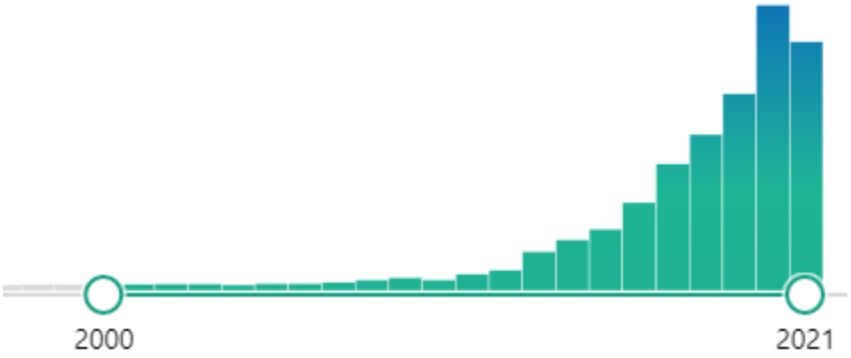

Figure 1. Interest in the role of the microbiome in aging has snowballed over the past two decades. Histogram of the number of PubMed search results for "microbiome and aging" by year from 2000 to 2021. Figure generated in PubMed.

The Firmicutes/Bacteroidetes ratio is used to approximate microbiome conditions. Mariat et al. found that the Firmicutes/Bacteroidetes ratio was decreased in individuals 70-90 years old, compared to adults between 25 and 45 years old (Mariat et al. 2009). This result was confirmed by others (Claesson et al. 2011), but Biagi et al. was not able to confirm this trend (Biagi et al. 2010). Among age-related changes to Firmicutes, *Clostridium* cluster XIVa and *Clostridium* cluster IV were found to be decreased in elderly individuals by some researchers (Hayashi et al. 2003; Mueller et al. 2006; Mäkivuokk et al. 2010). However, some of these trends appear geographically based, and the opposite trends were found in different countries (Mueller et al. 2006; Claesson et al. 2011). Decreases in *Clostridium* cluster XIVa and Bacteroidetes have been linked to frailty (Biagi et al. 2016). Another health-promoting bacteria, Bifidobacteria, is also believed to decrease in aging (Vaiserman et al. 2017). Expansions of populations of "pathobionts" accompany the loss of some of these populations that are crucial to human health." "Pathobionts" are bacteria that are commonly present at low amounts in a healthy state; however, overgrowth of these species leads to pathology and increases inflammation (Biagi et al. 2012). Enterobacteria are among the potentially pathogenic bacteria that increase in aging (Pédron and

Sansonetti 2008). Overall, aging is associated with a decline in health-promoting microbiota and a progressive increase in inflammatory and potentially pathogenic bacteria.

The mechanism by which the microbiome changes in aging is incompletely understood. Changes in diet may offer one mechanism, as diet has a significant impact on microbiome composition (Buford 2017). Age-related changes in taste and smell likely alter dietary patterns in elderly individuals (Fukunaga et al. 2005). Diet may also explain the inconsistencies in studies looking at the human microbiome in aging (Buford 2017). However, diet alone cannot explain age-related changes to the microbiome since animal models of aging consistently report similar changes to the microbiome (Kim and Jazwinski 2018). Immunologic changes are another possible mechanism for age-related shifts in microbiome diversity (Buford 2017; Vaiserman et al. 2017; Kim and Jazwinski 2018), and the effect of immunosenescence and inflammaging on the microbiome may be bidirectional (Buford 2017). Changes to host immunity may alter the PRR response to commensal microorganisms and pathogens, altering the microbiome (Rakoff-Nahoum et al. 2004; Buford 2017). However, changes to microbiome diversity may play a causal role in age-related changes to immunity. Fransen et al. found that the transfer of gut microbiota from 17 month-old mice to young germ-free mice induces an inflammatory immune response similar to inflammaging (Fransen et al. 2017). Increased antibiotic use in the elderly population also plays a role in the loss of diversity in the microbiome (Buford 2017). Use of antibiotics and other drugs and age-related physiologic changes to the intestines may also affect the microbiome (Buford 2017).

The Microbiome in Sepsis

Disruptions in microbiome diversity, known as dysbiosis, increase the risk of developing sepsis (Prescott et al. 2015; Baggs et al. 2018). Furthermore, sepsis has a profound impact on the microbial communities within the gut (Fay et al. 2017; Adelman et al. 2020; Miller et al. 2021). In sepsis, altered immune response, along with critical care intervention such as the use of antibiotics and total parenteral nutrition, leads to the development of a "pathobiome" (Krezalek et al. 2016; Fay et al. 2017; Miller et al. 2021). The pathobiome is characterized by a loss in health-promoting Firmicutes populations and a relative increase in the number of potentially pathogenic gram-negative Proteobacteria (Fay et al. 2017). Sepsis-related changes to the microbiome also reduce SCFA concentrations. SCFAs are associated with protection in sepsis, and a decrease in SCFA concentrations is likely to contribute to organ damage (Adelman et al. 2020). Additionally, intestinal permeability is altered in sepsis, allowing for the dissemination of microorganisms and other inflammatory stimuli, which leads to local and distant injury (Fay et al. 2017). The impact of the microbiome on sepsis outcomes has made microbiome research a priority in sepsis research. Yet, much is still unknown about the impact of the microbiome in sepsis (Coopersmith et al. 2018).

Trained Immunity and the Microbiome

There is mounting evidence that the immune system and microbiome communicate and influence each other (Wannemuehler et al. 1982; Kiyono et al. 1982; Ivanov et al. 2008a; Gaboriau-Routhiau et al. 2009; Maslowski et al. 2009; Molloy et al. 2012; Tang et al. 2015;). Trained immunity may also influence the microbiome (McCoy et al. 2019; Negi et al. 2019). Dectin-1, the primary receptor for β-glucan, has been found to be an essential receptor in the

regulation of the gut microbiome (Iliev 2015; Li et al. 2019). Tang et al. found that Dectin-1 signaling promotes *Lactobacillus* in the gut, regulating T-cell expansion and contributing to colitis (Tang et al. 2015). Additionally, β-glucan signaling through Dectin-1 is involved in regulating inflammation in the intestines (Iliev 2015). Thus, β-glucan induced trained immunity may modulate microbial communities in the gut (Iliev 2015). The microbiome also alters bone marrow progenitors, which provides a potential mechanism for immune training on hematopoiesis (Negi et al. 2019). While a link between trained immunity and the microbiome has been speculated (McCoy et al. 2019; Negi et al. 2019), the effect of immune training on gut microbiota has not been directly addressed.

Hypothesis and Specific Aims

In the present study, we investigated the effects of trained immunity in aging and critical illness. Using animal models and human cells *ex vivo,* we identified the effects of β-glucan-induced immune training in innate immune cells in human aging and in a clinically relevant *in vivo* model of polymicrobial sepsis. Additionally, we uncovered the effects of trained immunity on the microbiome in aging and critical illness. The following are specific aims in our study:

Aim 1: Identify the effects of β-glucan-induced trained immunity on monocytes from aging individuals. These results are presented in Chapter 2.

Aim 2: Investigate the effect of trained immunity on sepsis and sepsis-induced immunosuppression. These results are presented in Chapters 2 and 3.

Aim 3: Identify the effects of trained immunity on the microbiome in aging and critical illness. These results are presented in Chapters 4 and 5.

CHAPTER 2. TRAINED IMMUNITY ENHANCES MONOCYTE FUNCTION IN AGING AND SEPSIS

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Abstract

It is well established that as humans age, our immune system becomes progressively weaker through a process called immunosenescence. Aging plays a critical role in the incidence of infection, with age emerging as an independent predictor of mortality in sepsis. Trained immunity reprograms immunocytes to respond more rapidly and effectively to pathogens. However, there is very little evidence of trained immunity in the aging immune system or in the presence of sepsis. In this study, we examined the impact of innate immune training on monocytes from aging healthy humans as well as sepsis patients. Using an *ex vivo* model, we found that the trained immunity phenotype can be induced in monocytes isolated from healthy subjects ≥60 years old as well as sepsis patients. We observed increased metabolic capacity, upregulated IL-6 and TNF-α secretion, increased H3K27 acetylation, and upregulation of crucial intracellular signaling in trained monocytes from healthy aging subjects. The response to trained immunity in healthy aging subjects was equivalent to the response of younger, i.e., 18 – 59 years, individuals. Additionally, we found that trained immunity induced a unique expression pattern of

cell surface markers in monocytes that was consistent across age groups. Trained monocytes were less likely to express CD16 and expressed lower levels of HLA-DR and CD40, which is associated with the M2 macrophage phenotype. Trained monocytes from sepsis patients also displayed enhanced metabolic capacity and increased production of TNF-α and IL-6. This result has implications for the prevention and treatment of infection and disease in the aging population.

Introduction

It has been estimated that over the next thirty years the number of individuals ≥60 years of age will more than double, increasing by over a billion; and the number of individuals over age 80 may increase by as much as 300 million (Sadighi Akha 2018). This dramatic increase in the number of aging individuals will result in a significant increase in age related diseases. It is well established that as humans age our immune system becomes progressively weaker through a process called immune senescence(Sadighi Akha 2018). This age-related decrease in immune function increases susceptibility to infection and chronic diseases (Cunha et al. 2020; Jackaman et al. 2017; Sadighi Akha 2018; Ventura et al. 2017a).

Sepsis is the leading cause of death in non-cardiac intensive care units (ICU) and accounts for 40% of ICU expenditures(Mantovani et al. 2011). Patients that survive sepsis have long-term physical and cognitive disabilities and are frequently re-admitted to the hospital with recurrent infections (Delano and Ward 2016a; 2016c; Fernandes Jr. and de Assuncao 2012; Wang et al. 2014). Over the past two decades there has been an increased incidence of sepsis(Mayr et al. 2014) and this trend is likely to continue due to our aging population, increased use of immunosuppressive drugs and invasive procedures and the emergence of antibiotic resistant opportunistic pathogens(Mantovani et al. 2011). Aging plays a critical role in the incidence of infection, with age emerging as an independent predictor of mortality in sepsis(Kline and Bowdish 2016; van Vught et al. 2016). Indeed, 60% of sepsis cases occur in patients over 65 years of age(Gs et al. 2006; Kline and Bowdish 2016). Despite the importance of aging in human disease there is still much that is not known about the relationship between aging and sepsis. However, it is generally accepted that age related immune senescence increases susceptibility to sepsis (Gs et al. 2006; Kline and Bowdish 2016). A major question is whether it

is possible to modulate the aging immune system such that it would be more resistant to infection. One possible approach to enhancing immune function during aging is innate immune training(Cheng et al. 2014b; Divangahi et al. 2020).

There is a substantial literature demonstrating that the innate immune system can be trained to respond more rapidly and effectively to a variety of infectious insults(Cheng et al. 2014a; Kleinnijenhuis et al. 2012; Moorlag et al. 2020a; Netea et al. 2020; Saeed et al. 2014). This is referred to as "trained immunity" or "innate immune memory"(Netea et al. 2020). Trained immunity is characterized by increased leukocyte metabolism and epigenetic reprogramming(Cheng et al. 2014a; Saeed et al. 2014). There is very limited information available on the effect of trained immunity in aging and/or sepsis. In 2011 Wardhana and colleagues reported that BCG vaccination prevented respiratory infections and improved cytokine production in individuals 60-75 years of age (Wardhana et al. 2011). It is now known that BCG induces the immune trained phenotype in humans(Cirovic et al. 2020; Moorlag et al. 2020c), thus it is reasonable to speculate that the effect of BCG on respiratory infections in aging subjects may be mediated, in part, by trained immunity. In this study, we examined innate immune training in monocytes isolated from healthy aging subjects and compared and contrasted their response to immune training with monocytes isolated from younger healthy individuals. We also examined innate immune training in monocytes derived from patients diagnosed with sepsis. We found that trained immunity increases metabolism and functionality of monocytes isolated from healthy aging subjects as well as in sepsis patients.

Materials and Methods

Isolation of human monocytes from healthy subjects. Whole blood containing EDTA was purchased from Biological Cell Specialty (Gray, Tennessee). The blood samples were de-identified except for age, gender, and race. Blood was collected from healthy individuals in 3 age groups, *i.e.* 20-30, 31-59, ≥60 years of age. The age range for the entire cohort was 20-71 years. A detailed description of the subjects is provided in **Table 1**. Monocytes were isolated from whole blood using immunomagnetic negative selection with the EasySep™ Direct Human Monocyte Isolation Kit (StemCell 19669). Monocyte purity was assessed by flow cytometry.

Table 1. Age, sex, and race breakdowns for healthy human subjects in each age group.

Group	Mean Age (stdev)	Female (%)	Male (%)	Caucasian (%)	Black (%)	Hispanic (%)	Asian
20-30	25.4 (3.0)	16 (45.7%)	19 (54.3%)	29 (82.9%)	2 (5.71)	4 (11.4%)	0 (0%)
31-59	41.8 (8.3)	13 (37.1%)	22 (62.9%)	31 (88.6%)	3 (8.6%)	1 (2.9%)	0 (0%)
60+	65 (3.1)	23 (65.7%)	12 (34.3%)	33 (94.3%)	0 (0%)	0 (0%)	2 (5.7%)
Combined	44.1 (17.2)	52 (49.5%)	53 (50.5%)	93 (88.57%)	5 (4.8%)	5 (4.8%)	2 (1.9%)

Immune training protocol. Monocytes were adjusted to 5 x 10^5 cells/mL in RPMI 1640 and treated with 10 μg/mL β-glucan, isolated from *Candida albicans* as previously described (Lowman et al. 2014). Isovolumetric PBS was used as a control. Glucan was washed out after 24h on day 1 (**Figure. 2A**), and media was replaced with RPMI 1640 supplemented with 10% human serum. The media was replenished at day 3. On day 7, the media volume was reduced, and cells were treated with 10ng/mL LPS (*Escherichia coli* O55:B5, Sigma L6529-1MG) for 24 hours. On day 8, the supernatants were harvested for cytokine analysis, and the cells were stained with antibodies for cell surface markers to be analyzed by flow cytometry.

Metabolic assays. Metabolic analysis of isolated monocytes was performed using the Agilent Seahorse XFp Analyzer (Santa Clara, CA). Cells were seeded at 1 x 10^5/well, and monocytes

were treated with 10 μg/mL β-(1,6)-Glucan for 24h on day 0. On day 1, the glucan was washed out, and media was replenished with RPMI 1640 supplemented with 10% human serum. On day 3, media was replaced. The assay was performed on day 7. The XFp Cell Energy Phenotype test template was used for the experiment. Oxygen consumption rate (OCR) was measured to approximate respiration, and Extracellular acidification rate (ECAR) was measured to approximate glycolysis.

Cytokine assays. TNF-α and IL-6 expression were analyzed by ELISA (TNA-α: BioLegend 430204, IL-6: BioLegend 430504). Cell culture supernatants were collected and stored at -80°C prior to analysis. ELISAs were performed following manufacturer instructions.

Cell surface markers. Immune trained and control cells were harvested, blocked using block buffer (PBS, 5% rabbit serum, 0.5% goat serum albumin, 5 mM EDTA, 0.1% NaN_3) and stained for flow cytometry using the following mouse anti-human antibodies: CD14 FITC, Clone TÜK4 (Bio-Rad MCA1568F); HLA-DR PerCP-Cy5.5, Clone G56-6 (BD Biosciences 560652); CD123 PE, Clone 9F5 (BD Biosciences 555644); CD11b/Mac-1 PE-Cy7, Clone ICRF44 (BD Biosciences 557743); CD40 APC, Clone 5C3 (BD Biosciences 555591); CD16 Pacific Blue™, Clone 3G8 (BD Biosciences 558122). All flow cytometry experiments were performed on a BD LSRFortessa flow cytometer. Cells were gated by forward scatter, side scatter, and CD14 positivity (**Figure 10b**). Cell gating was adjusted to account for glucan particles that remained from the initial treatment, given that some residual particles remain after multiple wash steps (**Figure 10a**). Mean fluorescence of isotype controls was subtracted from sample mean fluorescence to normalize between experimental replicates.

Intracellular signaling in immune training monocytes. Immune trained and control monocytes were adjusted to a density of 5 x 10^6 cells/mL in 12 x75 mm tubes in RPMI 1640. Cells were

treated with 10 μg/mL β-Glucan or RPMI as a control for 1 or 2 hours at 37°C. Cells were fixed, permeabilized and stained for 1 hr with the following antibodies: Alexa Fluor® 488 Mouse anti-Akt1, Clone 55/PKBa/Akt (BD Biosciences 560048; Alexa Fluor® 647 Mouse anti-Akt (pS473), Clone M89-61 (BD Biosciences 560343); PE Mouse Anti-mTOR (pS2448), Clone O21-404 (BD Biosciences 563489); PerCP/Cyanine5.5 anti-ZAP70 Phospho (Tyr319)/Syk Phospho (Tyr352) Antibody, Clone 1503310 (Biolegend 683710). Cells were gated for CD14 positivity (**Figure 9**). Mean fluorescence from fluorescence-minus-one controls was subtracted from the sample mean fluorescence to normalize between experimental replicates.

Monocyte histone modification in response to immune training. To assess H3K4 methylation, human monocytes were isolated and adjusted to a density of $1x10^6$ cells/mL in RPMI 1640, and $2\ x10^5$ monocytes were plated per well in a 96-well plate. Wells were treated with 10ug/mL β-glucan or PBS. Glucan was washed out after 24h on day 1, and media was replaced with RPMI 1640 supplemented with 10% human serum. The media was replenished at day 3. On day 7, cells were assayed for H3K4 methylation using the EpiQuik In Situ Histone H3K4 Methylation Assay Kit (EpiGentek P-3015-096). To assess H3K27 acetylation, $1\ x10^6$ human monocytes were plated in a 12-well plate at $0.5\ x\ 10^5$ cell/mL in RPMI 1640 as previously described. Wells were treated with 10ug/mL β-glucan or PBS. Glucan was washed out after 24 hrs on day 1, and media was replaced with RPMI 1640 supplemented with 10% human serum. The media was replenished at day 3. On day 7, histones were isolated using the EpiQuik Total Histone Extraction Kit (EpiGentek OP-0006-100) and frozen at -80°C. H3K27 acetylation was assayed using the EpiQuik Global Acetyl Histone H3K27 Quantification Kit (EpiGentek P-4059-96).

Monocytes from sepsis patients. Peripheral blood was obtained from sepsis patients at our participating hospital, Johnson City Medical Center, following informed consent (IRB study #

098-98s-MSHA). All patients met the Sepsis-3 criteria(Singer et al. 2016b). Peripheral blood was collected in BD Vacutainer® CPT™ (BD #362760) tubes. Mononuclear cells were isolated following manufacturer protocols. Monocytes were isolated from total mononuclear cells using the EasySep™ Human Monocyte Isolation Kit (StemCell #19359) following manufacturer instructions. Monocytes were cultured at 1 x 10^5 cells/mL in Agilent Seahorse XFp Cell Culture Miniplates in RPMI 1640 supplemented with 10% human serum. Monocytes were treated with 10 μg/mL β-glucan or vehicle control for 24 hours on day 0. After 24 hours, β-glucan was washed out and media replaced with fresh RPMI 1640 with 10% human serum on day 1. On day 3, the media was refreshed. On day 6, some wells were treated with 10 ng/mL LPS (*Escherichia coli* O55:B5, Sigma #L6529-1MG) for 24 hours. On day 7, supernatants were harvested for cytokine analysis, and the XFp Cell Energy Phenotype test was performed. The first 3 measurements taken were average to calculate baseline metabolism. After the three baseline measurements, 1um oligomycin (EMD Millipore #495455) and 1μm FCCP (Sigma #C2920) were injected, and the 5 subsequent measurements were averaged to calculate stressed metabolism. A detailed description of the included sepsis patients is provided in **Table 2.**

Table 2. Description of sepsis patients.

Patient	Age	Race	Sex	Type of Infection	SOFA	APACHE
1	48	White	Male	Staphylococcus aureus	0	Apache II score: 6 points (3% postoperative mortality, 8% nonoperative mortality)
2	68	White	Female	Staphylococcus aureus	2	Apache II Score: 9 points (3% estimated postoperative mortality, 8% estimated nonoperative mortality)
3	76	White	Male	Citrobacter species	3	Apache II Score: 13 points (7% est postoperative mortality)
4	78	White	Male	Enterobacteriaceae; Klebsiella species	3	Apache II Score: 8 points
5	23	White	Female	Sepsis due to Enterococcus faecalis **(12 days prior to consent)**	0	Apache II Score: 6 points

Statistical analysis. All statistical analysis was performed in GraphPad Prism 9. All data are presented as individual values or mean ± SEM represented by error bars unless otherwise specified. The effect of age and immune training was evaluated using a mixed-effect model in which all treatment conditions of a blood sample were paired. Šídák's multiple comparisons test was used for all post hoc tests. P=0.05 was used to determine the statistical significance of all measurements.

Results

Increased monocyte metabolism in response to immune training.

Cellular metabolism plays a significant role in immune cell function (Deleidi et al. 2015; O'Neill et al. 2016; Pence 2020). Decreases in respiration and glycolysis have been linked to declining immune systems in aging individuals (Pence 2020; Pence and Yarbro 2018; Weyand and Goronzy 2016). We found that immune training with β-glucan increased baseline and

stressed OCR in each age group (**Figure 2B**). Immune training also significantly increased baseline and stressed ECAR in all three age groups (**Figure 2C**). There was no significant difference in the metabolic response of immune trained monocytes across the age groups. We also examined the effect of immune training on monocyte metabolism as a function of sex (**Figure 7A** and **7B**) or age (**Figure 7CD)**. We observed no significant correlation as a function of age or sex (**Figure 7A-F**).

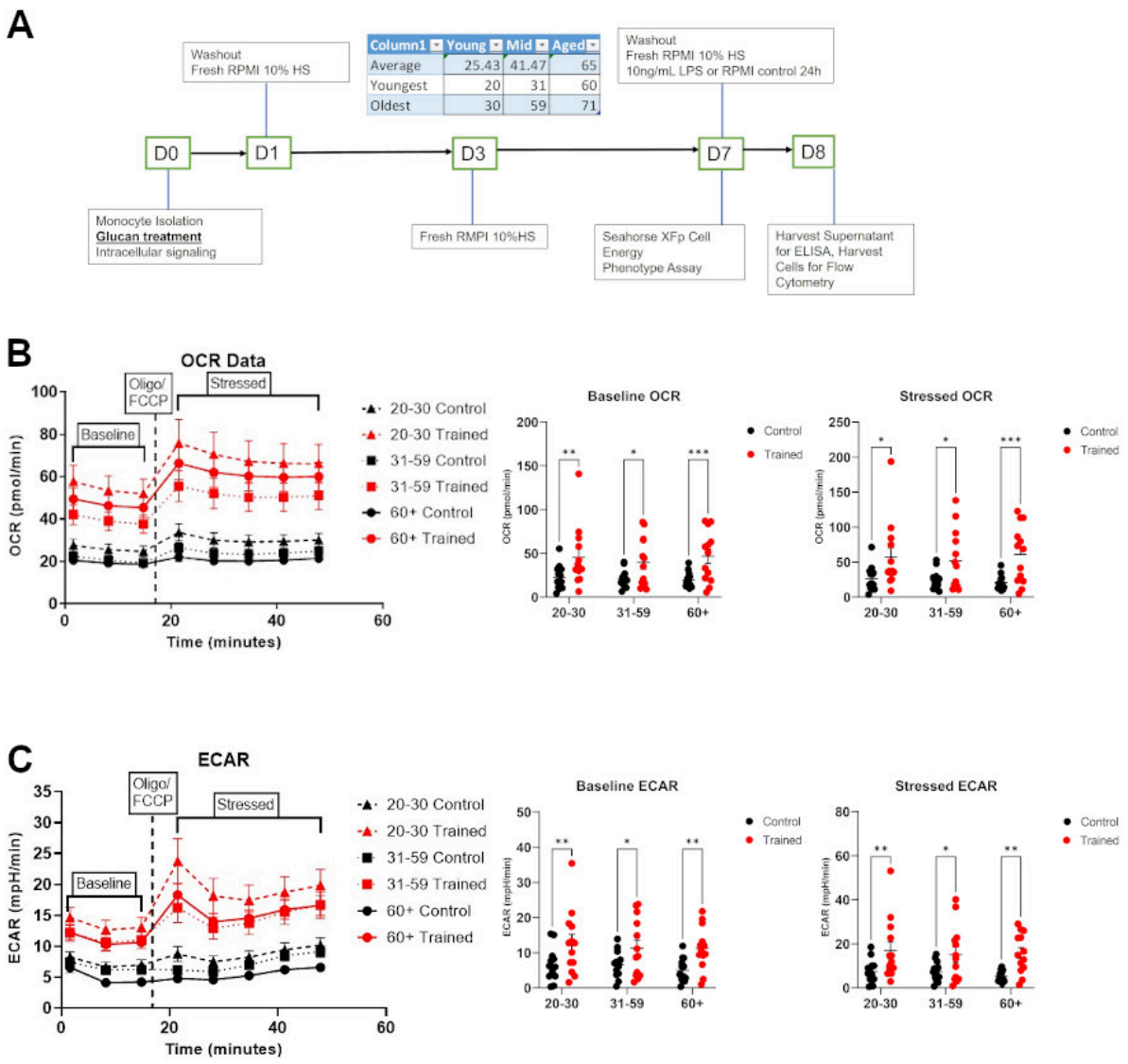

Figure 2. Immune training increases overall metabolic rate of monocytes across all age groups. (A) *Ex vivo* trained immunity protocol. **(B)** Baseline and stressed oxygen consumption rate (OCR) 7 days after β-glucan or PBS treatment. **(C)** Baseline extracellular acidification rate (ECAR) and stressed ECAR 7 days after β-glucan or PBS treatment. Mixed effect model and Šídák's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$) were used to compare **B** and **C** (N=13).

Effect of monocyte immune training on cytokine production.

To assess monocyte function, we measured IL-6 and TNF-α secretion following immune training. Increased cytokine production in response to immune training with β-glucan is hallmark of the immune trained phenotype (Cheng et al. 2014a; Ifrim et al. 2014; Saeed et al. 2014). We

found that immune training with β-glucan increased TNF-α expression in all three age groups (**Figure 3A**). There was no significant effect of age on TNF-α expression (**Figure 8C**), but TNF-α expression in trained cells was higher in males compared to females (**Figure 8A).** IL-6 expression was also increased by immune training across all age groups (**Figure 3B**). IL-6 expression positively correlated with age in untrained cells; however, this effect was abolished by immune training (**Figure 8D**). There was no significant effect of sex on IL-6 expression (**Figure 8B)**.

β-glucan induced immune training induced epigenetic changes in monocytes. Increased H3K4 methylation and H3k27 acetylation are associated with immune training and improved response to infection (Saeed et al. 2014). We found that immune training significantly altered monocyte H3K4 methylation (**Figure 3C**). However, only H3K4 methylation in the 20-30 age group was significantly increased (**Figure 3C**). We also observed that immune training had a significant effect on H3K27 acetylation (**Figure 3d**). Interestingly, H3K27 acetylation was significantly increased in immune-trained cells in the 60+ age group, but not in the younger groups (**Figure 3d**). Thus, we observed a differential effect of β-glucan induced histone modification in that H3K4 methylation was increased in monocytes derived from healthy young individuals, while H3K27 acetylation was significantly increased in monocytes from aging individuals.

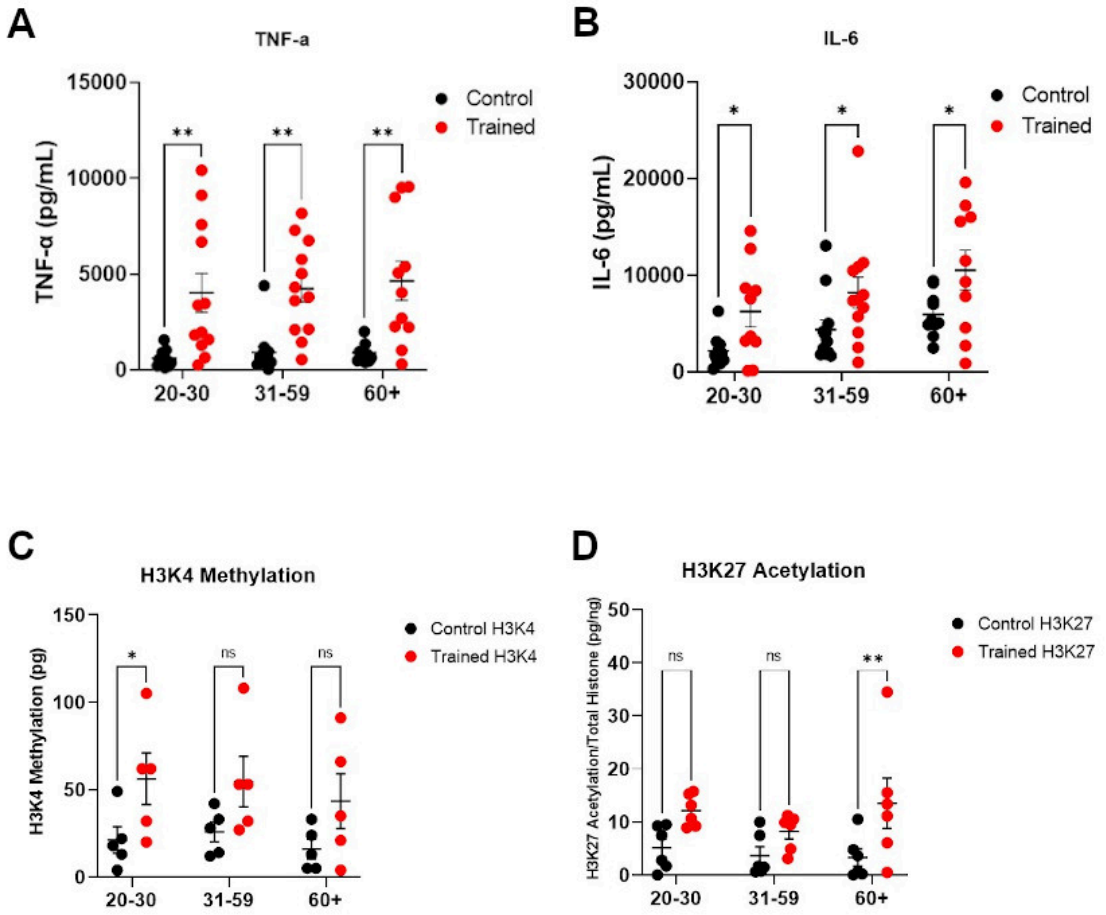

Figure 3. Immune training stimulated increased monocyte cytokine expression and histone modifications. (A) Scatter plot of TNF-α expression 7 days after β-glucan or PBS treatment, followed by LPS (N=11,12). **(B)** IL-6 expression (N=10-12). **(C)** H3K4 methylation measured *in situ* 7 days after immune training with β-glucan (N=5). **(D)** Histone H3K27 methylation 7 days after immune training with β-glucan (N=6). All measured were analyzed with a mixed effect model and Šídák's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$).

Activation of intracellular signaling pathways in response to immune training.

Cheng and colleagues have shown that β-glucan induced immune training is mediated by an Akt/mTor/HIF1-α dependent mechanism (Cheng et al. 2014b). Dectin-1 is one of the primary receptors for β-glucan (Brown et al. 2003; Goodridge et al. 2009) and may be required for β-glucan mediated trained immunity (Cheng et al. 2014a). Syk tyrosine kinase is an important

downstream mediator of Dectin-1 dependent signaling and is critical in Dectin-1 mediated immune responses (McSai et al. 2010). We examined the effect of immune training, as a function of age, on Syk, Akt and mTor dependent signaling. We found that phosphorylated Syk was increased in monocytes treated for 1 hour with β-glucan across all age groups (**Figure 4A**). However, only trained monocytes from young individuals expressed significantly higher levels of phosphorylated Syk at 2 hrs stimulation (**Figure 4A**). We found that AKT phosphorylation was upregulated in monocytes treated for 1 hour with β-glucan across all age groups. Monocytes from the 20-30 and 31-59 age groups that were trained with β-glucan for 2 hrs showed significantly higher phosphorylated AKT (**Figure 4B**). However, this effect was not observed in the 60+ age group. Phosphorylated mTOR was significantly increased in monocytes treated with β-glucan for one or two hours across all age groups (**Figure 4C**). Total AKT expression was significantly increased in β-glucan trained monocytes at 1 and 2 hrs (**Figure 4D**). The flow cytometry gating strategy for the intracellular signaling studies is shown in **Figures 9 and 10**.

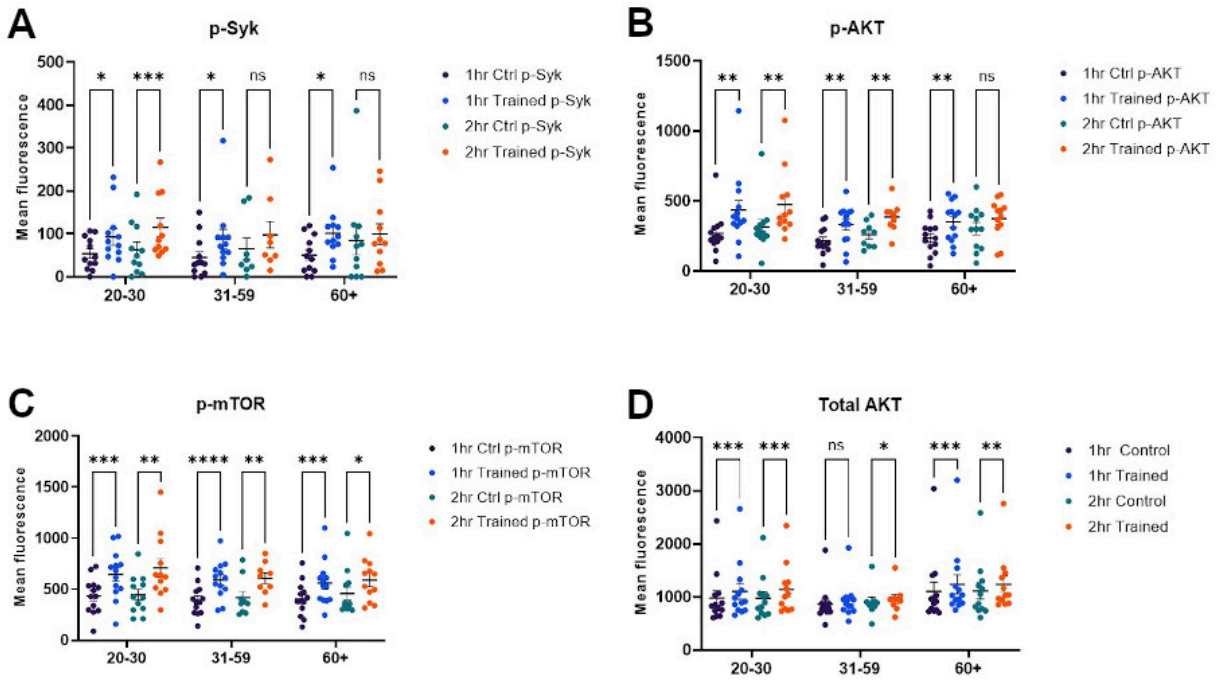

Figure 4. Immune training activates intracellular signaling pathways in monocytes. (A) Scatterplot depicting phosphorylated-Syk 1 and 2 hours after β-glucan or PBS treatment (N=8-12). **(B)** Scatterplot depicting phosphorylated-AKT expression (N=9-13). **(C)** Scatterplot depicting phosphorylated-mTOR (N=9-13). **(D)** Scatterplot depicting total AKT expression (N=9-13). All results analyzed using a mixed-effect model and Šídák's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

Effect of immune training on monocyte phenotype as a function of age.

Previous studies have demonstrated that age plays a significant role in the phenotype of CD14+ monocytes (Crooke et al. 2019; Metcalf et al. 2015; Metcalf et al. 2017). In addition, immune training with β-glucan has been reported to alter the expression of cell surface markers on monocytes (Ifrim et al. 2014). We measured the expression of cell surface markers in trained and control monocytes in the presence and absence of LPS secondary stimulation. We found that immune-trained cells in the 20-30 and 60+ age groups showed lower levels of CD16+ cells, indicating a higher relative abundance of intermediate or non-classical monocytes (CD14+, CD16+) (Seidler et al. 2010). Interestingly, this effect was not observed after LPS stimulation

(**Figure 5a**). CD11b/Mac-1 expression was only significantly altered in trained monocytes from the 20 – 30 age group and only following LPS stimulation (**Figure 5B**). CD123 expression was not altered in immune trained monocytes (**Figure 5C**). In contrast, immune training significantly decreased monocyte cell surface HLA-DR (**Figure 5D**). CD40 was significantly decreased by immune training in monocytes from the 60+ age group (**Figure 5E**). In the presence of LPS secondary stimulation, CD40 was decreased in all three age groups (**Figure 5E**).

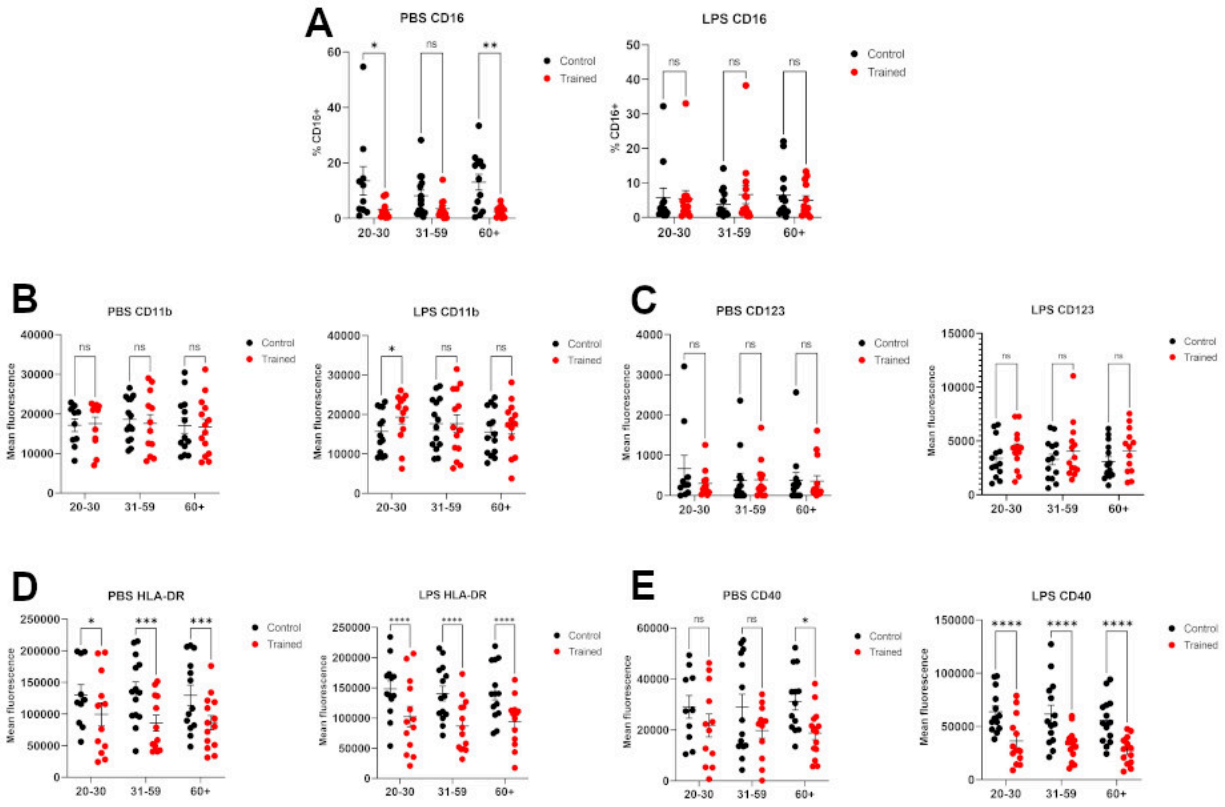

Figure 5. Immune training alters monocyte phenotype. Isolated human monocytes were trained with β-glucan or vehicle control and allowed to rest for 7 days. After 7 days, monocytes were treated with LPS (10ng/mL) or PBS for 24 hours. Cell surface markers were analyzed by flow cytometry. **(A)** Scatterplot depicting the percent of isolated CD14+ monocytes expressing CD16 in LPS-stimulated and unstimulated cells. **(B)** Mean fluorescence of CD11b/Mac-1. **(C)** Mean fluorescence of cell surface CD123. **(D)** Mean fluorescence of cell surface HLA-DR. **(E)** Mean fluorescence of cell surface CD40. All results analyzed using a mixed effect model and Šídák's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) (N=10-14).

Training monocytes from sepsis patients.

The data above clearly show that monocytes from healthy aging individuals can be trained in response to β-glucan. However, there is no data available on the ability of monocytes from septic patients to exhibit the immune trained phenotype. Therefore, we isolated monocytes from patients meeting the broad criteria of Sepsis-3. Monocytes isolated from septic patients were trained with β-glucan employing the protocol in Fig. 1A and allowed to rest for 7 days. After 7 days, monocytes were assayed for OCR to approximate respiration and ECAR to approximate glycolysis using the Agilent XFp Cell Energy Phenotype Test protocol. Trained septic monocytes had significantly higher baseline and stressed OCR than control septic monocytes (**Figure 6A**). Baseline and stressed ECAR were also increased in trained septic monocytes (**Figure 6B**). Additionally, immune trained monocytes from sepsis patients upregulate TNF-α (**Figure 6C**) and IL-6 (**Figure 6D**) upon LPS stimulation. These data clearly show that monocytes from septic patients are capable of exhibiting the immune trained phenotype and their functionality is increased, in a fashion similar to healthy aging subjects.

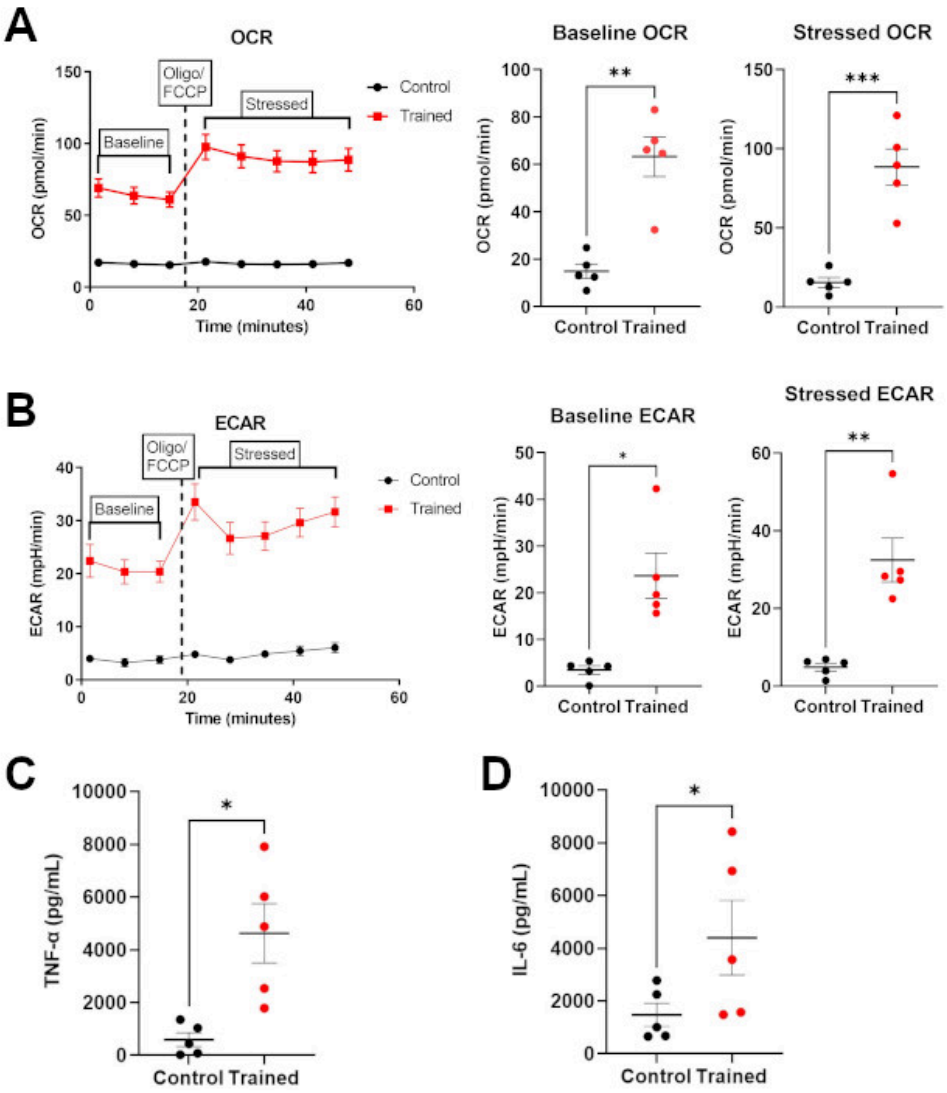

Figure 6. Immune training with β-glucan induces metabolic changes and cytokine expression in monocytes from sepsis patients. (**A**) Baseline and stressed OCR 7 days after β-glucan or PBS treatment. (**B**) Baseline and stressed ECAR 7 days after β-glucan or PBS treatment. (**C**) TNF-α expression 7 days after β-glucan or PBS treatment. **(D)** IL-6 expression. All measures were analyzed using a paired t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) (N=5).

Discussion

In the present study, we utilized an *ex vivo* approach to demonstrate that the aging immune system can exhibit immune training. We found that monocytes from healthy subjects ≥60 years old display the trained immunity phenotype after exposure to β-glucan. Immune-trained monocytes in aging individuals showed higher metabolic capacities, increased IL-6 and TNF-α production, and increased expression of epigenetic markers associated with the trained immunity phenotype (Cheng et al. 2014a; Saeed et al. 2014). In addition, we also found that monocytes from sepsis patients displayed the immune trained phenotype following β-glucan exposure. To the best of our knowledge, this is the first documentation of innate immune training in aging and sepsis. Our results suggest that the aging immune system can be trained to respond to infection more rapidly and effectively.

In addition to characterizing the trained immunity phenotype in β-glucan treated monocytes from aging individuals, we also examined the intracellular signaling pathways associated with the trained phenotype in aging monocytes. Previous studies have found that Dectin-1 signaling and mTOR activation are crucial in eliciting immunologic protection provided by trained immunity (Cheng et al. 2014a). Increased Syk phosphorylation after β-glucan exposure is consistent with Dectin-1 signaling (Goodridge et al. 2009). We also found up-regulation of total AKT, p-AKT, and p-mTOR, which supports this signaling pathway's role in inducing trained immunity across all age groups. There was no effect of age on the expression levels of these key signaling molecules. When considered alongside the previously described results, this indicates that pathways crucial to the induction of trained immunity remain viable in healthy aging individuals.

Lastly, immune training led to a relative reduction in $CD14^+/CD16^+$ cells. This population of monocytes is known to be expanded in elderly individuals and is thought to represent a less differentiated population of cells (Safi et al. 2016). Additionally, we found that immune training decreased expression of the activation markers HLA-DR and CD40 regardless of age. Decreased expression of HLA-DR and costimulatory molecules is suggestive of the M2 macrophage phenotype (Leonhardt et al. 2018b). This result is supported by a study by Leonhardt et al., which found that β-glucan induced monocyte differentiation towards an M2 phenotype (Leonhardt et al. 2018b). Furthermore, this may help to explain the upregulation of the anti-inflammatory cytokine, IL-10, in trained immunity which has been previously described (Garcia-Valtanen et al. 2017), since IL-10 expression is a hallmark of the M2 macrophage. This result suggests that trained macrophages may also play a role in limiting host damage during infection (Leonhardt et al. 2018b).

There are some limitations to our study. We employed an *ex vivo* monocyte model. Thus, we cannot know how immune training would affect other cell types in elderly or septic individuals. We also did not address how long trained immunity may last in the 60+ age group. Our model is not suited for longer-term studies as monocyte viability decrease after 7 days in culture (Patel et al. 2017; Safi et al. 2016). Finally, the sample size in our studies were small, which may partially explain the lack of statistical significance concerning epigenetic modifications in some age groups. However, we did find that monocytes from aging subjects and sepsis patients were capable of displaying the trained phenotype.

In conclusion, this study confirms that innate immune training can be induced in elderly individuals as well as critically ill sepsis patients. We found that innate immune training can be induced regardless of age. This suggests that innate immune training may be a viable

prophylactic and/or therapeutic approach to preventing and treating infection in vulnerable populations, such as the elderly.

Supplementary figures

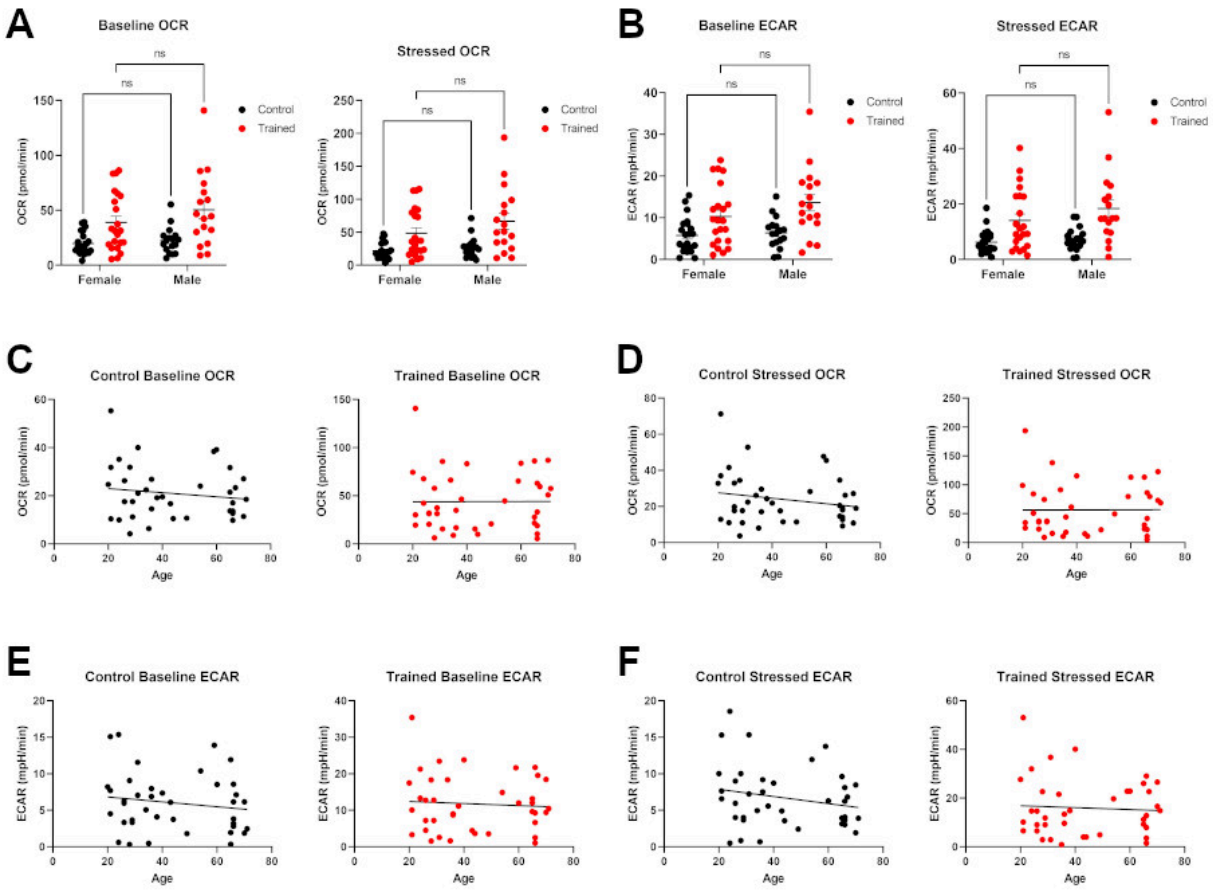

Figure 7. Effect of sex and age on cell metabolism (supplementary). Scatter plot of baseline and stressed oxygen consumption rate 7 days after β-glucan or PBS treatment (Female: N=22, Male: N=17). **(B)** Baseline extracellular acidification rate (ECAR) and stressed ECAR 7 days after β-glucan or PBS treatment (Female: N=22, Male: N=17). **(C-F)** Simple linear regression of baseline and stressed OCR and ECAR. No correlations are significant (N=13). **A** and **B** were analyzed by 2-way ANOVA and Šídák's multiple comparisons test for sex and training.

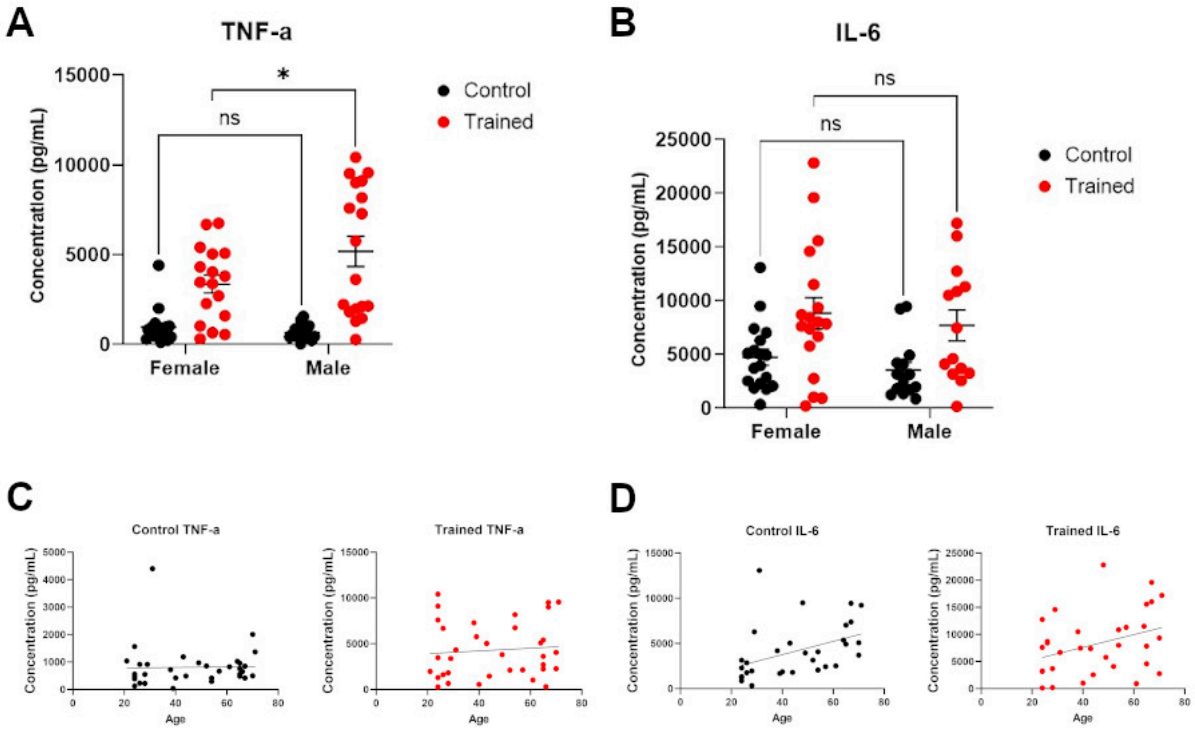

Figure 8. Effect of sex and age on cytokine expression (supplementary). (A) Scatter plot of TNF-α expression after LPS stimulation (10ng/mL) 7 days after β-glucan or PBS treatment (* $P \leq 0.05$, Female: N=18, Male: N=14). **(B)** IL-6 expression after LPS stimulation (Female: N=17, Male: N=14). **(C)** Scatter plot and simple linear regression of TNF-α expression by age in control and trained cells. Correlations are not significant (N=35). **(D)** Scatter plot and simple linear regression of IL-6 expression in trained and control cells. Control cells: R^2=0.1792, P=0.0158 (N=32). Trained cells: R^2=0.0.1203, P=0.0518 (N=32). **A** and **B** were analyzed by 2-way ANOVA and Šídák's multiple comparisons test for sex and training.

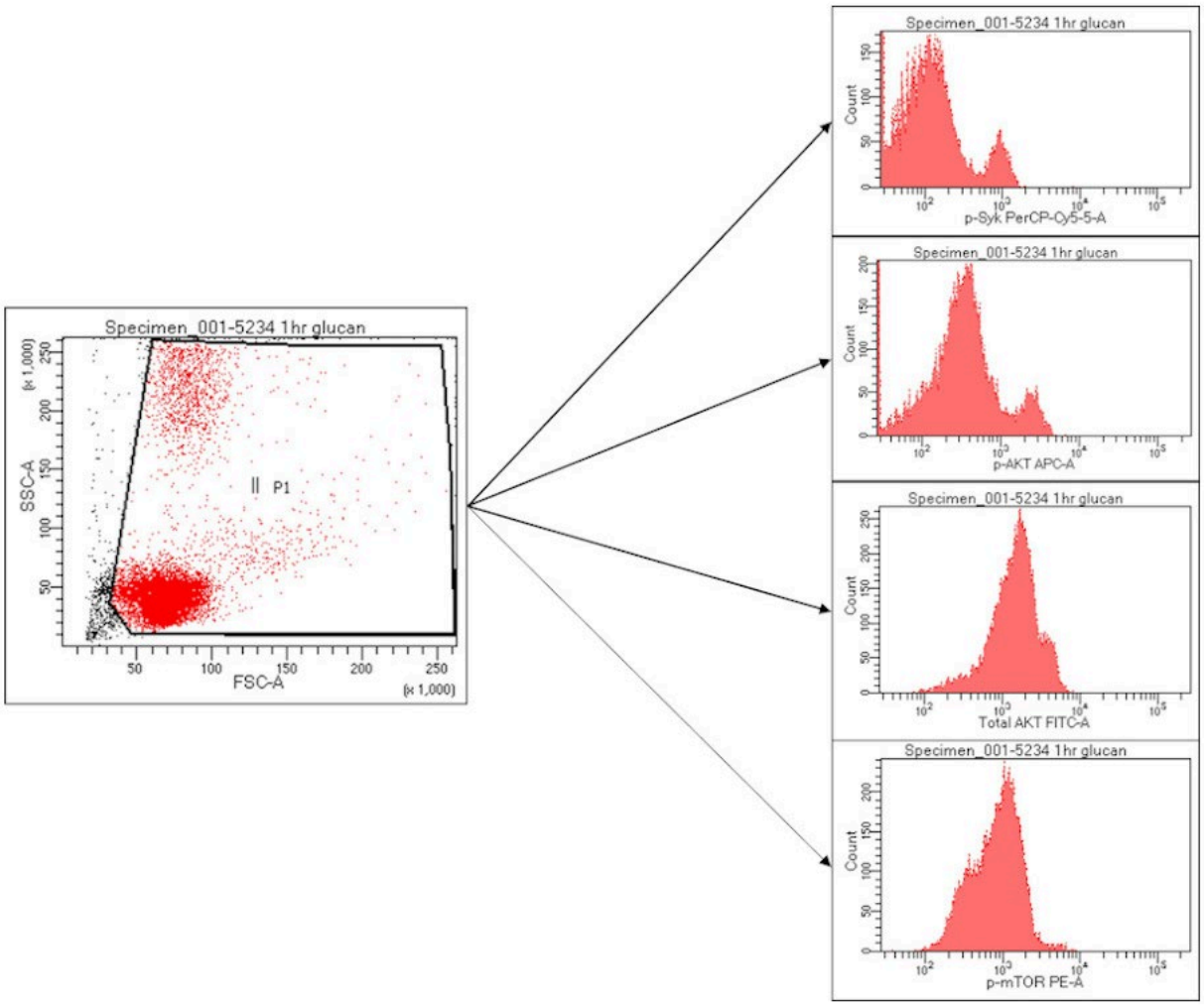

Figure 9. Flow cytometry gating strategy for intracellular signaling experiments (supplementary). Cells isolated using magnetic separation for CD14+ monocytes were gated by FSC and SCC to remove debris. Mean fluorescence of intracellular signaling proteins in this population was used as an experimental measure.

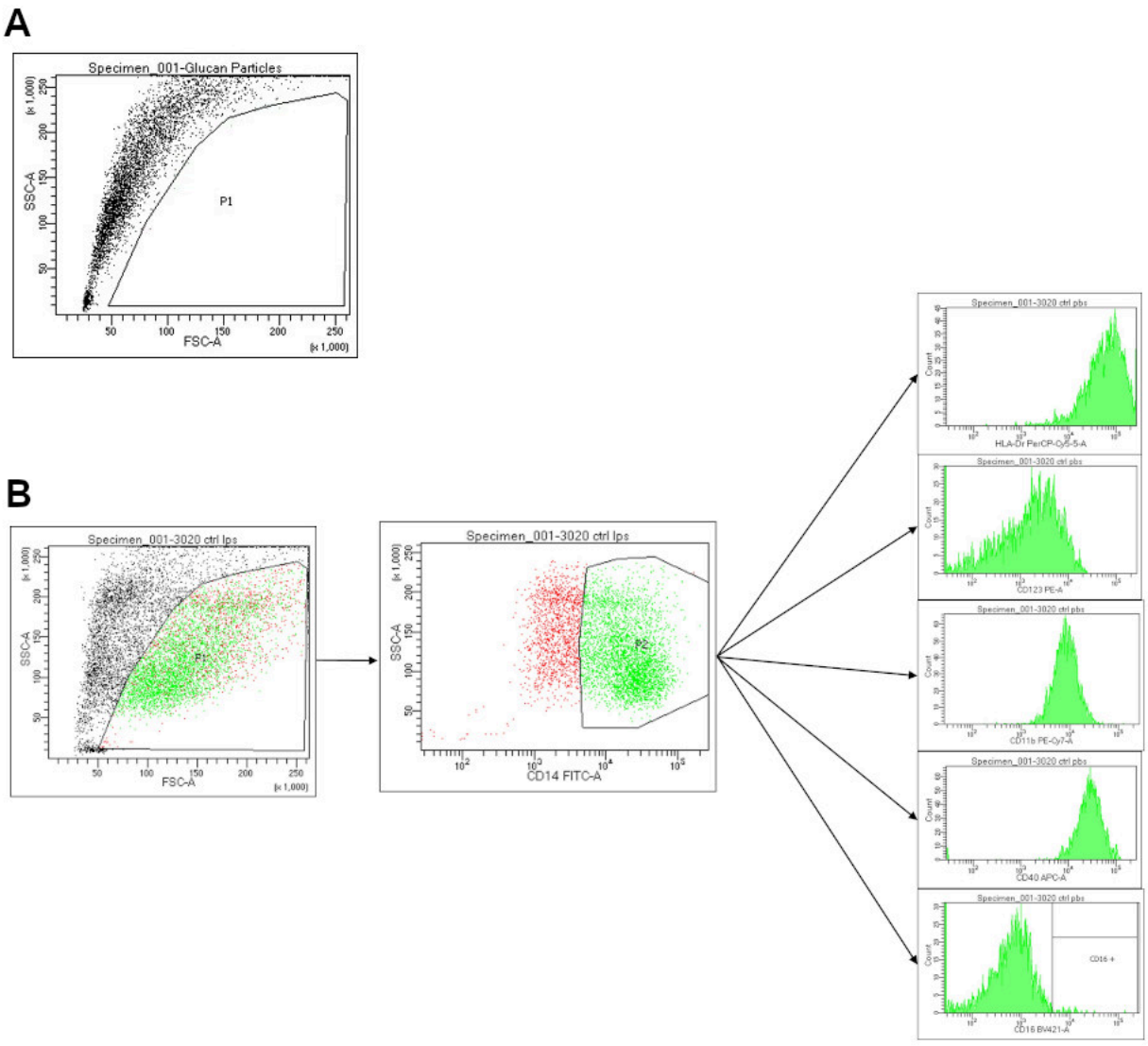

Figure 10. Flow cytometry gating strategy for cell surface phenotype experiments (supplementary). (A) P1 population was set to exclude residual β-glucan particles that could not be removed in wash steps. **(B)** After excluding β-glucan particles, cells were gated for CD14 positivity using fluorescence minus one and isotype controls. Mean fluorescence was used to measure HLA-DR, CD123, CD11b/Mac-1, and CD40. CD16 positive cells were also measured, and gaiting for positivity was determined using FMO and isotype controls.

Acknowledgements

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CHAPTER 3. IMMUNE TRAINING INDUCES A SUBSET OF LARGE CD11B^{+}, GR-1^{HI} CELLS IN A CHRONIC MODEL OF SEPSIS

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Abstract

Sepsis is among the leading causes of death in the US and around the world, presenting a significant challenge to healthcare systems. Sepsis is characterized by organ dysfunction resulting from a dysregulated immune response to infection. Patients who survive the initial hyperinflammatory phase of sepsis often develop long-lasting immunosuppression that increases their vulnerability to recurring infections. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells that have immunosuppressive functions. MDSCs are known to increase in sepsis patients; however, the role of MDSCs in sepsis is incompletely understood. Recent studies have found that treatment with β-glucans "trains" the innate immune system to respond more quickly and effectively to infection. This phenomenon is known as trained immunity, and it is associated with improved immune responsiveness to infection. Using a chronic polymicrobial animal model of sepsis, we found that immune training increases a population of CD11b^{+}, GR-1^{hi}, FSC^{hi} cells in the blood of septic mice 15 days following cecal ligation and puncture (CLP). CD11b^{+}, GR-1^{hi}, FSC^{hi} cells composed 5.6% of total blood leukocytes in trained septic mice compared to only 3.4% of total blood leukocytes in control septic mice. Immune training did not increase CD11b^{+}, GR-1^{hi}, FSC^{hi} cells in sham mice. Additionally, CD11b^{+}, GR-1^{hi}, FSC^{hi} cells express high levels of PD-L1 and low levels of MHC

class II, suggesting an immunosuppressive phenotype. Our results indicate that MDSCs may play a role in immune training and an immunomodulatory role in sepsis.

Introduction

The Sepsis-3 conference defines sepsis as "life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al. 2016b)." Sepsis incidence is increasing due to an aging population and better clinical recognition of the condition (Dellinger et al. 2013; Singer et al. 2016b). According to a 2017 study, sepsis is the most costly condition in US hospitals, costing nearly $40 billion, accounting for almost 9% of all hospital costs(Liang et al. 2006). Globally, sepsis presents an especially critical healthcare issue. There were an estimated 48.9 million sepsis cases and 11 million deaths from sepsis worldwide in 2017(Rudd et al. 2020). Over the past four decades, there have been over 100 Phase II and Phase III clinical trials aimed to improved sepsis outcomes(Marshall 2014). However, these studies have largely failed to identify new treatments for sepsis, and improved sepsis mortality can be attributed to improved recognition of the condition and more timely treatment(Cavaillon et al. 2020).

Sepsis patients experience a hyper-inflammatory immune response (D'Elia et al. 2013; Huang et al. 2019) and prolonged immunosuppression (Hotchkiss et al. 2013b; Venet and Monneret 2018) that make treatment and recovery difficult. The hyper-inflammatory phase of sepsis is characterized by activation of pattern recognition receptors (PRR), such as toll-like receptors (TLRs), Nod-like receptors, and C-type lectin receptors (Anwar et al. 2013; Raymond et al. 2017). Activating PRRs by pathogen-associated molecular patterns (PAMPs) induces the secretion of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Medzhitov 2007). This pro-inflammatory response is crucial to the clearance of infection; however, in sepsis, this response is dysregulated. Additionally, cellular damage caused by the inflammatory response in

sepsis releases damage-associated molecular patterns (DAMPs), further stimulating an inflammatory response in sepsis (Raymond et al. 2017). However, treatments aimed at suppressing the inflammatory cytokine response have been ineffective and more harmful in some cases (Cohen et al. 2012; Hotchkiss et al. 2013a). Over 70% of sepsis deaths occur more than three days after onset(Hotchkiss et al. 2013a). While the leading cause of death in sepsis is organ failure, those who die from sepsis often have active infections in many organ systems (Torgersen et al. 2009), which indicates a dysfunctional immune response. Furthermore, survivors of sepsis have long-lasting immunosuppression, including reduced cytokine secretion (Arens et al. 2016), leukocyte depletion (Hotchkiss et al. 2013b), and an increase in myeloid-derived suppressor cells (MDSCs) (Delano et al. 2007). Sepsis-induced immunosuppression leaves patients susceptible to recurring nosocomial infections long after the initial insult has subsided (Boomer et al. 2011).

Recent breakthrough studies have illustrated that the innate immune system can be "trained" to better respond to infection (Cheng et al. 2014a; Ifrim et al. 2014; Saeed et al. 2014). Termed trained immunity or innate immune memory, this phenomenon is characterized by an increased reliance on aerobic glycolysis, epigenetic remodeling, and increased expression of pro-inflammatory cytokines that occurs upon exposure to specific pathogens or PAMPs (Cheng et al. 2014a). Immune training can result in a more effective immune response to a broad range of pathogens (Netea et al. 2020). One of the most potent inducers of trained immunity is β-glucan, which induces trained immunity through a Dectin-1 dependent mechanism (Cheng et al. 2014a). Rieber et al. found that Dectin-1 signaling also induces a subset of MDSCs that is protective against fungal infection by *C. albicans(Rieber et al. 2015)*. Thus, we hypothesize that immune training with glucan will induce MDSCs in sepsis.

Materials and Methods

Animals: Male c57BL/6J mice were purchased from Jackson labs and housed in the East Tennessee State University Division of Laboratory Animal Resources. Mice were fed a standard chow diet and given water *ad libitum*. Mice were between 15 and 26 weeks old at the time of experiments.

Glucan phosphate: Glucan phosphate was prepared from *Saccharomyces cerevisiae* as previously described(Williams et al. 1991). Lyophilized glucan-phosphate was depyrogenated, solubilized and filter sterilized in phosphate-buffered saline (PBS) and further diluted in dextrose 5% in water (D5W). Diluted glucan phosphate was administered by intraperitoneal (IP) injection at 40mg/kg 1 hour before cecal ligation and puncture (CLP). An equal volume injection of 0.5 mL D5W was given as a control.

Cecal Ligation and Puncture: Mice were anesthetized using isoflurane, and cecal ligation and puncture was performed as previously described (Baker et al. 1983) with modifications. In this cecal ligation and puncture model, one quarter of the cecum was ligated, a single puncture was made to the cecum using a 25g needle and the incision was closed in two layers. Imipenem antibiotics (5 mg) in 1 mL saline were administered subcutaneously immediately after the procedure and were given daily for 3 days following CLP. For sham mice, mice were anesthetized, an incision was made, and the cecum was exteriorized from the abdomen and then replaced without ligation or puncture. Sham mice received antibiotics and saline immediately after the procedure. The 14-day survival was monitored. Body weight and temperature were monitored daily for the duration of the study.

Flow cytometry: Blood and spleens were harvested from mice at 7 and 15 days after CLP (**Figure 11A**). Red blood cells were lysed and leukocytes stained with 2 panels of antibodies. Set

1 consisted of CD-3 Pacific Blue (BD #558214), CD19-FITC (BD #553785), PD-1 PE (Biolegend #114118), IL7R APC (BD #564175), and PD-L1 BV-711 (Biolegend #124319). Set 2 consisted of Ly-6B.2 FITC (BioRad #MCA771FB), MHC-II PE (Biolegend #107608), GR-1 PerCP-Cy5 (BD #552093), CD11b PE-Cy7 (BD #552850), F4/80 AF647 (BD #565853), and PD-L1 BV-711 (Biolegend #124319). Myeloid cells and lymphocytes were differentiated by forward scatter (FSC) and side scatter (SSC). Cells were then gated into the following distinct populations: $CD11b^{+}$, $GR\text{-}1^{hi}$, MDSC ($CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi}) (**Figure 11B**), Neutrophils ($Ly6\text{-}B.2^{+}$, $GR\text{-}1^{+}$), Monocytes/Macrophages (mono/mac) ($F4/80^{+}$, $CD11b^{+}$) (**Figure 11E**), T-cells ($CD3^{+}$, $CD19^{-}$), and B-cells ($CD19^{+}$, $CD3^{-}$) (**Figure 11D**). Cells from each treatment group were also stained with isotype control antibodies matched to each marker antibody. Flow cytometry gates were set using isotype controls.

Co-culture experiments: 22-week-old male C57BL/6J mice were used for these experiments. Mice underwent an accepted trained immunity protocol (Cheng et al. 2014a). Mice were injected with 1 mg β-glucan isolated from *C. albicans* as previously described (Lowman et al. 2014) or an equal volume of D5W on days -7 and -4. On day 0, all mice were injected with 1mg/kg of LPS to generate MDSCs. 1 mg/kg was chosen because an inflammatory immune response is induced, but 100% of mice survived that dose(Lew et al. 2013; Sardari et al. 2020). 7 days after LPS injection, mice were humanely euthanized, and their spleens were harvested. $CD11b^{+}$, $GR\text{-}1^{+}$ cells were isolated using the EasySep™ Mouse MDSC isolation kit (StemCell #19867). FSC^{hi} cells were selected using FACS. Cells density was adjusted in DMEM containing 9% NCS, 1% FBS, and 1% Penicillin/Streptomycin (Fisher #BW17-602E). CD11b+, GR-1+ cells, and MDSCs were pooled by treatment group. Additionally, T-cells were isolated from spleens harvested from untreated, age-matched mice using EasySep™ Mouse T Cell Isolation kit

(StemCell #19851A) and cultured in DMEM. T-cells were stimulated by seeding 100,000 T-Cells in 96-well cell culture plates coated with LEAF anti-mouse CD3 (Biolegend #100331) and treating cells with 1ug/ml LEAF anti-mouse CD28 (Biolegend #102112)(Unprimed t cell activation - antibody stimulation methods | bio-rad). $CD11b^{+}$, $GR\text{-}1^{hi}$ cells and MDSCs were seeded along with T-cells at the following ratios: $CD11b^{+}$, $GR\text{-}1^{hi}$ 1:2, 1:5; MDSCs 1:5 and 1:10. Cells were allowed to incubate at 37°C for 24 hours, and supernatant IFN-ɣ was assayed by ELISA.

Cytokine assays. IFN-ɣ expression was analyzed by ELISA (Biolegend 430804). Cell culture supernatants were collected and stored at -80°C prior to analysis. ELISA was performed following manufacturer instructions.

Statistical analysis: All statistical analysis was conducted using GraphPad Prism 9. The survival plot (**Figure 11E**) was analyzed using a log-rank test. All comparisons of more than 2 groups were conducted using an ordinary one-way ANOVA and Tukey's multiple comparisons test. Comparison of $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cell populations 7 and 15-days after CLP were conducted using a paired-T test. Comparisons of treatments in co-culture assays were not compared statistically due to the low sample size. All data are presented as means ± SEM unless otherwise specified.

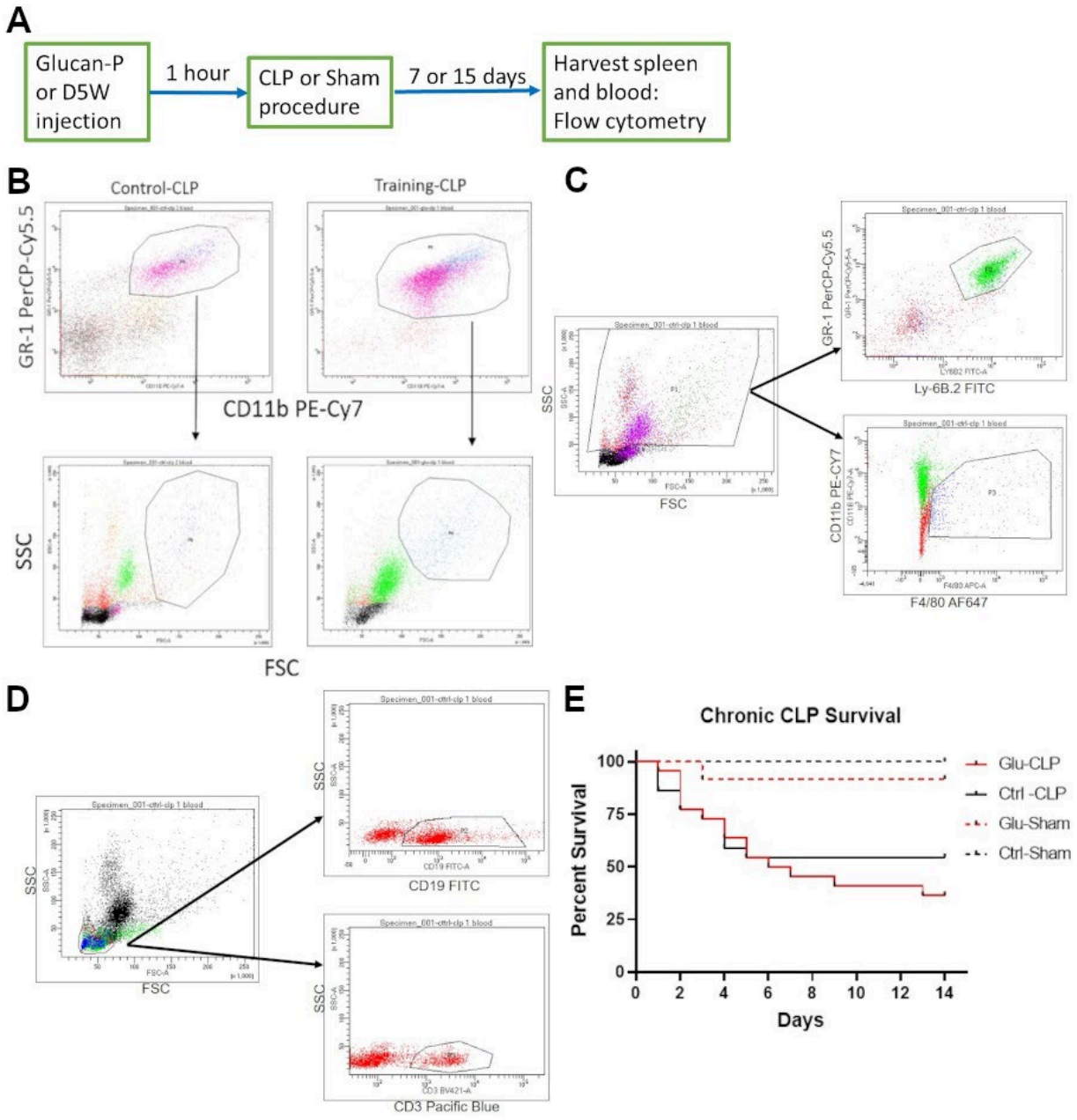

Figure 11. Methods and CLP model survival. (**A**) Male c57BL/6J mice were administered 40mg/kg glucan-phosphate or D5W 1 hour before CLP or sham procedure. Blood and spleens were harvested for analysis 7 or 15-days following CLP. (**B**) Gating strategy for $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells. (**C**) Gating strategy for neutrohpils ($Ly6\text{-}B.2^{+}$, $GR\text{-}1^{+}$) and monocytes/macrophages ($F4/80^{+}$, $CD11b^{+}$). (**D**) Gating strategy for B-cells ($CD19^{+}$) and T-cells ($CD3^{+}$). (**E**) Survival plot for the chronic model of CLP utilized in this study. CLP: N=22; Sham: N=12 **C** analyzed by Log-rank test.

Results

Sepsis and trained immunity increase CD11b+, GR-1hi, FSChi cells in a chronic model of polymicrobial sepsis.

Sepsis survivors often experience prolonged organ and immune dysfunction and experience recurring infection(Hotchkiss et al. 2013b). To assess the effect of immune training on sepsis survivors, we utilized a chronic model of polymicrobial sepsis in adult-aged mice. This model has a 54% survival rate in control mice. We did not find a difference in survival rate between the glucan-phosphate treated mice and control CLP mice (**Figure 11E**). We harvested spleens and blood and assessed leukocyte populations by flow cytometry to assess the cellular response to sepsis and immune training. We found that CLP significantly increased a unique population of myeloid cells that are $CD11b^{+}$, $GR\text{-}1^{hi}$, and FSC^{hi} (**Figure 12A**). The same population of cells was also significantly increased in circulation, and this effect was further enhanced by immune training with glucan-phosphate (**Figure 12B**). $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells were significantly more abundant 15 days after CLP in animals that received glucan-phosphate than they were at 7 days (**Figure 12C**). Corresponding with increased abundance of $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells and neutrophils, splenic T-cell abundance was significantly decreased in septic mice 15 days following CLP. This effect was not present in circulation (**Figure 12D**).

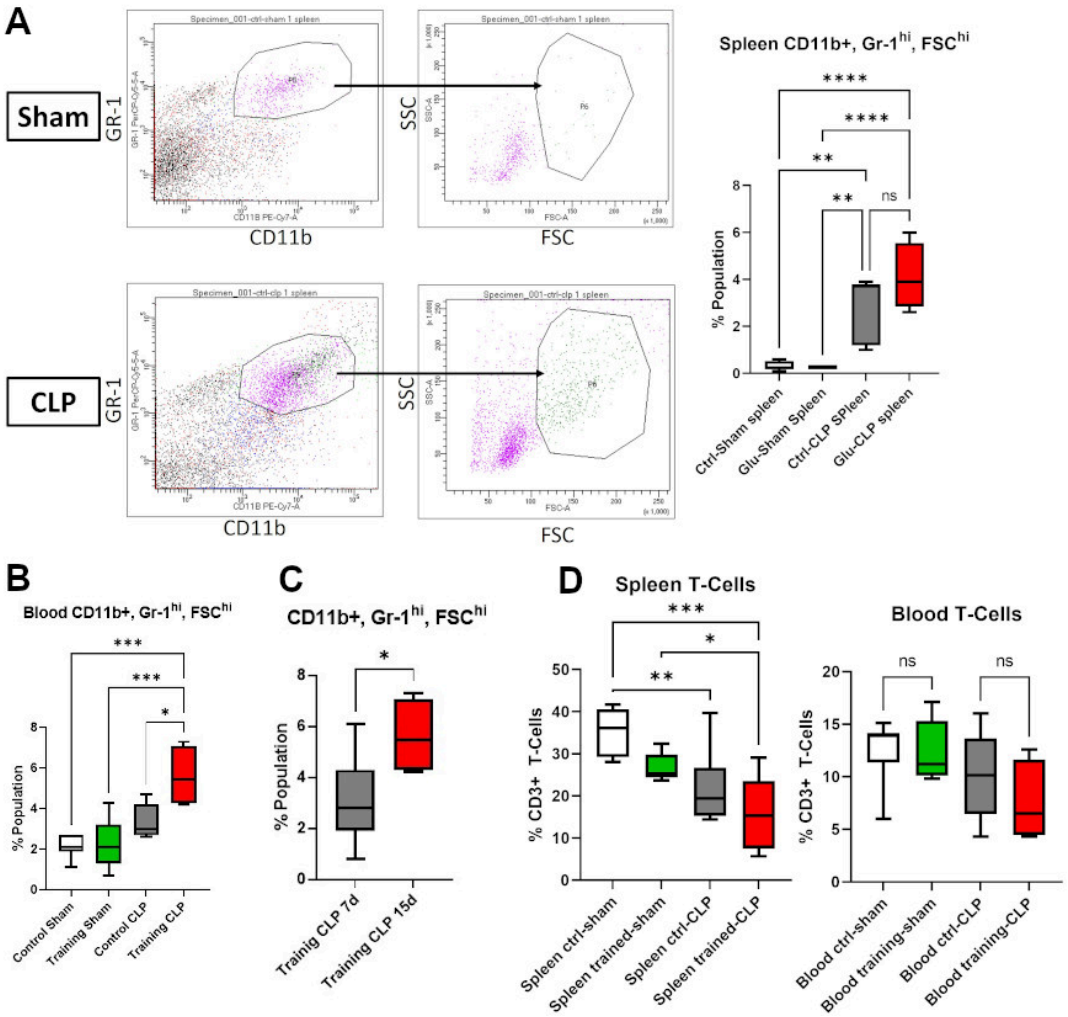

Figure 12. Sepsis and trained immunity increase CD11b+, GR-1hi, FSChi cells in a chronic model of polymicrobial sepsis. (**A**) % $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells in the spleen 15-days following CLP. N=5-8. (**B**) % $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells in the blood of mice 15-days following CLP. N=4-7. (**C**) Comparison of % $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells in the blood 7 versus 15-days after CLP in immune trained mice. N=4-5. (**D**) $CD3^{+}$ T-cells in spleen and blood 15-days after CLP. N=5-8. Ordinary one-way ANOVA followed by Tukey's post hoc test was used for **A**, **B**, and **D**. Paired T-test was used for **C**.

$CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells from immune trained mice following CLP have an immunosuppressive phenotype.

To assess the phenotype of cell populations, we analyzed MHC II and PD-L1 expression. MHC II expression has previously been used to help differentiate MDSCs from

monocytes(Veglia et al. 2021). $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells express low levels of MHC II (**Figure 13A**). MHC expression on this population is significantly less than untrained monocytes/macrophages but not significantly different from the population of neutrophils or the CD11b+, GR-1hi. PD-L1 is expressed by MDSCs and neutrophils and inhibits T-cell function (Zhang et al. 2017), and it is often used as a marker of immunosuppression (Patil et al. 2017; Wang et al. 2015a). We found that $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells from immune-trained septic mice express high levels of PD-L1. This population of cells expresses significantly higher levels of PD-L1 than the $CD11b^{+}$, $GR\text{-}1^{hi}$ population, monocytes/macrophages, and neutrophils (**Figure 13B**). However, when co-cultured with stimulated T-cells, $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells from immune trained and control mice did not reduce IFN-γ production (**Figure 13C**). Additionally, the total $CD11b^{+}$, $GR\text{-}1^{hi}$ population did not reduce IFN-γ production by stimulated T-cells (**Figure 13D**).

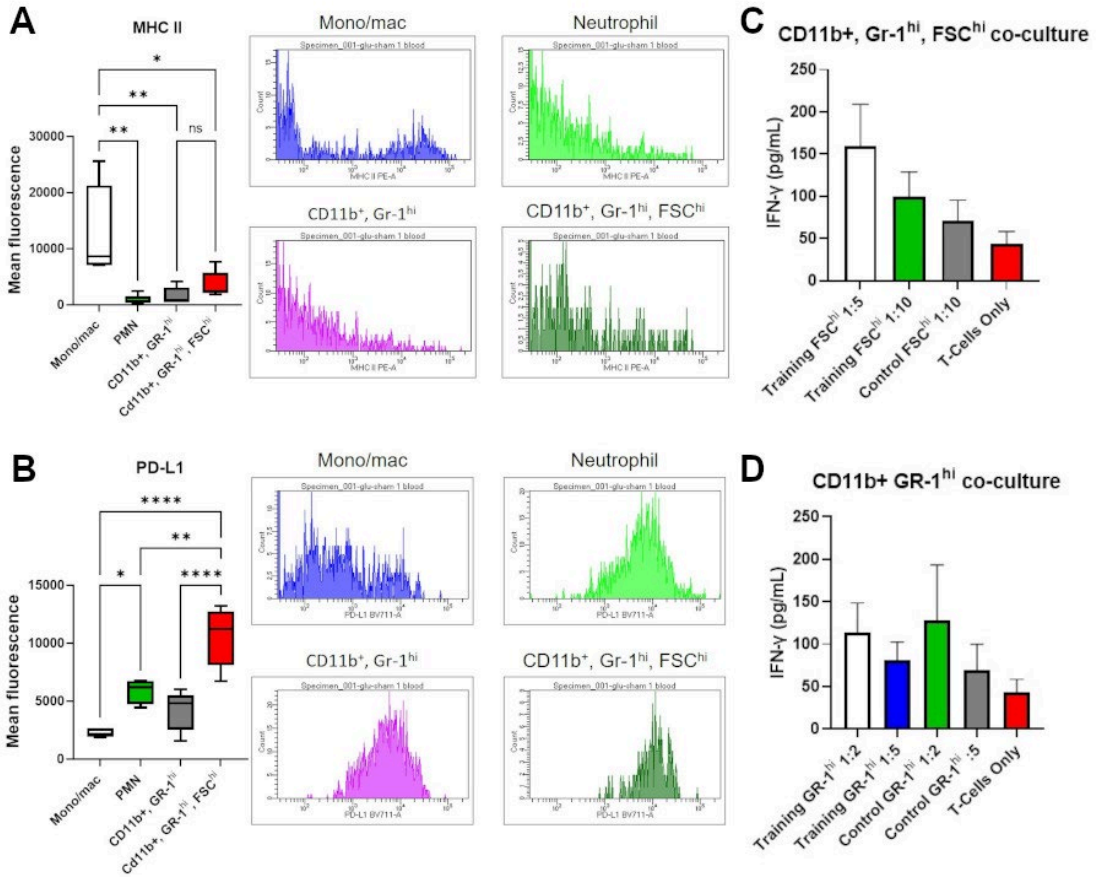

Figure 13. CD11b^{+}, GR-1^{hi}, FSC^{hi} cells from immune trained mice following CLP have an immunosuppressive phenotype. (**A**) Mean fluorescence of MHC II-PE by myeloid cell population 15-days following CLP. N=5. (**B**) Mean fluorescence of PD-L1-BV711 by myeloid cell population 15-days following CLP. N=5. (**C**) CD11b^{+}, GR-1^{hi}, FSC^{hi} cells were isolated from mice treated with LPS and co-cultured with stimulated T-cells from healthy mice at several concentrations. IFN-ɣ secretion was measured by ELISA. N=2-4. (**D**) CD11b^{+}, GR-1^{hi} cells were co-cultured with stimulated T-cells from healthy mice at several concentrations, and ELISA measured IFN-ɣ secretion. N=2-4. **A** and **B** were statistically compared using an ordinary one-way ANOVA followed by Tukey's post hoc test.

Discussion

The immune response to sepsis is complex, dysfunctional, and incompletely understood(Angus 2011; Chaudhry et al. 2013; Cohen et al. 2012; Hotchkiss et al. 2013a). Patients who survive the early hyperinflammatory stage of sepsis often develop prolonged

immunosuppression that makes them susceptible to recurring nosocomial infections(Hotchkiss et al. 2013b). Advances in the field of trained immunity have demonstrated its promise as a potential prophylactic for vulnerable populations, such as sepsis patients and patients with sepsis-induced immunosuppression(Cheng et al. 2014a; Fensterheim et al. 2018; Netea et al. 2020; O'Neill and Netea 2020). Thus far, no studies have examined the effect of trained immunity in a polymicrobial model of sepsis. To evaluate the effect of trained immunity on sepsis, we used an accepted animal model of polymicrobial sepsis(Dejager et al. 2011) and *in vitro* immune training with β-glucan(Cheng et al. 2014a).

In this study, we utilized a chronic model of CLP-induced sepsis to examine the effect of immune training on sepsis survivors. Using flow cytometry, we assessed cell populations in mice that lived for 7 and 15-days following CLP. We found that immune training increased the abundance of $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells in the blood of surviving mice. Rieber et al. found that Dectin-1 signaling also increased MDSCs in mice exposed to *C. albicans(Rieber et al. 2015)*, indicating that Dectin-1 signaling is likely the mechanism responsible for increased $CD11b^{+}$, $GR\text{-}1^{hi}$, FSChi cells in our study. The same population was significantly increased in the spleens of septic animals, but immune training did not affect it. In mice, $CD11b^{+}$, $GR\text{-}1^{+}$ cells have historically been considered MDSCs (39). However, we find that population overlaps significantly with $Ly6\text{-}B.2^{+}$, $GR\text{-}1^{+}$ neutrophils. Large (FSC^{hi}) cells may represent immature leukocytes(Rincón et al. 2018) which are thought to be one source of MDSCs (Gabrilovich 2017; Medina and Hartl 2018). MDSCs are a heterogeneous population of myeloid lineage cells. The definition of MDSCs has been expanded to include 2 subtypes: monocytic $CD11b{+}Ly6C^{+}Ly6G^{low}$ (M-MDSCs) and granulocytic $CD11b^{+}$, $Ly6C^{low}$, $Ly6G^{+}$ (PMN-MDSCs) (Medina and Hartl 2018). While much of the early research conducted on MDSCs was

conducted in the paradigm of cancer(Tam et al. 2014; Venet and Monneret 2018), more recently, the role of MDSCs in infection and sepsis has become a topic of increasing interest(Hotchkiss et al. 2013a; Schrijver et al. 2019). The main function of MDSCs in infection is to suppress the immune system. The immunosuppressive effect is thought to be mediated through the production of iNOS and ROS(Nagaraj et al. 2007), secretion of IL-10(Medina and Hartl 2018) and expression of PD-L1(Gabrilovich 2017), COX2(Veglia et al. 2021). Many of these mechanisms function to inhibit or modulate T-cell function; however, MDSCs can also impact myeloid cell function (Heim et al. 2015), which may be underrepresented in the literature.

Additionally, we found that $CD11b^{+}$, $GR\text{-}1^{hi}$, FSC^{hi} cells from trained mice expressed high levels of PD-L1, which supports their status as immunosuppressive cells. However, we were unable to confirm immunosuppressive function using a T-cell co-culture assay. A study by Aarts et al. reported that isolating MDSCs or neutrophils by FACS impairs their functions (Aarts et al. 2019). Thus, FACS may not be ideal for isolating these populations. Future studies should investigate the immunosuppressive effects of MDSCs using alternative isolation methods, such as those described by Aart et al. (Aarts et al. 2019). Once a suitable isolation method has been established, future studies should also investigate the effects of this cell population in *in vivo* infection models, using techniques such as adoptive transfer or cell depletion.

The failings of clinical trials in sepsis demonstrate that it can be difficult to discern which components of the immune response are beneficial and which are harmful to the patient. MDSCs are no exception. MDSCs are largely considered detrimental in cancer since their suppressive functions allow for tumor growth(Law et al. 2020). However, the role of MDSCs in infection and sepsis remains unclear. MDSCs appear to be beneficial in the immune response in infections from pathogens like *Salmonella typhimurium (Tam et al. 2014)*, *Pseudomonas aeruginosa*

(Rieber et al. 2013), and *Candida albicans (Rieber et al. 2015)*. In contrast, MDSCs are detrimental to the host in infections caused by *Mycobacterium tuberculosis (du Plessis et al. 2013)*, *Staphylococcus aureus (Skabytska et al. 2014; Tebartz et al. 2015),* and HIV-1(Qin et al. 2013). In sepsis, MDSCs may play a dual role. MDSCs might exert immunomodulatory effects in sepsis by limiting some of the hyper-inflammation experienced in the earlier stages of sepsis (Schrijver et al. 2019). However, in patients that survive the initial infection and hyperinflammatory response, MDSCs may have a prolonged effect on the immune system and contribute to the immunosuppression and recurring infection experienced in these patients (Hotchkiss et al. 2013b; Venet and Monneret 2018). Also, MDSC function may be impacted by the disease stage. In an animal model, Gr-1^{+}, CD11b^{+} cells have been shown to stimulate the immune response early in the disease, but suppress the immune system in the later stage of illness(Brudecki et al. 2012), making MDSCs a complex component of the immune response in sepsis that is incompletely understood. Understanding the effect of trained immunity on MDSCs in sepsis will help to illuminate their function in the illness and allow further understanding of the application of trained immunity in the case of sepsis.

Acknowledgements

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CHAPTER 4: IMMUNE TRAINING ALTERS THE GUT MICROBIOME OF AGING MICE TO MORE CLOSELY RESEMBLE THE MICROBIOTA OF YOUNGER MICE

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Abstract

The gut microbiome plays an essential role in health and disease. However, age related changes to the microbiome contribute to immune dysfunction, metabolic disorders and frailty. An important and rapidly growing area of immunology is innate immune memory or trained immunity. We investigated age-related changes to the gut microbiome in mice and whether trained immunity impacted microbiome diversity in aging. We found that trained immunity, induced by systemic administration of β-glucan, reversed several age-related changes to the microbiome. Specifically, immune training restored the Firmicutes/Bacteroidetes ratio of aging mice to levels consistent with younger mice and increased the relative abundance of *Clostridia*. Our results indicate that innate immune training alters the gut microbiome of aging mice to more closely resemble the microbiota of younger mice. We conclude that trained immunity may be an effective approach to modulating the aging gut microbiome in a manner that may prove beneficial to the aging host.

Introduction

The microorganisms in the gastrointestinal tract play a critically important role in health and the response to disease(Buford 2017; Lloyd-Price et al. 2016). The majority of the gut microbiota are bacteria, and around 90% of all species within the gut microbiome fall into two bacterial phyla, *i.e.* Firmicutes and Bacteroidetes(Kim and Jazwinski 2018). Bacteria in the gut influence host health, and the bacterial composition of the gut microbiome plays a critical role in host well-being throughout life(Salazar et al. 2020). Notably, the microbiome directly impacts immune function, host metabolism, and physiology and is thought to play a significant role in longevity(Vaiserman et al. 2017). The microbiome acquired at birth remains relatively stable throughout life, but the microbiome can be altered by diet, environment, and overall health (Kim and Jazwinski 2018). Disruptions in the diversity of the microbiome, *i.e.* dysbiosis, have been linked to a number of disease states, including cancer, obesity, cardiovascular disease, and poor outcomes in critically ill patients(Goulet 2015; Lloyd-Price et al. 2016; Steves et al. 2016).

Aging is accompanied by a natural decline in many systems throughout the body(Sadighi Akha 2018). Age-related changes in metabolism, nutrition and immunity make aging individuals increasingly susceptible to disease and cognitive dysfunction (Salazar et al. 2020). Aging is also associated with low-grade inflammation, also known as inflamm-aging(Buford 2017; Fulop et al. 2018). Inflamm-aging has been linked to various age-related diseases such as diabetes, dementia, cancer, and frailty. Recent evidence suggests that the microbiome may play a role in inflamm-aging(Buford 2017). By way of example, the microbiome is involved in the production of anti-inflammatory short-chain fatty acids (SCFAs) and the synthesis of essential vitamins and amino acids(Biagi et al. 2012). Age-related decreases in these microbial products are associated with inflammation and increased susceptibility to infection(Biagi et al. 2012). Age-related loss in

diversity in the gut microbiome may impact essential digestive functions leading to increased inflammation and frailty in aging individuals(Vaiserman et al. 2017).

The changes that occur in the gut microbiota with advanced age are incompletely understood. However, a 2011 study found that elderly subjects had lower proportions of Firmicutes and altered Firmicutes/Bacteroidetes ratios when compared to younger adults(Claesson et al. 2011). Zwielehner et al reported that institutionalized elderly individuals show a significantly higher abundance of Bacteroidetes, a lower proportion of *Bifidobacterium,* and a lower abundance of certain *Clostridia,* when compared to younger individuals(Zwielehner et al. 2009). Additionally, the Firmicutes/Bacteroidetes ratio is decreased in elderly individuals(Mariat et al. 2009). It is important to note that loss of microbiota diversity correlates with biologic age, but not necessarily chronologic age. This suggests that aging individuals that retain gut microbial diversity tend to remain healthier (Kim and Jazwinski 2018).

Several reports indicate that the immune system and microbiome communicate and influence one another (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2008b; Kiyono et al. 1982; Maslowski et al. 2009; Molloy et al. 2012; Wannemuehler et al. 1982). However, the relationship between the immune system and the microbiome is complex and poorly understood (McCoy et al. 2019; Negi et al. 2019). We and others have shown that the innate immune system can be trained to respond more rapidly and effectively to a variety of insults(Cheng et al. 2014a; Cheng et al. 2014b; Kleinnijenhuis et al. 2012; Moorlag et al. 2020a; Netea et al. 2020; Saeed et al. 2014). This is referred to as “trained immunity” or “innate immune memory”(Netea et al. 2020). Trained immunity is characterized by increased leukocyte metabolism and epigenetic reprogramming(Cheng et al. 2014a; Saeed et al. 2014). β-glucan is the most well studied inducer of the immune trained phenotype (Cheng et al. 2014a; Hamada et al. 2018; Ifrim

et al. 2014; Negi et al. 2019; Netea et al. 2020; Saeed et al. 2014). β-glucan signals through the C-type lectin, Dectin-1 (Cheng et al. 2014a). Tang et al. reported that Dectin-1 promotes gut *Lactobacillus,* which regulates T-cell expansion in the gut and contributes to colitis(Tang et al. 2015). β-glucan signaling through Dectin-1 is also involved in regulating intestinal inflammation(Iliev 2015). We speculated that the gut microbiota may be modulated by β-glucan induced trained immunity (Iliev 2015). Thus, we investigated the effect of β-glucan induced trained immunity on the composition and diversity of the gut microbiome in young and aging mice.

Methods

Mice. Male C57BL/6J mice were purchased from Jackson Laboratory and housed by the East Tennessee State University Division of Laboratory Animal Resources. Mice were housed in pairs and fed a standard chow diet. Mice were separated into three age groups: 1.5-3 months (young), 6-9 months (middle), and 12-18 months (aging). All mice were maintained under 12-h light/dark cycle conditions in plexiglass cages in a controlled atmosphere. During all experiments, mice had free access to standard laboratory chow and water

β-glucan. β-1,3-(D)-glucan was isolated from *Candida albicans* SC5314 as previously described (30). Mice were injected (IP) with 1 mg/mouse of β-glucan suspended in 0.5 mL of dextrose 5% (w/v) and water (D5W). In control mice, dextrose 5% w/v and water (D5W) was administered as the vehicle control.

In vivo immune training and sample collection. One mg of *C. albicans* β-glucan or an equal volume of D5W was administered intraperitoneally to mice on day -7 and day -4. A baseline fecal sample (**F0**) was acquired before the first injection. Fecal samples were then collected weekly, starting at day 0 (**F1**) and continuing through day 21 (**F4**) (**Figure 14A**). Fresh fecal

samples were collected and immediately snap-frozen in liquid nitrogen and stored at -80°C. DNA was isolated from fecal samples using the Qiagen PowerSoil DNA isolation kit (Qiagen #12888). Amplification of the V3/V4 region of the 16S ribosomal RNA gene and preparation of DNA library was performed following the Illumina 16S Metagenomic Sequencing Library workflow(Illumina 2013). DNA sequencing was performed on the Illumina MiSeq. Data analysis was performed using the Microbial Genomics Module in the CLC Biogenomics Workbench, SigmaPlot 14, and Qiime Virtualbox (PICRUSt).

Bone marrow-derived macrophages (BMDM). Bone marrow cells were washed from femurs of male C57/BL6J mice between 12 and 18 months old. BMDMs were filtered through a 70 μm cell strained and cultured in RPMI 1640 with 25mM HEPES (ThermoFisher 22400071), 10% FBS, 1% Gibco antibiotic-antimycotic (ThermoFisher 15240062), and 10 ng/mL M-CSF (R&D systems 416-ML-050) for seven days. At the end of the incubation (D7), the cells were washed, and media was changed. Cells were then treated with 10 μg/mL *C. albicans*-derived β-glucan or PBS for 24 hours. Cells were then allowed to rest for 7 days, with media being changed every other day. Cell metabolism and cytokine production was measured, and RNA was extracted from BMDMs 7 days after β-glucan or PBS administration.

Metabolic studies. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using the Agilent XFp analyzer (Agilent Santa Clara, CA). Trained or control BMDMs (1×10^5 cells/well) were seeded and allowed to rest overnight. The following day, metabolism was assayed following the XFp Cell Energy Phenotype test template. In-well concentrations of 1 μm FCCP (Sigma C2920-10mg) and 1μM oligomycin (Sigma 495455-10mg) were injected after the third baseline measurement. The three baseline measurements were

averaged to calculate baseline OCR/ECAR, and the five measurements taken after oligomycin/FCCP injection were averaged to calculate stressed OCR/ECAR.

RNA Sequencing. RNA was isolated from BMDMs using the RNeasy Mini Kit (Qiagen# 74104). RNA samples were screened for quantity and quality using the Qubit and Agilent 2100 bioanalyzer. Sequencing was conducted on the Illumina NovaSeq 6000 Sequencing System by Novogene (Durham NC). Only samples meeting Novogene's sample requirement standards of at least 0.8 μg of RNA and an Agilent 2100 quality score of at least 6.3 were used for sequencing.

Data analysis. All data, excluding RNA sequencing data, were analyzed using GraphPad Prism 9. Unless otherwise specified, all data are presented as mean ± SEM. Baseline comparisons of F0 samples were analyzed using the Kruskal-Wallis test and Dunn's multiple comparisons test. Comparisons of fecal samples within an age-group and treatment group were analyzed using a two-way ANOVA and Šídák's multiple comparisons test. PICRUSt data were analyzed using a one-way ANOVA and Holm-Šídák's multiple comparisons test. Gene expression levels were estimated using FPKM. Statistical significance of differential expression of individual genes was determined using the DESeq2 R package, and p-values were adjusted using Benjamini and Hochberg's approach to control for multiple comparisons. GO statistics were calculated using clusterProfiler R package. Seahorse metabolism assay data and cytokine data were analyzed using paired *t*-tests. $P \leq 0.05$ was considered statistically significant for all measures.

Results

Aging mice show changes in the gut microbiome.

We compared fecal samples from young, middle, and aging groups of mice at baseline (F0) to assess age-related changes to the microbiome. The 0.5 UniFrac distance was calculated to

compare the populations present in each age group at baseline F0 (D-7). UniFrac was significantly different in all comparisons of age groups (**Figure 14B**). Differential abundance of operational taxonomic units (OTUs) was also significantly different between each age group at baseline, as displayed using a Venn diagram. The largest number of significantly altered OTUs were between the 1.5 - 3 month and 12–18-month age groups with 347 OTUs (**Figure 14B**).

At the phylum level, Bacteroidetes, Verrucomicrobia, and Proteobacteria were significantly altered between the age groups. Aging mice had a significantly higher relative abundance of Bacteroidetes at 71.33% than young (1.5-3-month-old) mice at 53.67% and middle (6-9-month-old) mice at 59.92%. Aging mice (12-18 months) also had a significantly higher abundance of Proteobacteria at 1.88% than young mice at 0.72%- and middle aged mice at 0.66%. Verrucomicrobia were significantly decreased in aging mice at 0.29% compared to young mice at 9.5%. In post hoc tests, there were no significant differences in the relative abundance of bacterial phyla between young and middle aged mice (**Figure 14C**). Additionally, the Firmicutes/Bacteroidetes ratio was significantly decreased in aging mice at 0.37 compared to young mice, which had a Firmicutes/Bacteroidetes ratio of 0.71 (**Figure 14D**).

At the class level, the relative abundances of Bacteroidia, Verrucomicrobiae, Betaproteobacteria, and Erysipelotrichi were significantly altered with age. Relative abundance of Bacteroidia was significantly elevated in the aging group at 71.33% compared to the young group at 53.67% and middle group at 59.92%. Betaproteobacteria was also significantly increased in the aging group at 1.71% compared to the young group at 0.70% and middle group at 0.55% Verrucomicrobiae were significantly less abundant in aging mice compared to young mice. Erysipelotrichi were significantly increased in aging mice at 2.26% compared to the middle group at 0.10% (**Figure 14E**). Interestingly, there were no significant differences in

bacterial class between young mice and middle-aged mice. When taken together, these data clearly show that the aging mouse gut microbiome is significantly different from that found in younger mice.

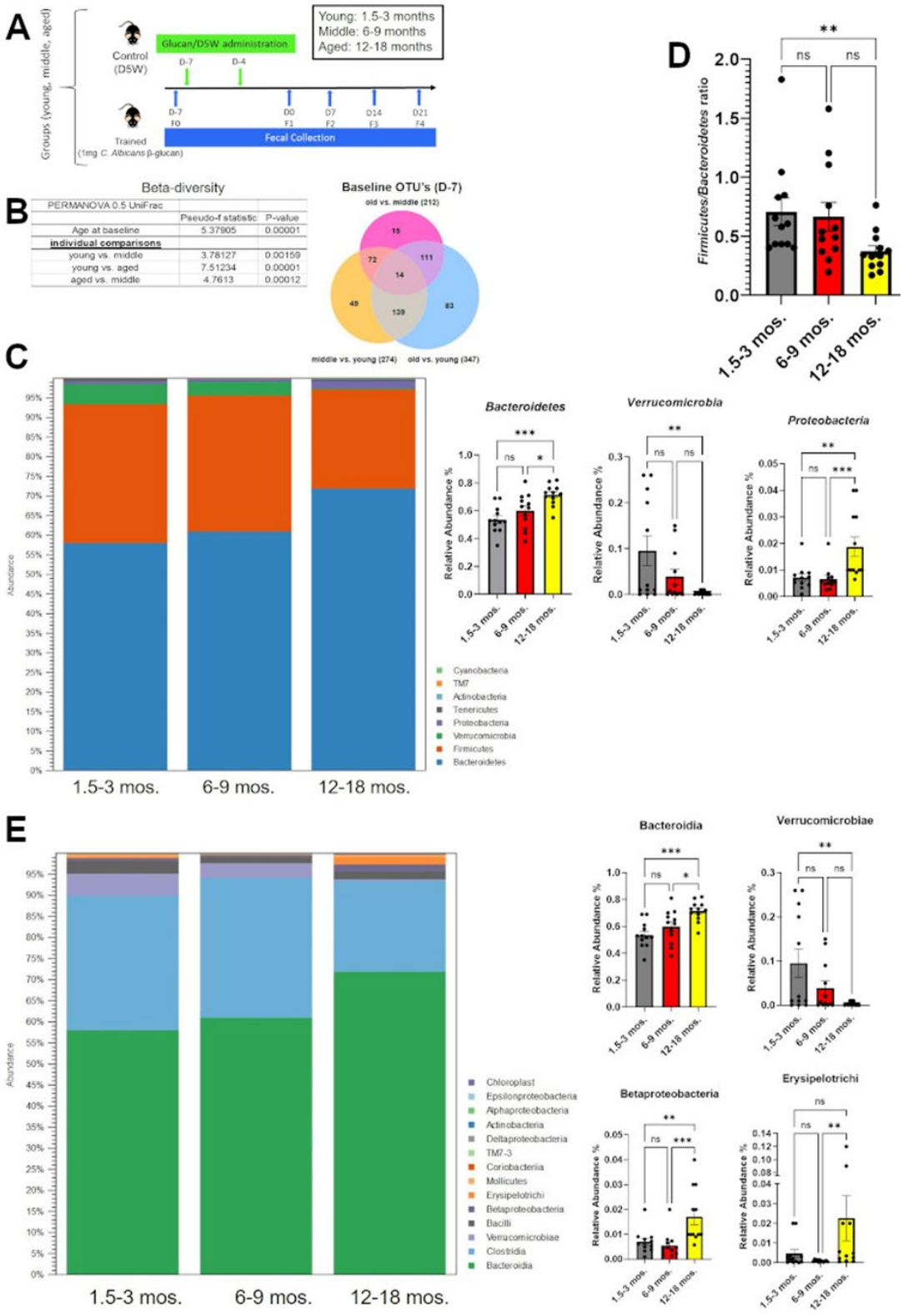


Figure 14. Aging mice show changes in the gut microbiome when compared to younger mice. (A) A baseline fecal sample (F0) was taken from male C57Bl/6J mice before treatments.

Mice were injected twice 3 days apart with 1mg *Candida albicans* β-glucan or an equal volume of D5W. Fecal samples were taken weekly following the first injection (F1, F2, F3, F4) for a total of four weeks. **(B)** 0.5 UniFrac PERMANOVA measure of beta diversity comparing all age groups at baseline and Venn diagram of significantly altered OUT's between each age group. **(C)** Relative abundance of bacterial phylum at F0. **(D)** Firmicutes/Bacteroidetes ratio as a factor of age. **(E)** Relative abundance of bacterial class at F0. Data displayed as mean ± SEM. N=12 for all measures. The statistical significance of the Venn diagram in **B** was calculated using a general linear model. Kruskal-Wallis and Dunns multiple comparisons tests were used for **C-E**; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Trained immunity alters the gut microbiome in aging mice.

To evaluate the effect of immune training on the microbiome, we analyzed differences between baseline (F0) and fecal samples collected after administration of β-glucan. Firmicutes/Bacteroidetes ratio is often used to approximate the overall status of the microbiome(Mariat et al. 2009). Firmicutes/Bacteroidetes ratio was significantly elevated 14 days following immune training in aging mice from a baseline (F0) level of 0.37 to 0.84 (F3) (**Figure 15A**). In immune trained aging mice, the relative abundance of Firmicutes was significantly higher at F3 at 42.2% compared to baseline (F0) at 22.5%. Bacteroidetes were significantly decreased at the same time point from 74.5% at baseline to 54.4% at F3 (**Figure 15B)**. This effect was not present in the young or middle groups. We also found that *Clostridia* were significantly increased by immune training in aging mice. The relative abundance of *Clostridia* was significantly increased at F3 with 37.6% when to compared to baseline which was 18.67%. This effect was dissipated at F4, at which time clostridia returned to a relative abundance of 25.83% (**Figure 15C**).

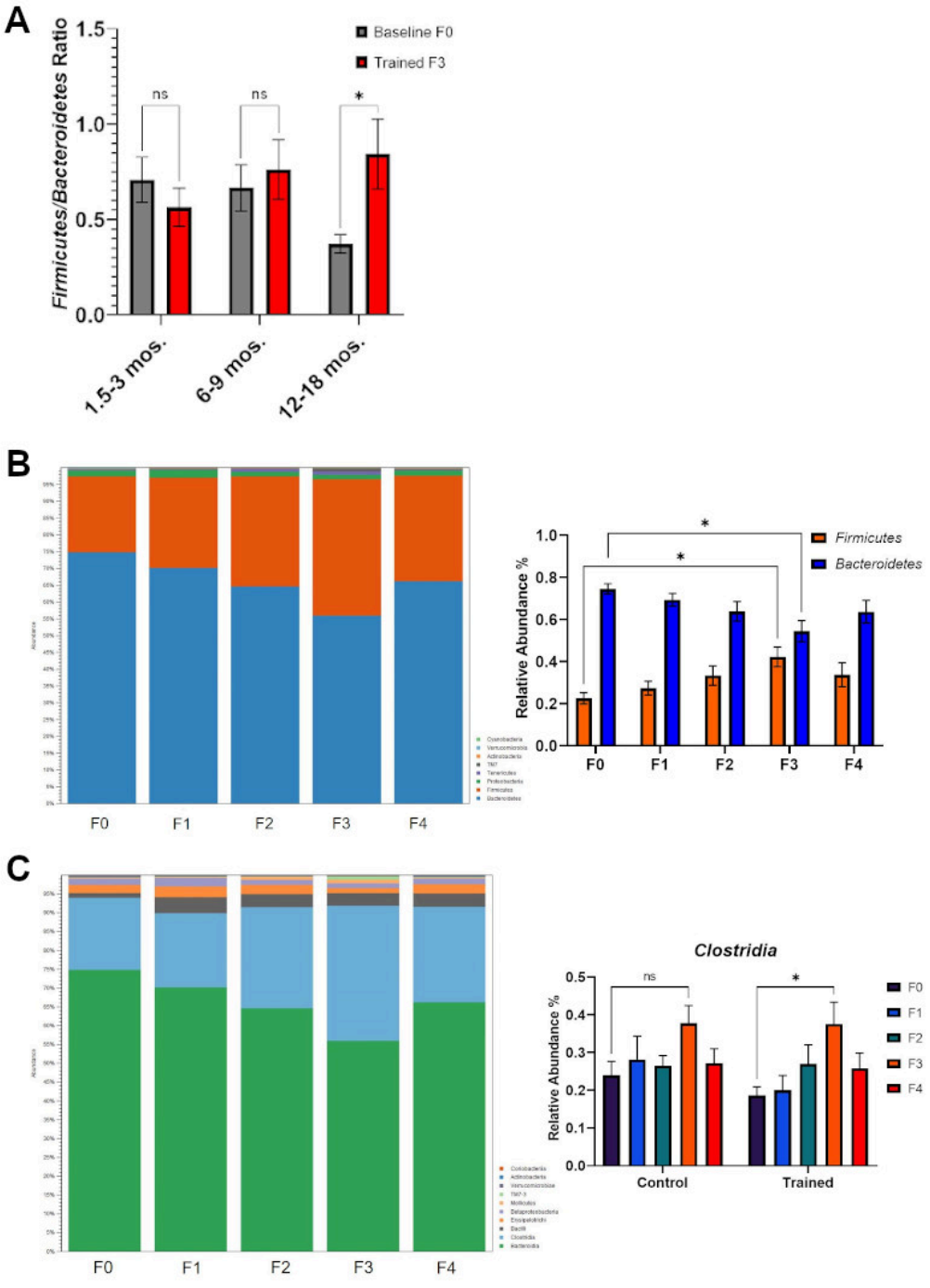


Figure 15. Trained immunity alters the gut microbiome in aged mice. (A) Firmicutes/Bactoidetes ratio as a function of age and immune training. **(B)** Relative abundance of bacterial phylum in 12-18-month-old immune trained mice. **(C)** Relative abundance of bacterial classes in 12-18-month-old immune trained mice and comparison of *Clostridia* abundance across fecal samples in 12-18-month-old control and immune trained mice. All results were analyzed by 2way ANOVA and Šídák's multiple comparisons test post hoc. N=6-12, *P<0.05

Trained immunity modulates predicted function (PICRUSt) of microbial communities in aged mice.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, or PICRUSt, software uses 16s rRNA sequencing data and a computational approach to predict metagenome function(Langille et al. 2013). This approach utilizes KEGG Orthology to infer the gene content of the bacterial genomes present. **Figure 16A** lists level 2 Kegg pathways present at significantly different levels in the metagenome of each age group at baseline (F0). Comparison of young and aging mice produced the most significant differences, including several pathways involved in metabolism. We observed that enzyme families of genes were significantly different between middle and aging mice (**Figure 16A**). Bacterial invasion of epithelia, calcium signaling pathway, cell motility and secretion level 3 KEGG pathways were significantly altered by trained immunity in aging mice (**Figure 16B-D**). No significant differences between fecal samples were detected in post hoc tests of bacterial invasion of epithelia genes and calcium signaling pathway genes (**Figure 16B, C**). However, cell motility and secretion genes were significantly decreased at F3 compared to baseline (**Figure 16D**).

A

F0's by age group (Kruskal Wallace)

Category	Young vs old (P-value)	Young vs middle (P-Value)	Middle vs old (P-Value)
Cell processes and signaling	0.014	0.067	0.419
Enzyme families	<0.001	0.040	0.045
Immune system diseases	<0.001	0.001	0.487
Lipid metabolism	0.003	0.082	0.136
Metabolic disease	0.020	0.028	0.772
Metabolism	0.021	0.034	0.712
Metabolism of other amino acids	0.002	0.012	0.388
Nucleotide metabolism	<0.001	0.006	0.273
Poorly characterized	0.035	0.025	0.978
Replication and repair	0.010	0.019	0.682
Signal transduction	0.020	0.023	0.840
Transcription	0.029	0.022	0.960
Translation	0.006	0.015	0.610
Xenobiotics biodegreadation	0.004	0.067	0.209

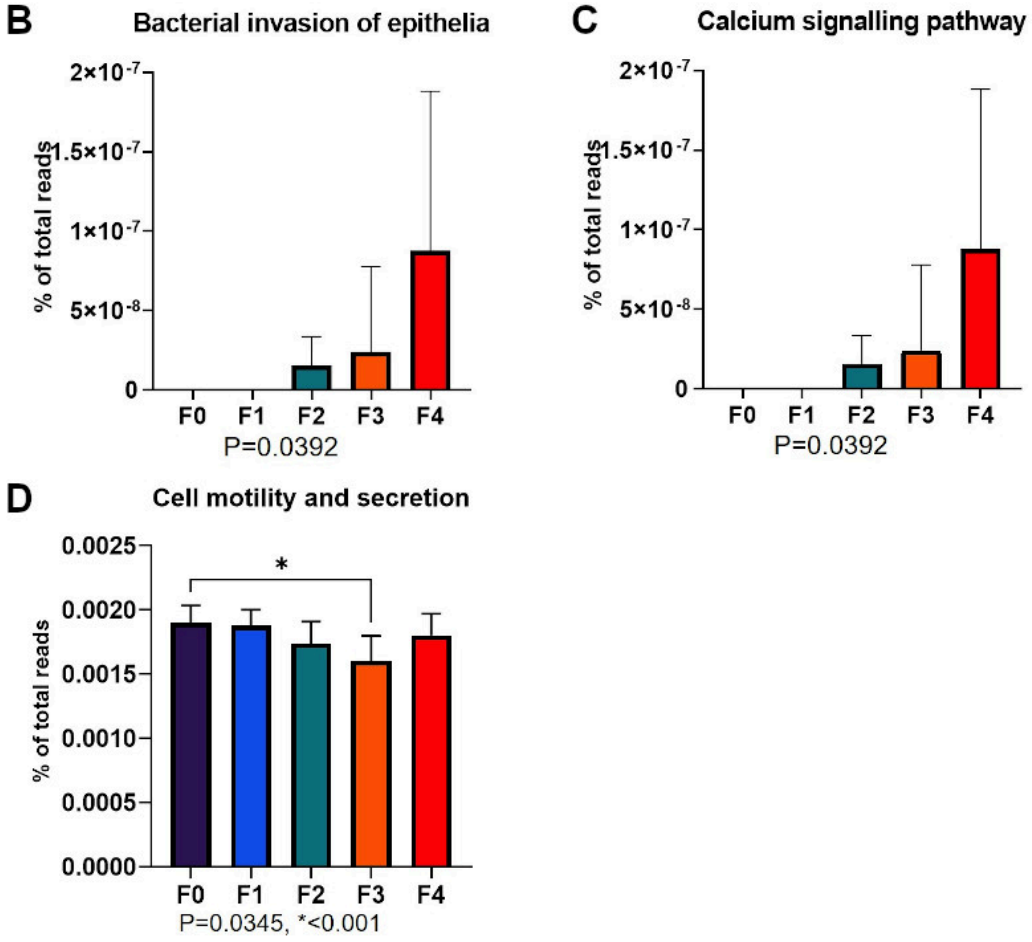
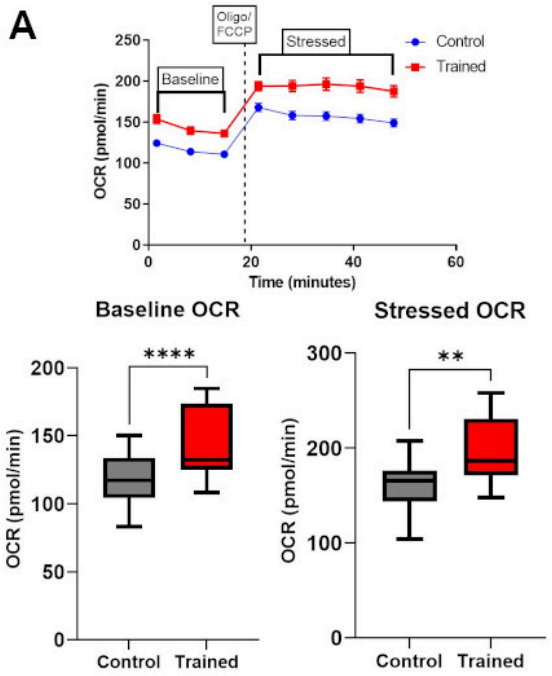


Figure 16. Trained immunity modulates predicted function (PICRUSt) of microbial communities in aged mice. (A) List of level 2 KEGG pathways significantly altered in the microbiome as a function of age. **(B** and **C)** Level 3 Kegg pathways significantly altered in the microbiome of 12-18-month-old immune trained mice as a function of fecal sample. **A** was

analyzed using Kruskal Wallace. **B-D** were analyzed by one-way ANOVA and Holm-Šídák's multiple comparisons test. N=5-6 *P<0.05.

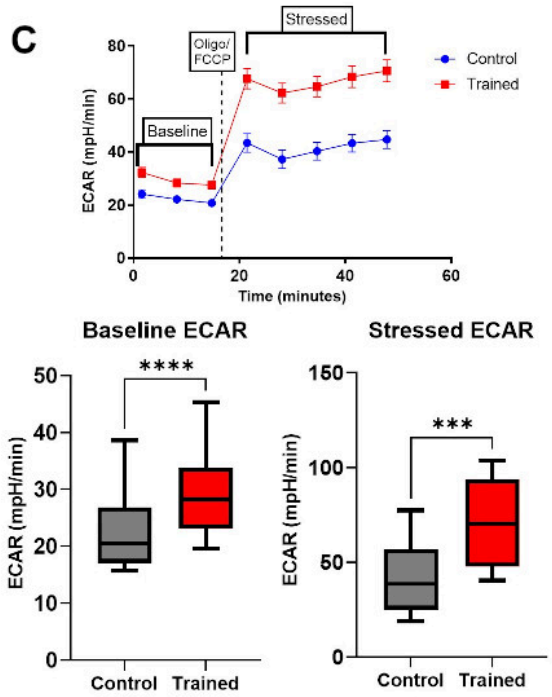
Trained immunity increases cellular metabolism of BMDMs in aging mice

We confirmed the induction of trained immunity in the aging group by assessing cellular metabolism of BMDMs from mice between 12 and 18 months of age. Cellular metabolism is often decreased in aging individuals(Pence 2020; Pence and Yarbro 2018; Weyand and Goronzy 2016). However, we confirmed the trained immunity phenotype in BMDMs from aging mice. Both baseline and stressed OCR were significantly increased in immune trained cells (**Figure 17A**). Transcriptomic analysis revealed that 19 genes involved in oxidative phosphorylation were upregulated, and only 1 was downregulated (**Figure 17B**). Using the Agilent Seahorse, we assayed OCR to functionally assess cellular respiration. We found that cellular respiration was increased in trained BMDMs. ECAR was assessed to functionally approximate glycolysis. Baseline and stressed ECAR were significantly increased in immune trained BMDMs (**Figure 17C**). Immune training resulted in nine upregulated genes and two downregulated genes, all of which were involved in the glycolysis pathway (**Figure 17D**). These data confirm the β-glucan induced trained immune phenotype.



B

Gene Name	log2FoldChange	Adjusted P-Value
Atp6v0d2	1.358221	0.022423
Ppa1	0.907892	9.99E-05
Atp6v1b2	0.531366	0.000834
Atp6v1c1	0.474175	1.18E-05
Ndufa9	0.463124	0.000706
Ndufb8	0.452792	0.021259
Ndufv2	0.409937	0.035154
Atp6v1h	0.402559	0.000492
Ndufv1	0.370334	0.002831
Sdhb	0.358785	0.038477
Atp6v1d	0.310895	0.002247
Atp6v1e1	0.306355	0.037501
Lhpp	0.298085	0.016719
Uqcrc1	0.27938	0.036238
Ndufa10	0.250413	0.040896
Uqcrc2	0.242227	0.022629
Atp5b	0.241103	0.016982
Atp5a1	0.212139	0.01067
Atp6v1a	0.18876	0.048412
Atp6v1g2	-0.76696	0.021825



D

Gene Name	Log2FoldChange	Adjusted P-value
Aldh1b1	0.59363	1.83E-05
Tpi1	0.432853303	0.00994769
Ldha	0.425554	0.019555
HK3	0.38440338	0.02660485
Dld	0.343627	0.000255
Pgk1	0.339271	0.026629
Pgam1	0.299159	0.003971
Dlat	0.209194	0.038242
Minpp1	0.0326708	0.012369
PFKM	-0.484964307	0.000776
HK2	-0.591380739	0.03860874

Figure 17. Trained immunity increases metabolism of BMDMs in aged mice. (A) Oxygen consumption rate (OCR) of BMDMs from 12 – 18 month old mice before and after administration of Oligomycin and FCCP. Three measurements before injection were averaged to calculate baseline OCR. Five measurements after injection were averaged to calculate stressed OCR. **(B)** Oxidative phosphorylation genes in the KEGG pathway that were significantly altered

by immune training with β-glucan. Red indicates increased expression, and green indicates decreased expression as a result of immune training. **(C)** Baseline and stressed extracellular acidification rate (ECAR) of BMDMs. **(D)** Glycolysis/gluconeogenesis genes in the KEGG pathway that were significantly altered by immune training with β-glucan. N=13 for all metabolic measures. N=8 for RNA sequencing data. **A** and **C** were analyzed using a paired T-test ***P<0.001, ****P<0.0001. Significance in **B** and **D** was determined using the DESeq2 R package, and p-values were adjusted using Benjamini and Hochberg's approach.

Trained immunity induces transcriptional changes in BMDMs derived from aging mice.

Previous studies have shown that trained immunity induces significant transcriptomic changes in immune cells (Cheng et al. 2014a; Saeed et al. 2014). We employed the method of Cheng et al to induce the trained phenotype (**Figure 18A**)(Cheng et al. 2014c). We found a unique transcriptomic profile after immune training with β-glucan in BMDMs isolated from aging mice. The principal component analysis (PCA) shows tighter clustering in trained BMDMs compared to control cells. (**Figure 18B**). We found that 173 genes were uniquely expressed in trained mice, while 497 were expressed in untrained control mice (**Figure 18C**). Ultimately, more genes were downregulated as a result of immune training than were upregulated, *i.e.* 1079 genes were upregulated, and 1992 genes were downregulated after immune training (**Figure 18D**). Nineteen (19) gene ontology pathways were significantly altered by trained immunity. Proteasome regulatory particle and proteasome accessory complex were the most significantly altered GO terms as determined by adjusted P-value. Nuclear envelope and nucleoside-triphosphatase regulator activity were the two most altered GO terms based on gene ratio. Multiple GO terms involving the proteasome and GTPase activity were significantly altered by immune training. Interestingly, the GO term ruffle was significantly altered by immune training (**Figure 18E**). Ruffle formation is a crucial function in macrophages, where it contributes to cell motility, receptor internalization, and phagocytosis (Condon et al. 2018; Patel and Harrison

2008; Wickramarachchi et al. 2010). Additionally, we found that immune training induced upregulation of 21 genes and downregulated transcription of 9 genes in the phagosome pathway in the KEGG database (**Figure 19A, 19B**). We also found that transcription of 6 genes was upregulated, and 2 genes were downregulated in the KEGG antigen presentation pathway due to immune training. (**Figure 19B**). These results indicates that these functions may be enhanced in immune-trained leukocytes in aging mice.

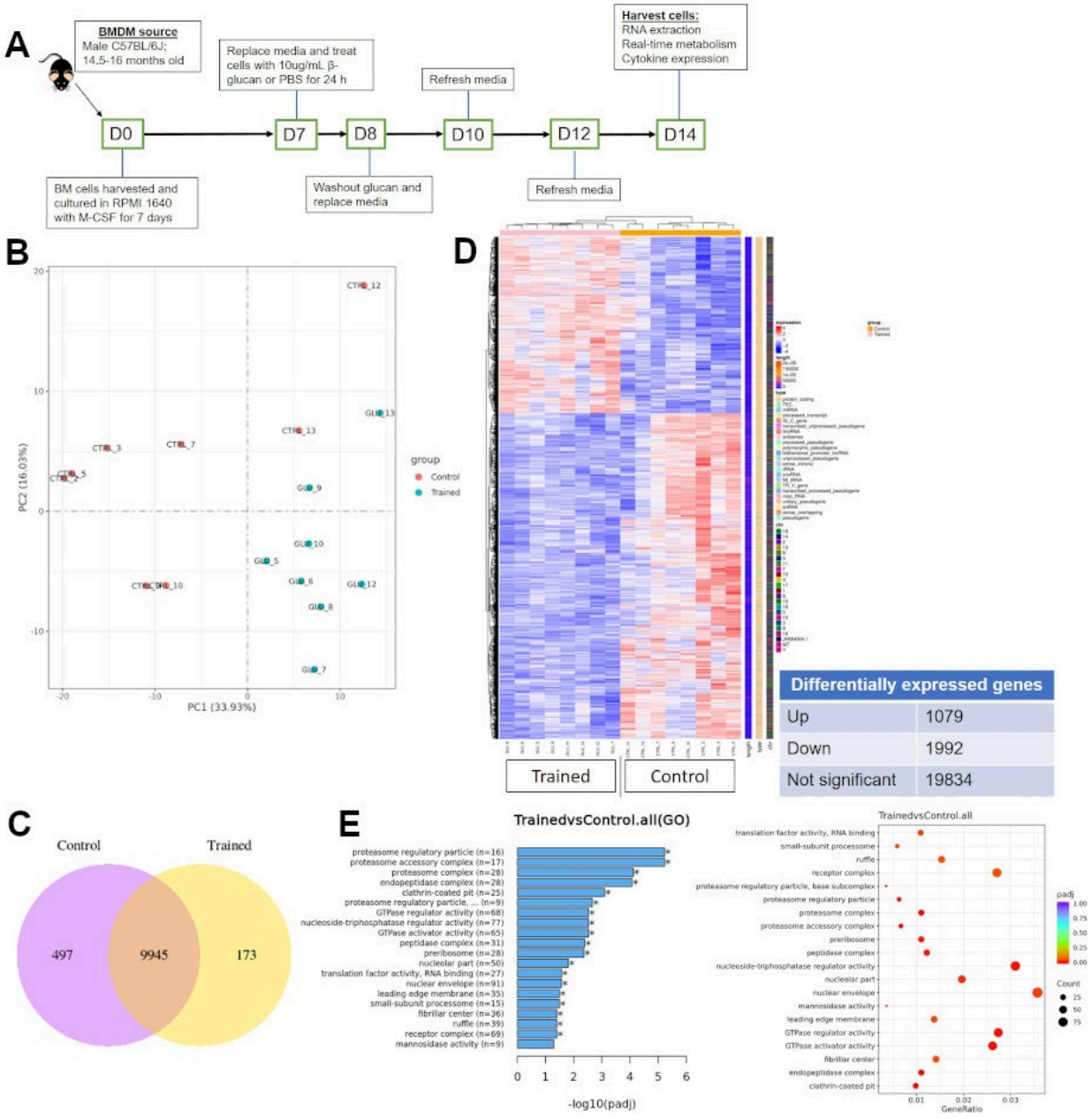
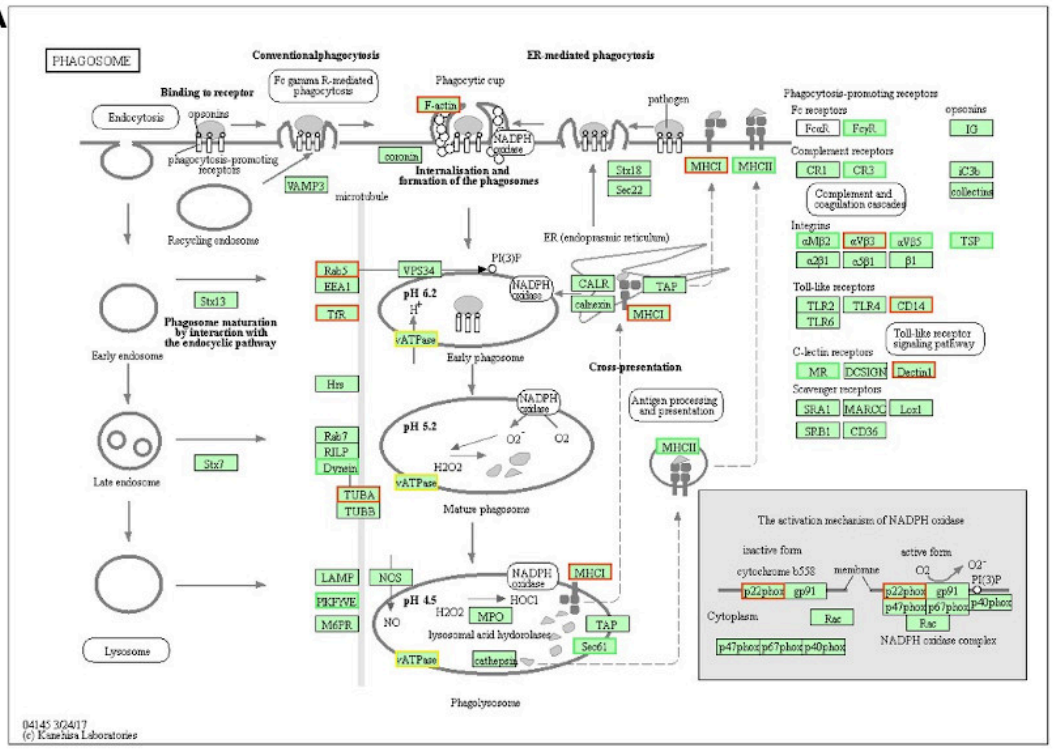


Figure 18. Trained immunity induces transcriptional changes in BMDMs derived from aged mice. (A) Bone marrow (BM) cells were harvested from male C57Bl/6J between 12 and 18 months old. BM cells were maintained in RPMI 1640 with 10% FBS and M-CSF for 7 days. After 7 days, cells were treated with β-glucan or an equal volume of PBS and allowed to incubate for 24 hrs. Cells were maintained for 7 days, and then RNA was extracted and sequenced. **(B)** Principal component analysis (PCA) of BMDM as a function of immune training. **(C)** Venn diagram representing genes that are uniquely expressed within each group. **(D)** Cluster analysis of differentially expressed genes as a function of immune training with β-glucan. (**E)** Gene ontology (GO) enrichment analysis. Histogram and scatter plot of top 20 significantly increased GO terms as a function of immune training. N=8 for all measures. Statistical significance for **C** and **D** were calculated using the DESeq2 R package, and p-values were

adjusted using Benjamini and Hochberg's approach to control for multiple comparisons. GO statistics calculated using clusterProfiler R package.

A



B

Phagocytosis

Gene Name	log2FoldChange	Adjusted P-Value
H2-M2	2.798881856	7.29E-08
Atp6v0d2	1.358220704	0.022423119
H2-Q6	0.936845158	0.009588461
Clec7a	0.841923262	0.001531428
Itgb3	0.804029238	0.000460029
Tuba4a	0.721938825	6.37E-08
Actg1	0.546310395	9.40E-05
Atp6v1b2	0.531365886	0.000833941
Cd14	0.523568416	0.004893874
Tuba1a	0.51916554	0.005374705
Actb	0.495701218	0.009213128
Atp6v1c1	0.474175466	1.18E-05
Cyba	0.473439781	0.008535756
Atp6v1h	0.402559352	0.000491743
Tuba1c	0.382608537	0.005974501
Tuba1b	0.37171307	0.031327224
Atp6v1d	0.310895422	0.002247006
Atp6v1e1	0.306355214	0.037501334
Tfrc	0.291717974	0.040052133
Rab5a	0.24952294	0.036374939
Atp6v1a	0.188760378	0.048412471

Phagocytosis

Gene Name	log2FoldChange	Adjusted P-Value
Itgb2	-0.308	0.025075
Itgam	-0.50077	0.033078
Itgb5	-0.55866	0.00796
Atp6v1g2	-0.76696	0.021825
Mrc1	-0.80094	2.79E-05
Sec61a2	-0.87488	0.000997
Fcgr2b	-0.88888	1.75E-05
H2-Ob	-1.82518	0.008803
Thbs2	-3.65375	0.000882

Antigen Presentation

Gene Name	log2FoldChange	Adjusted P-value
H2-M2	2.798882	7.29E-08
H2-Q6	0.936845	0.009588
Cd74	0.725186	0.024219
Hsp90aa1	0.583623	0.000226
Psme2	0.437215	0.02915
Psme3	0.254732	0.004002
Lgmn	-0.3277	0.017254
H2-Ob	-1.82518	0.008803

Figure 19. Immune training modulates the immune response in BMDMs in aged mice. (A) Kegg pathway of phagocytosis. Genes highlighted in red are increased with immune training, green highlighted genes are decreased, yellow highlighted genes contain both increased and decreased genes. **(B)** Genes within the KEGG pathways for phagocytosis and antigen presentation that were altered by immune training. Significance in **A** and **B** was determined using the DESeq2 R package, and p-values were adjusted using Benjamini and Hochberg's approach.

Discussion

McCoy, Negi, and colleagues have speculated on the potential relationship between trained immunity and the microbiome (McCoy et al. 2019; Negi et al. 2019). However, empirical data addressing the influence of immune training on the microbiome is lacking. To the best of our knowledge, this is the first study to address the effect of immune training on the microbiome as well as the microbiome in aging. Previous studies have investigated the effect of aging on the microbiome and the effects that this may have on immunity in aging individuals(Amsterdam and Ostrov 2018; Biagi et al. 2012; Cattaneo et al. 2017; Han et al. 2017; Jackson et al. 2016; van Tongeren et al. 2005). Some of the reported changes to the microbiome are thought to contribute to immunosenescence(Amsterdam and Ostrov 2018) and inflamm-aging (Buford 2017) in aging populations. Using a combination of *in vivo* and *ex vivo* approaches, we found that innate immune training reverses some, but not all, age-related changes to the microbiome, thus altering the microbiome in aging mice to more closely resemble the microbiome of younger mice.

We used 16s rRNA gene sequencing to assess bacterial populations across 3 age groups before and after induction of immune training. We found that prior to immune training, the baseline Firmicutes/Bacteroidetes ratio was decreased in aging mice compared to younger mice. This result reflects the decrease in the Firmicutes/Bacteroidetes ratio observed in aging humans(Claesson et al. 2011; Mariat et al. 2009). To assess the effect of immune training on the microbiome, we administered *C. albicans* β-glucan, which is among the most well-studied and strongest inducers of the trained immunity phenotype [24, 25, 28]. After immune training with β-glucans, the Firmicutes/Bacteroidetes ratio in aging mice returned to a level that is more consistent with younger mice. This increase in the Firmicutes/Bacteroidetes ratio was achieved by significantly reducing the relative abundance of Bacteroidetes and a corresponding increase in

Firmicutes that peaked 14 days after immune training. Changes to the microbiome, primarily to the Firmicutes/Bacteroidetes ratio, have been principally linked to diet (Lochlainn et al. 2018; Turnbaugh et al. 2009), indicating that changes in diet in aging individuals may play a role in alterations to the microbiome. However, diet cannot explain our results since all mice in our study were fed the same diet.

Additionally, our study found an increase in the relative abundance of Proteobacteria, a bacterial phylum that contains opportunistic "pathobionts"(Vaiserman et al. 2017), in aging mice at baseline. This finding is supported by a previous study that found an increase in Proteobacteria in centenarians(Biagi et al. 2012). After immune training, the relative abundance of *Clostridia* was significantly increased in aging mice. While the *Clostridia* class contains species that are pathogenic in humans, such as *Clostridia difficile*, *Clostridia* are considered beneficial in the gut and contribute to microbiome homeostasis(Lopetuso et al. 2013) and brain function(Labus et al. 2017). There is growing support for a link between the immune system and the microbiome(Biagi et al. 2012; Molloy et al. 2012). Thus, it is reasonable to conclude that immune training might affect the microbiome.

Previous studies of trained immunity have focused on *ex vivo* analysis of circulating monocytes(Cheng et al. 2014d). We selected monocytes for our study because this cell population may have a substantial impact on the microbiome(Thaiss et al. 2016). Humans and mice obtain their initial intestinal resident macrophage populations from embryonic precursors (Bain and Schridde 2018). As we age this population is replenished by circulating monocytes that originate from bone marrow precursors(Bain et al. 2014). To confirm the induction of immune training, we generated BMDMs from aging mice using an established protocol. We assessed the effect of immune training on BMDMs from aged mice using real-time cell

metabolic assays and transcriptome sequencing. After immune training with β-glucan, BMDMs from aging mice showed higher baseline and stressed ECAR consistent with increased glycolysis observed in trained immunity(Cheng et al. 2014a). Interestingly, BMDMs from aging mice also showed higher oxygen consumption rates, indicating higher levels of oxidative phosphorylation. While this result is in contrast to some previous reports (Cheng et al. 2014a; Netea et al. 2020), we (manuscript submitted) and others(Arts et al. 2016; Leonhardt et al. 2018a) have observed a similar response previously.

In addition to metabolic changes induced by immune training, we also observed broad and numerous changes to the transcriptome in immune-trained BMDMs from aging mice. Previous studies have observed similar levels of transcriptional changes after immune training with β-glucan(Cheng et al. 2014a; Saeed et al. 2014), but not in aging mice. We observed significant changes in the transcription of genes involved in membrane ruffle, phagocytosis, and antigen presentation, all of which are critical macrophage functions(Bain and Schridde 2018; Condon et al. 2018; Henson 2017; Patel and Harrison 2008; Wickramarachchi et al. 2010). These functions interface the immune system and gut microbiome(Bain and Schridde 2018); therefore, alterations to transcription in these pathways might affect microbial communities in the gut. However, the specific mechanism by which immune training may influence the microbiome remains unclear.

While we have made a number of significant observations, our study does have limitations. We employed 12 – 18 month old mice as the aging group. Mice are generally considered "aged" or "old" at ~18 months of age and beyond. However, survival in the $\geq$18 month old mice is problematic and can impact the study. We confirmed that the microbiome of 12–18 month old mice showed changes consistent with aging (**Figure 1**). Thus, we opted to

employ 12-18 month old aging mice as our model. In addition, we did not elucidate a mechanism of action by which glucan induced immune training modulated the gut microbiota. We speculate that Dectin-1 signaling may be responsible for the immune training-induced changes observed in this study. However, the relationship between the immune system and microbiome is complex(Buford 2017; Lloyd-Price et al. 2016; Maffei et al. 2017; Rakoff-Nahoum et al. 2004; van Tongeren et al. 2005), and multiple mechanisms may be involved. Additional studies will be required to elucidate the mechanisms by which immune training impacts the microbiome.

This study provides new and novel insights into the effect of immune training in aging and in the aging microbiome. We found that immune training with β-glucan increased the Firmicutes/Bacteroidetes ratio of aging mice to levels consistent with younger mice and increased the relative abundance of *Clostridia* in aging mice. We also observed metabolic and transcriptomic changes in immune-trained BMDMs from aging mice which are consistent with the trained phenotype(Cheng et al. 2014a; Ifrim et al. 2014; Saeed et al. 2014). Our data also suggest that the interaction between the immune system and the microbiome is bidirectional in that systemic induction of the immune trained phenotype alters the aging gut microbiome. Of potentially greater importance, our data also suggest that it may be possible to pharmacologically modulate the microbiome by training the innate immune system with compounds such as β-glucan. When considered as a whole, these results indicate that immune training can influence the aging microbiome in a manner that may prove beneficial to the aging host.

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Author contributions

PSG was involved in the conceptualization of the study, performed the experiments, analyzed data, drafted the manuscript. JVH assisted with the microbiomic studies, analyzed the microbiomic data and edited the manuscript. TRO assisted with the immune training BMDM studies, analyzed data and edited the manuscript. DLW was involved in the conceptualization of the study, assisted with the trained immunity studies, analyzed data, drafted and edited the manuscript and was responsible for overall supervision of the study.

Competing interests

The authors have no competing interests.

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CHAPTER 5: TRAINED IMMUNITY ATTENUATES SEPSIS-RELATED CHANGES TO THE MICROBIOME

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Abstract

Sepsis presents an immense challenge to healthcare systems and healthcare providers worldwide. In addition to immune dysfunction and organ failure, the host's gut microbiome is significantly impacted by sepsis and contributes to the severity of the condition. In sepsis, the host's commensal microbial communities are often replaced by a "pathobiome" that can disseminate into organ systems, causing multiple infections. A recent study found that Dectin-1 signaling is an essential mediator of intestinal immunity and directly impacts intestinal health and microbial communities. β-glucan "trains" the innate immune system to more quickly and effectively respond to infection through a Dectin-1 dependent mechanism. We utilized a clinically relevant model of polymicrobial sepsis to investigate the effect of β-glucan induced trained immunity on the microbiome in sepsis. We found that immune training with β-glucan prevented sepsis-induced changes to the microbiome. Bacteroidetes in control mice decreased from a relative abundance of 66.2% at baseline to 9% after cecal ligation and puncture (CLP). Additionally, Proteobacteria in control mice increased from 0.03% at baseline to 25% after CLP.

Immune training prevented all statistically significant shifts in bacterial phylum or family populations. Using a computational approach to predict microbiome gene content, we found 3-fold more predicted changes to microbiome function in control septic mice compared to glucan-treated septic mice. Our results indicate that trained immunity may conserve the microbial diversity and function of the microbiome in sepsis.

Introduction

Sepsis is characterized as a dysregulated host response to infection, often leading to organ dysfunction and failure (Singer et al. 2016b). Due to an aging population and improved diagnosis, sepsis incidence is increasing (Dellinger et al. 2013; Singer et al. 2016b). In 2017, sepsis cost the United States healthcare system $40 billion, making it the costliest condition treated in hospitals (Liang et al. 2006). Sepsis presents an even greater burden worldwide, where it accounts for almost 20% of all deaths, making it a leading cause of death (Rudd et al. 2020). While improvements in of the diagnosis of sepsis leading to earlier treatment have decreased mortality rates (Cavaillon et al. 2020), decades of clinical trials have primarily failed to identify effective therapies(Angus 2011; Cohen et al. 2012; Marshall 2014). Immunotherapies have shown some promise in treating sepsis (9, 10), there are still no FDA-approved drugs for sepsis (Zhang et al. 2016).

A healthy gut microflora helps to prevent infection by colonizing the intestines and outcompeting potentially pathogenic microbes (Miller et al. 2021). The interaction between the microbiome and immune system is also crucial in preventing allergies and controlling the inflammatory response (Buford 2017). For example, when gut microbiome homeostasis is disrupted by antibiotic treatment, individuals experience increased susceptibility to viral infections (Abt et al. 2012; Ichinohe et al. 2011). The gut microbiome also plays an

immunomodulatory role in the host (Buford 2017), and it contributes to limiting the acute inflammatory response that occurs after injury (Rakoff-Nahoum et al. 2004). Additionally, dysbiosis in the gut increases an individual's risk for a wide range of diseases, such as Crohn's disease (Dubinsky et al. 2006), Parkinson's disease (Sampson et al. 2016), diabetes (Barlow et al. 2015), and osteoporosis (Buford 2017).

In sepsis, the immune response is characterized by an initial hyperinflammatory phase (D'Elia et al. 2013; Huang et al. 2019), followed by prolonged immunosuppression that leaves patients vulnerable to secondary infections (Hotchkiss et al. 2013b; Venet and Monneret 2018). Gut permeability is also altered in sepsis (Fay et al. 2017). These aspects of the host response to sepsis, along with standard of care, antibiotics administration, can cause microbiome dysbiosis (Bassetti et al. 2020; Fay et al. 2017; Miller et al. 2021). When dysbiosis occurs in sepsis, commensal microbial communities in the gut are often replaced by a "pathobiome" that causes further illness, injury, and immune impairment (Bassetti et al. 2020; Fay et al. 2017; Miller et al. 2021). Ultimately, this leads to dissemination of infection to organ systems and worsens outcomes in critically ill patients (Kitsios et al. 2017; Krezalek et al. 2016).

There is increasing evidence that the innate immune system can be "trained" to more quickly and effectively respond to infection (Cheng et al. 2014a; Ifrim et al. 2014; Netea et al. 2020; Saeed et al. 2014). This concept is known as innate immune memory or trained immunity. Trained immunity is characterized by exposure to a pathogen or pathogen-associated molecular pattern (PAMP), resulting in a subsequent increase in cellular metabolism, pro-inflammatory cytokine production, transcriptomic changes, and specific epigenetic modifications (Cheng et al. 2014a; Netea et al. 2020). *Candida albicans*-derived β-glucan is one of the most well-studied and potent inducers of immune training (Cheng et al. 2014a; Ifrim et al. 2014; Negi et al. 2019; Netea

et al. 2020; Saeed et al. 2014). Dectin-1 is the primary receptor for β-glucan, and trained immunity is dependent on Dectin-1 signaling (Cheng et al. 2014a). Tang et al. found that Dectin-1 signaling contributes to colitis in the gut by promoting *Lactobacillus* growth (Tang et al. 2015). Dectin-1 is also known to regulate intestinal inflammation, and it has been speculated that trained immunity will modulate the microbiome through a Dectin-1 mediated mechanism (Negi et al. 2019). Additionally, data from our lab revealed that trained immunity alters the gut microbiome of aging mice (manuscript submitted). Thus, we investigated the effect of β-glucan-induced trained immunity on the microbiome in an animal model of chronic, polymicrobial sepsis.

Methods

Mice. Male C57Bl/6J mice were purchased from Jackson laboratory. All procedures and experiments involving mice were approved by the IAUC committee under protocol #190201. Mice were housed and cared for by the Division of Laboratory Animal Resources at East Tennessee State University. Mice were fed a standard chow diet and given food and water *ad libitum*.

β-glucan. β-1,3-(D)-glucan was isolated from *Candida albicans* SC5314 as described by Lowman et al. (Lowman et al. 2014). 18-week-old mice were injected (IP) twice with 1 mg (approx. 40 mg/kg) of β-glucan suspended in 0.5 mL of dextrose 5% in water (D5W). Control mice were injected with 0.5 mL D5W as the vehicle control.

Cecal Ligation and Puncture. β-glucan or D5W were injected on days -7 and -4 before cecal ligation and puncture (CLP) was performed on day 0 as previously described (Baker et al. 1983) (**Figure 20A**). For the model used in this study, half of the cecum was ligated, and a single puncture was made with a 20g needle. Mice were injected subcutaneously with warmed saline

solution immediately after the procedure for fluid resuscitation. Mice were monitored daily to assess their condition. Mice were euthanized before imminent death, and surviving mice were euthanized 14 days after CLP.

16s sequencing. 3 fresh fecal samples were taken from each mouse. F1 was collected before the first β-glucan or D5W injection. F2 was collected immediately before CLP. F3 was collected before death or euthanasia when possible (**Figure 20B**). Fecal samples were snap-frozen in liquid nitrogen immediately after collection and stored at -80°C. DNA was isolated from fecal samples using the Qiagen PowerSoil DNA isolation kit (Qiagen #12888). The V3/V4 region of the 16S ribosomal RNA gene was amplified, and a DNA library was prepared for each sample following the Illumina 16S Metagenomic Sequencing Library workflow (Illumina 2013). The Illumina MiSeq platform was used for DNA sequencing.

Statistical analysis. All data are presented as means unless otherwise specified. Data analysis was performed, and graphs were created using the Microbial Genomics Module in the CLC Biogenomics workbench (Qiagen), Minitab, and Microsoft Excel. Survival curves were analyzed using a Log-rank (Mante-Cox) test. Alpha diversity was used to assess phylogenetic diversity in the Microbial Genomics Module in CLC. Beta diversity was assessed by performing principal coordinate analysis and a PERMANOVA analysis using weighted and unweighted UniFrac. Differential abundance of OTUs was analyzed statistically in CLC using a generalized linear model and a likelihood ratio test to analyze differences across fecal samples. The false discovery rate corrected p-value (FDR p-value) was used to correct for multiple comparisons. Relative abundance of bacteria phylum and families were compared statistically using the Kruskal-Wallis test. Predicted pathways were constructed using PICRUSt, and F1 and F3 predicted pathways were analyzed statistically using an ANOVA and Tukey's posthoc test.

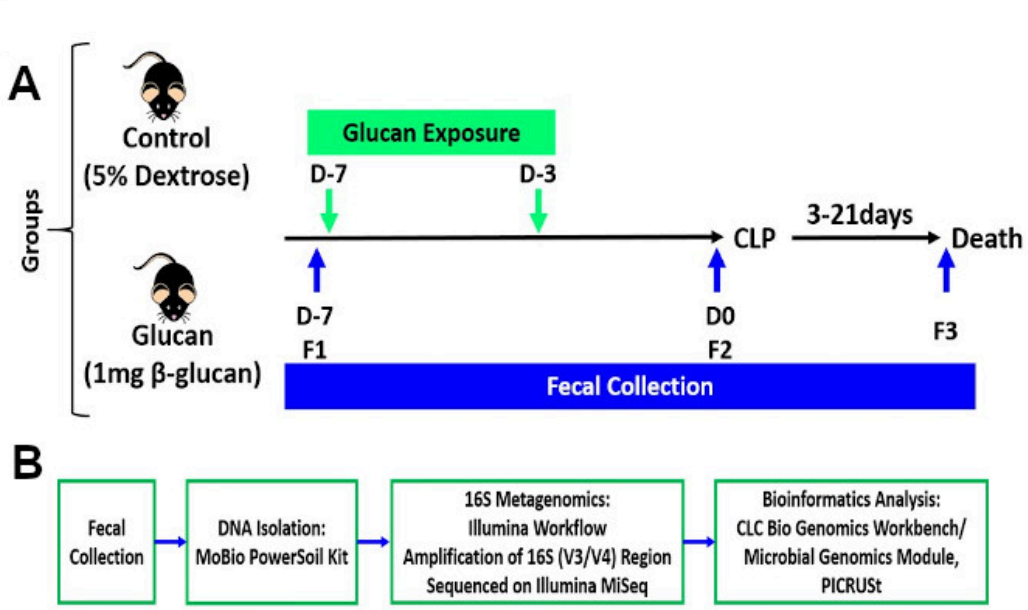


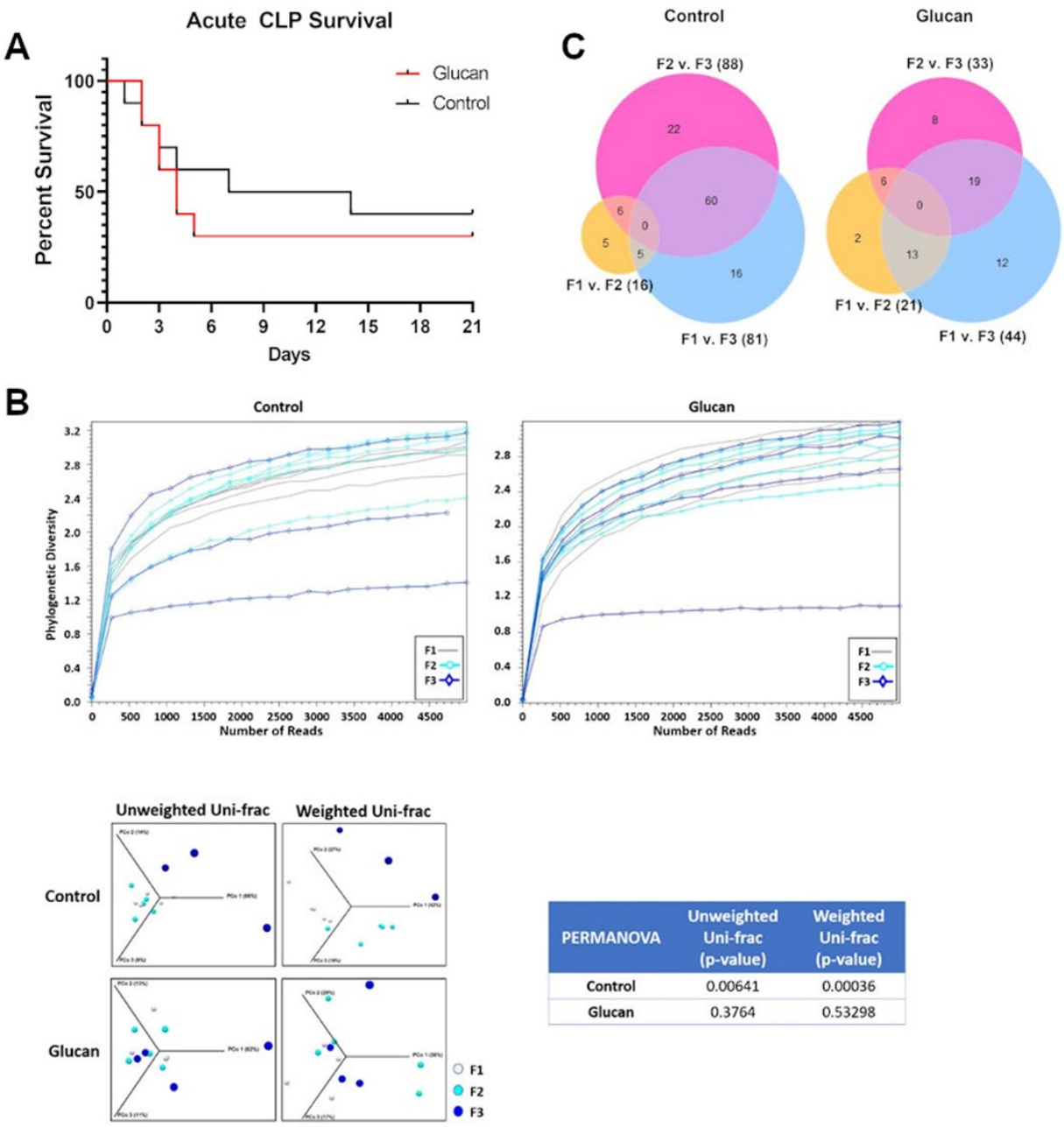
Figure 20. Immune training modulates the immune response in BMDMs in aged mice. (A) Fecal samples were collected from male C57Bl/6J mice. F1 was collected before injection of β-glucan or D5W injection. F2 was collected 7 days after F1 after β-glucan or D5W injections. F3 was collected after CLP before death. **(B)** 16s rRNA sequencing and analysis workflow.

Results

Trained immunity attenuates sepsis-induced changes to the microbiome.

Mice were injected with β-glucan or D5W 7 and 4 days before cecal ligation and puncture. There was no significant difference in survival rates between control and glucan-treated mice (**Figure 21A**). Fecal samples were collected before the first β-glucan or control injection, after immune training and control injections but before CLP, and after CLP before death or euthanasia. We did not observe significant differences in alpha diversity as a result of β-glucan or D5W injection; however, we observed increased variance of diversity after CLP in control mice and to a lesser extent in β-glucan treated mice (**Figure 21B**). Principle coordinate analysis (PCoA) was performed to measure beta-diversity, and a PERMANOVA was used to

analyze these results statistically. There was a significant difference in beta diversity between fecal samples in the control group using weighted and unweighted uni-frac. However, there was no significant difference in the beta diversity in the glucan-treated group (**Figure 21B**). **Figure 21C** displays Venn diagrams depicting numbers of operational taxonomic units (OTUs) present at significantly different abundances between fecal samples. There were significantly different relative abundances of OTUs between all fecal samples in both groups. However, these differences were 54% greater in the control group than in the glucan-treated group (**Figure 21C**).



PERMANOVA	Unweighted Uni-frac (p-value)	Weighted Uni-frac (p-value)
Control	0.00641	0.00036
Glucan	0.3764	0.53298

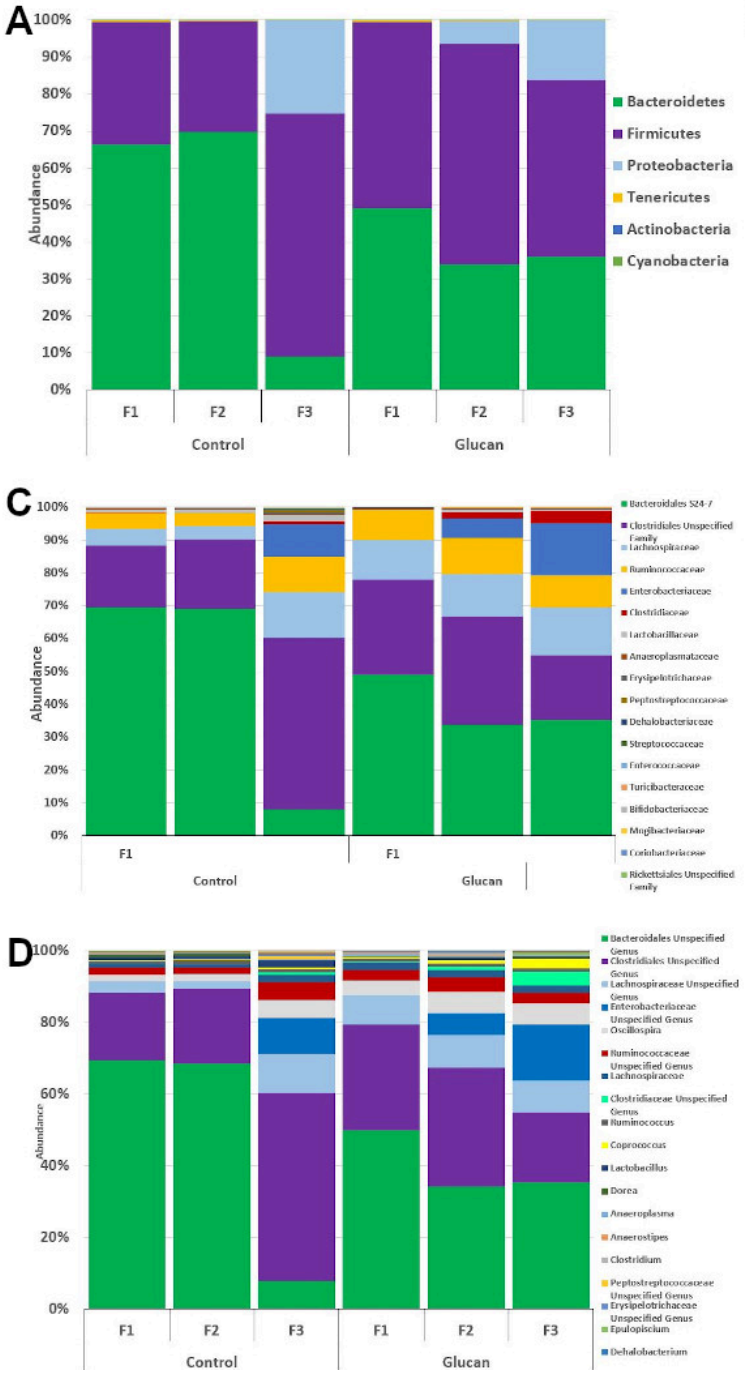
Figure 21. Trained immunity attenuates sepsis-induced changes to the microbiome. (A) Survival curve of CLP model comparing control and glucan treated mice. N=10. **(B)** Alpha (top panels) and Beta (bottom panels) diversity of 16s rRNA sequencing samples. Glucan: N=4-5, Control N=3-5; $P<0.05$.

Trained immunity prevents changes to the relative abundance of bacterial communities in the gut.

The relative abundance of bacterial phyla and families was assessed at each time point. The relative abundance of Bacteroidetes and Proteobacteria was significantly altered throughout the experiment in control mice. In control mice, Bacteroidetes composed on average 66.2% of the bacterial fecal microbiome at baseline (F1), 69.8% after D5W injection (F2), but only 9% after CLP (F3). Also, in control mice, Proteobacteria composed 0.03% of the bacterial fecal microbiome at baseline, 0.01% after D5W injection, and 25.2 % after CLP (**Figure 22A**). Decreased Bacteroidetes and increased Proteobacteria have been associated with worse outcomes in sepsis models (Bassetti et al. 2020), and increased Proteobacteria has been previously observed in human sepsis patients (Wan et al. 2018). Additionally, we observed an increase in the Firmicutes/Bacteroidetes ratio after CLP in control mice (**Figure 22B**). In contrast, we did not observe any significant shifts in bacterial phyla across the experiment in glucan-treated mice (**Figure 22A, B**).

Additionally, the relative abundance of 4 bacteria families was significantly altered in control mice, i.e. *Christensenellaceae*, *Carnobacteriaceae*, *Enterobacteriaceae*, and an unspecified family of *Bacteroidales* (**Figure 22C**). Relative abundance of *Christensenellaceae* and an unspecified family of *Bacteroidales* both decreased after CLP. *Christensenellaceae* decreased from approximately 0.02% before CLP (F1), to 0%, or below the detection level, after CLP (F3). The unknown family of *Bacteroidales* decreased from 68.5% at baseline (F1) and 66.8% after D5W injections but before CLP (F2) to only 9% after CLP (F3). *Christensenellaceae* has previously been associated with health (Waters and Ley 2019), and decreases in *Bacteroidales* are associated with changes to the microbiome in critically injured patients

(Howard et al. 2017). Relative abundance of *Carnobacteriaceae* and *Enterobacteriaceae* both increased after CLP. *Carnobacteriaceae* increased from 0% or below the detection level before CLP to 0.05% after CLP. *Enterobacteriaceae* increased from 0.008% at baseline and 0.004% after D5W injection to 25.2% after CLP. Little is known about the significance of *Carnobacteriaceae* in the microbiome. *Enterobacteriaceae* is a diverse family of bacteria of which expansion has been associated with inflammatory conditions such as Crohn's Disease (Zhang et al. 2015). In contrast, there were no significant shifts in bacterial families due to CLP or in immune-trained mice (**Figure 22C**).



B

	Firmicutes/Bacteroidetes Ratio		
	F1	F2	F3
Control	0.50	0.43	7.33
Glucan	1.02	1.76	1.33

Figure 22. Trained immunity prevents changes to the relative abundance of bacterial communities in the gut. (A) Relative abundance of bacteria phylum across fecal samples in glucan-treated and control mice. Glucan: N=4-5, Control N=3-5; P<0.05. **(B)** Firmicutes/Bacteroidetes ratio of F1-F3 fecal samples in control and glucan-treated mice. Glucan: N=4-5, Control N=3-5. **(C)** Relative abundance of bacteria families across fecal samples in glucan-treated and control mice. Glucan: N=4-5, Control N=3-5; P<0.05. **(D)** Relative abundance of bacteria genera across fecal samples in glucan-treated and control mice. Glucan: N=4-5, Control N=3-5; P<0.05.

Trained immunity attenuates changes to the predicted function of microbial communities in sepsis.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a software package and computation technique used to predict metagenome function using 16s rRNA sequencing data (Langille et al. 2013). For this analysis, we compared F1 (baseline) samples to post-CLP (F3) samples to assess the effect of immune training on predicted gene function of the microbiome in sepsis. Overall, we observed 3-fold more pathways were significantly altered after CLP in the control group, compared to the immune trained group (**Figure 23A, B**). Among the significantly altered pathways in the control group were 'glycan biosynthesis and metabolism' and 'environmental information processing,' neither were altered in the glucan-treated group (**Figure 23A**). Pathways involved in cell motility and flagellar assembly were significantly altered in both groups (**Figure 23A, B**). While changes to predicted function were observed in both control and immune training, β-glucan injection appears to have dampened sepsis-related changes to the microbiome.

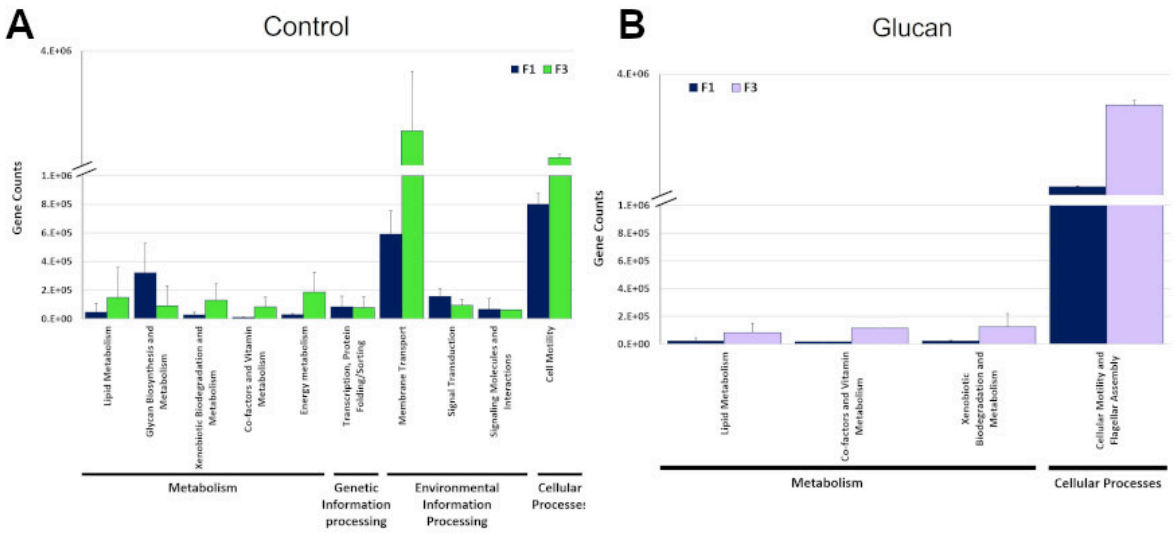


Figure 23. Trained immunity attenuates changes to the predicted function of microbial communities in sepsis. PICRUSt was used to approximate the gene content of bacterial communities in fecal samples from control and glucan-treated mice. **(A)** Level 2 KEGG terms significantly altered between F1 and F3 in control mice. N=3-5, P<0.05. **(B)** Level 2 KEGG terms significantly altered between F1 and F3 in glucan-treated mice. Glucan: N=4-5, P<0.05.

Discussion

Sepsis is among the costliest and most deadly diseases worldwide (Liang et al. 2006; Rudd et al. 2020). The systemic and dysfunctional host response to sepsis leads to damage and failure across many organ systems (Huang et al. 2019). The profound effect of sepsis on the host also critically impacts the microbiome, further damaging the host (Bassetti et al. 2020; Fay et al. 2017; Kitsios et al. 2017; Krezalek et al. 2016; Miller et al. 2021). Recent studies have found that the innate immune system can be trained to more quickly and effectively respond to infection (Cheng et al. 2014a; Ifrim et al. 2014; Netea et al. 2020; Saeed et al. 2014). It has been speculated that trained immunity will also impact the microbiome (McCoy et al. 2019; Negi et al. 2019), and work from our lab has found that trained immunity may prevent some age-related changes to the microbiome (manuscript submitted). We utilized a clinically relevant model of

polymicrobial sepsis and 16s rRNA sequencing to investigate the microbiome in the presence of sepsis and trained immunity. We found that β-glucan-induced trained immunity prevented sepsis-associated changes to the microbiome when compared to untrained control mice.

We used 16s rRNA sequencing to assess bacterial communities present in mice at baseline, after immune training or D5W injection, and after CLP before death or euthanasia. Overall, we found no effect of injection of D5W or immune training alone. However, sepsis induced many changes to the fecal microbiome. In control mice, we found a significant loss of Bacteroidetes, a shift in the Firmicutes/Bacteroidetes ratio, and a significant expansion of Proteobacteria. None of these shifts were observed in glucan-treated mice, indicating that trained immunity may prevent sepsis-associated changes to the microbiome. This result is noteworthy since sepsis-induced dysbiosis contributes significantly to pathophysiology of sepsis (Fay et al. 2017; Krezalek et al. 2016). Expansion of the Proteobacteria population in sepsis is linked to increased inflammation and development of antibiotic resistance in sepsis patients (Bassetti et al. 2020; Fay et al. 2017). Additionally, loss of Bacteroidetes and expansion of Proteobacteria lead to induction of bacterial dissemination and death in a mouse model of sepsis (Hyoju et al. 2019). Thus, prevention of this shift may prove beneficial in sepsis.

Similarly, we observed shifts in the control group at the bacterial family level that were not present in the glucan-treated group. Of particular note, the relative abundance of *Christensenellaceae* was decreased in control mice in sepsis. *Christensenellaceae* only make up about 0.01% of the human gut microbiome (Goodrich et al. 2014; Waters and Ley 2019). This bacterial family has been associated with longevity, low BMI, reduced risk of colorectal cancer, and overall health (Goodrich et al. 2014; Mancabelli et al. 2021; Waters and Ley 2019). This finding highlights the idea that even loss of bacterial communities present at low concentrations

can profoundly impact the host. Thus, dysbiosis that occurs in sepsis may impact the host in unforeseen ways. To further investigate potential functional changes to the microbiome, we performed PICRUSt analysis. We observed 3-fold more significantly predicted functional changes in the microbiome in sepsis. Among the changes observed only in control mice, we found sepsis-induced changes to predicted pathways involved in 'glycan biosynthesis and metabolism' and 'environmental information processing.' Loss or alteration of microbiome function contributes to sepsis severity (Krezalek et al. 2016; Miller et al. 2021). In our study, trained immunity prevented most predictive functional changes to the microbiome. Thus, trained immunity appears to preserve microbiome function in sepsis.

It is well established that bidirectional communication exists between the immune system and microbiome (Goulet 2015; Ivanov et al. 2008b; Lloyd-Price et al. 2016; McCoy et al. 2019; Negi et al. 2019). Tang et al. reported the impact of Dectin-1 signaling on the expansion of *Lactobacillus murins* and the development of colitis in mouse models (Tang et al. 2015). Others have also noted the role of Dectin-1 in intestinal immunity (Iliev 2015; Li et al. 2019). Dectin-1 is also the primary receptor for β-glucans (Brown et al. 2003) and is required for β-glucan-mediated induction of trained immunity (Cheng et al. 2014a). Thus, we hypothesize that β-glucan-mediated Dectin-1 signaling may be responsible for the results described in this study; however, additional studies are necessary to determine the cellular and molecular mechanisms responsible for the protective effect of β-glucan on the microbiome.

When considered as a whole, our data indicate that immune training prevented a multitude of sepsis-induced changes to the microbiome. While shifts in Bacteroidetes and Proteobacteria populations were observed in control mice due to sepsis, no significant change to bacteria phyla or families was observed in immune trained mice. Using a computational

approach, we also found 3-fold more significant predictive changes to microbiome function in control mice when compared to immune trained mice, thus indicating that trained immunity may help preserve the microbiome's function in sepsis. While trained immunity did not improve survival in this sepsis model, maintaining microbiome diversity and homeostasis may be beneficial for recovery in sepsis survivors. Our results indicate that trained immunity influences the microbiome in sepsis, which may prove therapeutically beneficial.

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CHAPTER 6. CONCLUSIONS

In conclusion, our study uncovered effects of trained immunity in aging and sepsis. Firstly, we found that β-glucan induces the trained immunity phenotype in monocytes from healthy aging individuals and sepsis patients. Additionally, found that trained immunity also induces monocyte differentiation towards an M2 macrophage phenotype across all age ranges. Secondly, we determined that trained immunity induces a subset of FSC^{hi}, $CD11b^{+}$, $GR\text{-}1^{hi}$ cells in a chronic, polymicrobial model of sepsis, and this cell population bears some of the phenotypic hallmarks of MDSCs. We also demonstrated that trained immunity modulates host gut microbiota in aging and sepsis. In aging, β-glucan-induced trained immunity reversed age-related changes to the microbiome. Most notably, the Firmicutes/Bacteroidetes ratio in aged-trained mice was reverted to a level consistent with younger healthy, and the relative abundance of *Clostrida* was increased in trained aged mice. Trained immunity also prevented sepsis-induced changes to the gut microbiome. While we found that control septic mice had an expansion of potentially pathogenic Proteobacteria, we did not find any significant changes to bacterial phyla in immune-trained septic mice.

While we identified that immune training enhances the innate immune response in healthy aging individuals, future studies should investigate the effect of trained immunity on individuals experiencing chronic-age-related diseases related to immunosenescence. In the future, we will also investigate the function of the FSC^{hi}, $CD11b^{+}$, $GR\text{-}1^{hi}$ cells that were expanded in trained septic mice through co-culture and adoptive transfer experiments. Lastly, the mechanism(s) by which the gut microbiome is influenced by trained immunity must be investigated. While Dectin-1 signaling is nearly certain to be involved, other potential mechanisms remain unclear.

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APPENDIX: Abbreviations

2-DG	2-Deoxy-D-glucose
ABCs	Atypical memory B Cells
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AKT	Protein kinase B
ANOVA	Analysis of variance
BCG	Bacillus Calmette–Guérin vaccine
BMDM	Bone marrow derived macrophage
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
D5W	Dextrose 5% in water
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ECAR	extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxy phenylhydrazone
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FPKM	Fragments Per Kilobase of transcript per Million mapped reads

FSC	Forward scatter
GO	Gene ontology
GSEA	Gene set enrichment analysis
H3K27ac	Histone 3 lysine 27 acetylation
H3K4me	Histone 3 lysine 4 methylation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1a	Hypoxia-inducible factor 1-alpha
HLA-DR	Human leukocyte antigen-DR
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IRF	Interferon regulatory factors
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
MPLA	Monophosphoryl lipid A
mTOR	Mammalian target of rapamycin
NAD+	Nicotinamide adenine dinucleotide
NCS	Newborn calf serum
NETs	Neutrophil extracellular traps

NK cells	Natural killer cells
NOD receptors	Nucleotide-binding and oligomerization domain receptors
OCR	Oxygen consumption rate
OUT	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCoA	Principle coordinate analysis
PD-L1	Programmed death-ligand 1
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal ribonucleic acid
SCFAs	Short-chain fatty acids
SCID	Severe combined immunodeficient
SEM	Standard error of the mean
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCA cycle	Tricarboxylic acid cycle
Th cells	T helper cells

TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells

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