

Beta-glucan Trained Immunity in Macrophages as Immunotherapy Against Infection

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To my mom, whose sacrifices have given me the opportunities she never had

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CHAPTER 1. INTRODUCTION

Overview

Once considered a conquered foe, a combination of factors is contributing to the resurgence of infectious diseases as a significant and urgent threat for the twenty-first century. Patients are older with more comorbidities, yet they undergo more complex medical and surgical procedures resulting in an immunosuppressed and vulnerable population. Pathogens are rapidly developing resistance to the antimicrobial armamentarium available to healthcare providers, yet the discovery of new drugs has ground to a halt. Therefore, novel strategies to prevent infections are greatly needed. Prophylactic immunomodulation to augment the host response to infection is a promising strategy to achieve this goal.

Historically, infection prophylaxis has been restricted to the realm of vaccines, which induce long-lived adaptive immune responses. However, mounting evidence suggests that the innate immune system can be trained to mount an enhanced response to infection after initial stimulation. Trained innate immunity, or innate immune memory, is an attractive strategy especially for hospitalized or immunosuppressed patients. Our lab and others have characterized several microbial-derived ligands that induce trained immunity against subsequent infection. Further study of these ligands will shed light on the mechanisms underlying trained immunity and inform their clinical development.

Beta-glucan, a complex polysaccharide that constitutes the most abundant component of the fungal cell wall, is the canonical innate training agent. However, several questions remain regarding the mechanism by which β -glucan enhances innate immunity. In this dissertation, I explore the efficacy of β -glucan training in a clinically relevant model of infection and identify key cellular mechanisms that underpin protection. In **Chapter 1**, I explore the history, severity, and contributing factors of rising infectious disease morbidity and mortality. Additionally, I outline the

host innate immune response to infection, as well as provide the current understanding of how β -glucan augments this response. In **Chapter 2**, I demonstrate the ability of β -glucan training to protect against *Pseudomonas aeruginosa*, detail the physiologic and immunologic parameters of β -glucan training, and identify macrophages as a key contributor to protection. In **Chapter 3**, I show the β -glucan-induced alterations in macrophage antimicrobial activity that contribute to protection from infection. Additionally, I characterize the metabolic phenotype of β -glucan-trained macrophages and explore how metabolism contributes to the antimicrobial activity. In **Chapter 4**, I demonstrate that β -glucan induces protection against infection and alterations in macrophage phenotype independently of the major receptors dectin-1 and Toll-like receptor-2. Finally, in **Chapter 5**, I discuss how these findings fit within the evolving concept of trained immunity, the limitations of the presented studies, and future directions for the work.

The Modern Infectious Disease Problem

Over the last 250 years, advances in our understanding of infectious disease count among the most consequential for human health. In the early 19th century, vaccination against the smallpox virus became routine across Europe despite a lack of knowledge of microbes and the causes of infectious disease. This was eventually followed by adoption of the germ theory of disease, which identified pathogens as the causative agents of a wide variety of illnesses. Finally, the characterization of antibiotics at the turn of the 20th century is undoubtedly one of the most significant milestones in medical science. Not long after Paul Ehrlich, Alexander Fleming, and others began to describe the first antimicrobial compounds, academic institutions and industry partners worked together for rapid development of mass production for penicillin. After these initial findings, the number of available antibiotics rapidly expanded as new classes and formulations were discovered. At the same time, sterilization practices in the healthcare setting were also rapidly improving [1]. These discoveries are credited with prolonging the life expectancy in developed nations by as long as two decades [2].

However, infectious diseases remain a substantial burden in modern healthcare. In the hospital setting alone, it is estimated that infections claim around 100,000 lives and cost the United States healthcare system 28 to 33 billion dollars annually [3, 4]. Outside of the hospital, community-acquired infections also cause significant morbidity and mortality. By itself, community-acquired pneumonia is responsible for over 60,000 deaths and costs 17 billion dollars annually while the hospitalization rate continues to rise [5].

Several factors contribute to this phenomenon. In general, the population in the United States is aging and undergoing medical procedures later in life [6]. The same antibiotics that were responsible for the vast improvement in the treatment of infections are now rapidly becoming obsolete. Together, inappropriate prescribing and usage, antimicrobial resistance among microbes, and a lack of discovery of new antibiotics are making infections harder to manage [7, 8]. Additionally, advances in other realms of medicine, such as chemotherapy and organ transplant, leave patients severely immunosuppressed [9]. Understanding these risk factors is essential to ultimately overcoming this growing problem.

Antimicrobial resistance

Perhaps the most pressing problem for the treatment of infectious disease is the development of antimicrobial resistance amongst pathogens [10, 11]. Less than half a century after the implementation of antibiotics, microorganisms began to display resistance to those drugs [12]. This is compounded by the fact that the discovery of new antibiotics has slowed significantly due to the increasing complexity and lack of financial viability of developing new drugs [13]. It is estimated that without a significant reorganization of research and development, global deaths from infectious diseases will surpass cancer and total near 10 million per year, with 350,000 in the United States alone [14].

In particular, bacteria possess many natural mechanisms to obtain and share individual genetic elements that can spread resistance genes across and between species [15]. For example, the mechanism of action of β -lactam antibiotics, such as penicillin, is to inhibit the

bacterial enzyme transpeptidase, which is essential for production of the bacterial cell wall. In the 1950s, individuals began characterizing β -lactamase enzymes present in *Enterobacteriaceae* family bacteria that degraded penicillin, allowing transpeptidase activity to continue [16]. By the early 21st century, more than 800 β -lactamase enzymes had been characterized. The genes for these β -lactamase enzymes are encoded on a plasmid that can be easily transferred between bacteria. This process, known as horizontal gene transfer, allows a single bacterium to generate an entire colony of resistant pathogens [17].

A selective pressure is applied to bacteria when an antibiotic is applied [18]. If the antibiotic is completely effective, then 100 percent of the bacteria will die when exposed. In the case where a mutation or mobile genetic element leads to resistance, the antibiotic will still be effective if it kills off enough bacteria that the host immune system can clear the rest. However, when an antibiotic is given in too low of a dose, or doses are skipped by a patient, then enough selective pressure will be applied to kill off all non-resistant bacteria but not enough that the bacteria can be completely cleared from the host. This gives the relatively smaller number of resistant bacteria significantly more nutrients and resources to then re-populate, thus leading to a much larger population of resistant bacteria [19].

Generally, antibiotic resistance is accomplished in one of three ways: 1) upregulation of bacterial enzymes to metabolize the drug, 2) efflux of antibiotics out of the bacterial cytosol by specialized protein pumps, or 3) mutation of the original antibiotic target [20]. Bacterial enzymes able to metabolize antibiotics often serve a dual role of metabolizing a chemically similar structure found in the environment. Resistance that occurs secondary to repurposing an existing gene product is referred to as intrinsic resistance [21]. This cross-reactivity is hypothesized to occur because many bacteria subsist in soil, where organisms producing the natural products that form the chemical backbones of antibiotics cohabit with the pathogens [22]. Because the bacteria likely come into contact with these natural products, they have evolved strategies to

metabolize and neutralize bactericidal compounds. In addition to intrinsic resistance, bacteria also adopt antimicrobial resistance phenotypes due to anthropogenic activities. Since the 1940s, antibiotics have been used to treat human illness, promote growth in livestock, and provide pesticide for agriculture. Inevitably, these products will enter into the biosphere, given that antimicrobials are designed to resist degradation. This is especially true in livestock farms, where animal waste allows these drugs to ultimately enter the water supply. As mentioned above, chronic exposure of microbes to low doses of antimicrobial drugs is a potent selective pressure for resistance [23].

Compounding the problem of antimicrobial resistance is the dramatic slowdown in the development of new antimicrobial agents to bypass current resistance mechanisms. In the last three decades, there have been no new antibiotics discovered that constitute a novel chemical structure or mechanism of action [24]. Due to a lack of financial incentive for pharmaceutical companies to invest significant resources in antimicrobials, they fall to a lower priority tier. Furthermore, the bulk of antibiotic development has been based on discovery of natural products from the environment. Discovery of mechanistically unique natural products has slowed significantly, necessitating investment in *de novo* chemical synthesis, which is laborious and expensive [7]. Ultimately, without significant monetary investment and shift of research priorities, relying solely on antibiotics as antimicrobial strategy will fail.

Aging population

It is predicted that by 2040, at least 1 in 5 Americans will be over the age of 65, and many of them will live well into their eighth decade. As the population ages, infections will become more common and deadly. There are numerous factors contributing to increased risk for infection in elderly patients (discussed below); they can be summarized into the categories of impaired host defense and increased exposure to pathogens. Not only are aged patients more susceptible to infection, but they also present atypically, with classic infection signs and symptoms, such as fever, malaise, and chills oftentimes absent from the initial presentation [25].

This is because fundamental aspects of the immune response deteriorate over time, both masking the infection and preventing adequate host defenses.

Deteriorating immunity associated with older age, a process known as immunosenescence, is a multifactorial and complex process [26]. Hematopoietic stem cells, which are responsible for maintaining the population of immune cells across the body, decrease in number and diversity with age. This is due, in part, to decreased ability to sustain a self-renewing population in the bone marrow. The cells that do remain are less effective at homing to the appropriate tissues because of diminished interactions with bone marrow stromal cells [27]. While most tissues have some form of resident immune system to detect invaders, recruitment of additional cells from the bone marrow is essential to the immune response for most infections. The thymus, which is critical for T cell development, begins to atrophy at a young age and continues for the remainder of one's life. Finally, the leukocytes that do develop in older patients show marked decreases in their ability to mount antimicrobial responses, such as antigen presentation, cytokine production, bacterial killing, and development of long-term immunologic memory [28]. Taken together, these changes lead to a diminished response to infection, decreased efficacy of vaccines in the elderly, and a wider range of pathogens potentially responsible for disease in the elderly.

In addition to immunosenescence, older age is associated with decreased mobility and increased time at rest, which hinders clearance of bacteria from the lung. Pulmonary infections are particularly common in aged patients and represent the 5th leading cause of death in individuals older than 65 [29]. This is especially true in elderly smokers and those with chronic obstructive pulmonary disease (COPD). This highly prevalent disease impairs mucocilliary clearance and proper function of the innate immune system of the lungs [30]. Immobilized patients also often require indwelling catheters, dramatically increasing the risk of urinary tract infections in these patients.

Older individuals also suffer from several comorbidities that, in and of themselves, increase the risk for infection. Diabetes mellitus (DM), for example, affects nearly every major organ system and predisposes individuals to a wide array of infections [31]. Longstanding hyperglycemia from untreated DM damages renal microvasculature, which impairs recruitment of leukocytes to renal parenchyma. Above a certain blood glucose level, known as the renal threshold, glucose will begin to spill into the urine. This combination of impaired defense and increased nutrients make urinary tract infections especially common in patients with DM [32]. Destruction of the microvasculature of the distal extremities also leads to hypoperfusion and nerve damage in the fingers and toes of DM patients. The resultant neuropathy causes loss of sensation in the hands and feet, which prevents DM patients from noticing minor abrasions or cuts. Ignored wounds develop into ulcers that quickly become infected. Indeed, foot ulcer infections are one of the most common causes of hospitalization for DM patients [33]. Other common comorbidities that predispose elderly patients to infections include obesity, peripheral vascular disease, and chronic kidney disease.

Finally, older individuals are at increased risk for exposure to pathogens in several locations. Older patients are far more likely to utilize hospital services [34]. This places them at increased risk for nosocomial infections (discussed above) that carry significant risks. Older age is also associated with assisted- or group-living environments, such as nursing homes, which congregate many susceptible individuals into one space [35]. Indeed, nursing homes are a common source of infectious outbreaks, including influenza virus, *Klebsiella pneumoniae*, group A Streptococci, and COVID-19 [36-39]. Communal activities, such as meals or social gatherings, in nursing homes facilitate the spread of pathogens. Furthermore, the decreases in functional status and ability to complete activities of daily living that require nursing home care are risks for infection themselves [40].

As the population ages, innovation in medical treatment for infectious diseases will be essential to address these risk factors. Because geriatric patients respond less effectively to

current treatments, strategies to prevent the onset of infection are especially attractive. This is also compounded by the fact that elderly patients are at a higher risk for opportunistic infections, which are often resistant to current antimicrobials, as mentioned above. However, old age isn't the only condition that can predispose individuals to these infections.

Immunosuppression

Immunosuppression from illness or medical procedures places individuals at extremely high risk for infection, including from pathogens that don't typically infect an immunocompetent host. Many illnesses cause immunosuppression, with some of the most severe forms arising from human immunodeficiency virus (HIV) infection, extensive burns or trauma, and hematologic cancers [41]. Iatrogenic (medically related) causes include immunosuppressive therapy after solid organ transplantation, bone marrow irradiation, glucocorticoid use, and other treatments for autoimmune disease [42]. Broadly, immunosuppression can be categorized into defects of physical barriers, innate immunity, or adaptive immunity. Because each functional aspect of the immune system is specialized to defend against specific pathogens, deficiencies in a particular aspect leads to predictable infections able to take advantage of that deficiency [43].

HIV infection directly infects CD4+ T cells, ultimately leading to their lysis [44]. Without antiretroviral treatment, a patient's CD4+ count can reach undetectable levels in 12 to 18 months. Depending on the patient's CD4+ count, an extensive number of infections can arise. Below a CD4+ count of 50 cells/mm³ blood, acquired immunodeficiency disease (AIDS)-defining illnesses begin to occur [45-47]. For example, *Pneumocystis jirovecii* is an opportunistic fungus that causes pneumonia in immunocompromised HIV patients [48]. It is the most common opportunistic infection in HIV/AIDS when prophylactic antimicrobial agents aren't given [49].

The causes of burn- or trauma-induced immunosuppression are multifactorial, but common themes emerge. The initial injury, such as a large full thickness burn, causes hyperactivation of the immune system, especially preprogrammed innate immunity. The flood of

proinflammatory cytokines, prostaglandins, and other inflammatory mediators eventually stuns subsequent leukocytes and prevents them from functioning properly [50]. Additionally, these injuries diminish oxygen delivery to tissues, either by physical destruction of blood vessels or loss of intravascular volume [51]. Patients with severe burns or trauma will also spend a significant amount of time in the hospital, which is a risk factor for acquiring infection itself.

Modern healthcare can induce immunosuppression from all three categories. By definition, surgical intervention disrupts the physical barrier that constitutes the first line of defense against infection. The combination of advanced sterilization procedures and prophylactic antibiotics in the operating room make intraoperative infections rare. Despite this, 1 in 20 surgical patients in the United States will ultimately develop a surgical site infection [52, 53]. As I will discuss in more detail below, surgical infections are a major contributor to nosocomial infections in the United States.

Iatrogenic causes of immunosuppression are often secondary to the purposeful targeting of harmful immune processes. Corticosteroids are a common treatment for autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis [54]. At increasing doses of corticosteroids, innate immune trafficking, T cell function, and antibody production by B cells can be suppressed [55]. Given these broad effects, patients with chronic corticosteroid use are at risk for infections with viruses, bacteria, and fungi. *P. jirovecii* is the most common opportunistic infection affecting chronic corticosteroid users [56]. Another medical intervention that induces severe immunosuppression is bone marrow irradiation in preparation for bone marrow transplant for hematologic cancers. Bone marrow irradiation eliminates the hematopoietic precursor stem cells that give rise to nearly all leukocytes [57]. Care must be taken to identify latent infections (such as herpes viruses or tuberculosis) or previous colonization (such as with *Candida* species) prior to irradiation to provide the proper prophylactic treatment [58].

One of the most significant advances in modern medicine has been the ability to transplant solid organs to restore vital function and extend life in recipients. However, suppression of T cell immunity is required to prevent rejection of the transplanted organ by the host immune system [59]. This is often achieved by treatment with cyclosporine or tacrolimus plus supplemental immunosuppressants as needed [60]. One to six months following transplantation, host immunity is at a nadir. During this time period, infection with herpes viruses, especially cytomegalovirus (CMV), is common [61]. CMV infection can cause organ damage, including of the newly transplanted allograft, and can also further suppress immune function, creating a potentially fatal positive feedback loop [62, 63]. Because T cell-mediated immunity is specifically targeted, opportunistic infections that require an intact cell-mediated immune response are also common. The risk for infection waxes over time as immunosuppressive drugs are tapered. However, chronic rejection can occur months to years following transplantation, requiring reinitiation of immunosuppressive drugs.

Summary

While the development of antimicrobial agents against infectious disease has been one of the crowning achievements of modern medicine, several factors are placing those advances in jeopardy. Antimicrobial resistance among pathogens is increasing at an alarming rate due to the combination of indiscriminate antimicrobial usage and slowing discovery of new drugs. In addition to this, people are living to older ages and the proportion of the world's population in the later decades of their life is growing. These older patients are undergoing increasingly complex medical and surgical procedures. Finally, even in younger patients, the incidence of immunosuppression is increasing due to both pathologic and iatrogenic causes. Taken together, these factors represent a major burden facing the future of healthcare. In order to tackle this problem, a nuanced understanding of major pathogens and the host defense response against them is required.

Epidemiology and Microbiology of Modern Infections

Nosocomial infections

Infections acquired while in the hospital, also called nosocomial infections, account for 1.7 million infections in the United States each year. Approximately 1 out of every 20 hospitalized patients will contract a nosocomial infection [4, 64]. Of these patients, 100,000 (1 out of every 17) will die from the nosocomial infection [65]. While impossible to estimate, it is likely that nosocomial infections also contribute to 100,000s of more deaths due to complicating the course of recovery from other illnesses. For example, a study found that contracting a nosocomial infection worsens outcomes from cardiac surgery, even when the infection is cleared [66]. As discussed in detail above, hospitalized patients are uniquely poised for pathogen exposure due to lowered defenses, increased exposure, and enhanced antimicrobial resistance among pathogens.

Lowered defenses stem from immunosuppression secondary to the condition bringing the individual to the hospital, or due to a comorbidity. The average hospital patient is getting older in the United States, which is independently associated with increased risk for infection [67]. Despite extensive sterilization practices to disinfect staff and surroundings, hospitals remain fertile breeding grounds for bacteria. This is due to the fact the complete sterilization of the environment is costly and unfeasible. Lankford and colleagues found that 24 hours after sterilization, antimicrobial resistance pathogens had recolonized 92.9% of surfaces tested [68]. Also, patients themselves represent a key source of bacteria that cause nosocomial infections [69]. This cross-contamination of patients with bacteria, along with chronic exposure to antimicrobial agents, produces higher rates of antimicrobial resistant pathogens in the hospital setting, increasing morbidity and mortality [70]. As I will explore in detail below, the most frequent causes of nosocomial infections share a common factor: invasive action performed on the patient that facilitates the passage of pathogens into otherwise sterile locations. This

includes mechanical ventilation, surgical wounds, continuous access to bloodstreams, and indwelling catheters.

Hospital-acquired pneumonia

Hospital-acquired pneumonia (HAP) is the development of pneumonia 48 hours or more after admission to the hospital without any known prior infection. HAP is one of the most common nosocomial infections, especially in those who are under mechanical ventilation. Indeed, HAP accounts for 25% of all nosocomial infections and ventilator-associated pneumonia alone accounts for 10% [71]. Several factors predispose individuals to HAP, including admission to the intensive care unit, older age, and preexisting lung disease [72]. Bacteria are typically inoculated into the lower respiratory tract of the host by micro-aspiration. At baseline, aspiration of oral content occurs in about 45% of the population and this number is significantly higher in critically ill patients [73]. Despite the fact that endotracheal tubes have inflatable cuffs to prevent aspiration, a recent study by Jaillette and colleagues found no decrease in micro-aspirations when compared to uncuffed controls [74].

The most common causative organisms for HAP include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida* spp. fungi, and *Klebsiella pneumoniae*, in order [71]. Once HAP is suspected, broad-spectrum antibiotics to cover *S. aureus*, *P. aeruginosa* and other gram-negative bacilli should be initiated immediately after acquiring cultures. Delaying treatment with a regimen specific to the causative pathogen is associated with increased mortality in HAP [75, 76]. Based on several randomized trials, patients typically receive antibiotics for at least 7 days. Mortality rates of HAP are varied, but can reach as high as 50%, especially in the case of severe underlying comorbid disease [77].

Surgical site infections

Surgical site infections (SSIs) result from the direct breakage of the patient's skin and soft tissue barriers due to surgical intervention. SSIs account for 20% of nosocomial infections,

making them second most common in the hospital setting. Similar to the overall rate of nosocomial infection, nearly 1 in 20 surgical patients will develop an SSI. Any infection that occurs within 30 days near the surgical incision or within 90 days near a prosthetic implant is considered an SSI. The risk of SSI scales with complexity of surgery and number of organs involved. Because the small and large intestines are colonized with commensal (but potentially pathogenic) bacteria, bowel surgery is a common precursor to SSI. While age and comorbidities are common risk factors for all nosocomial infections, they are particularly pertinent in SSIs as older patients and those with diabetes have significantly impaired wound healing.

Escherichia coli, *S. aureus*, and *Enterobacter* species are the most common causes of SSI, likely due to the fact that they colonize the host skin or GI tract [71]. Consistent and timely administration of preoperative antibiotics and sterile operative technique can reduce the incidence of SSI by 40% compared to institutions that don't implement these precautions [78]. However, common causes of SSIs are increasingly antimicrobial resistant, and innovative antibiotic delivery systems such as antimicrobial sutures, dressings, and irrigation do not seem to quell the rising incidence of SSIs [79-81].

Central line-associated bloodstream infections

For patients who need continuous venous access for long-term medication administration or monitoring of hemodynamic status central venous catheters (also known as central lines) are the preferred access point for patients in the hospital. While central lines have improved care for many conditions, they are also a nidus for infection. These infections, known as central line-associated bloodstream infections (CLABSIs), are common in the ICU where chronic illness, malnutrition, and extremes of age are common [82-84]. Interestingly, CLABSIs have been a point of success for the United States in terms of infection control; frequent replacement of the catheter, close monitoring of symptoms, and early treatment have reduced the rate of CLABSIs by 19% from 2015 to 2017 [85].

Gram-positive organisms are especially common in CLABSIs, with *Staphylococci* spp. and enterococci making up 60% of total infections [86]. Because *S. aureus* is often found with resistance to methicillin (known as MRSA), empiric treatment for CLABSI is broad. It is important to ultimately determine the appropriate therapy, as inadequate treatment increases mortality in nosocomial infections [87]. In the event of confirmed CLABSI, lines should be removed promptly, and antibiotics should be continued for 10-14 days [88].

Catheter-associated urinary tract infections

In hospitalized patients, urinary catheters provide convenience for patients who may have trouble arising from bed. Additionally, they can be placed for diagnostic and therapeutic reasons. The placement of a urinary catheter introduces a continuous connection between the environment and the typically sterile urinary tract. Bacteria that grow on the extraluminal surface of the catheter can migrate and colonize the bladder. Urinary tract infections (UTIs) are necessarily symptomatic and confirmed by growth of at least 10^3 colony forming units (CFUs) per milliliter of urine. UTIs are the most common nosocomial infections, accounting for over 30% of recorded cases in acute care hospitals [4]. UTIs increase the morbidity, mortality, cost, and length of stay for hospitalized patients [89-91].

The most common causes of UTI are overwhelming *E. coli* and *Candida* spp, which together comprise 50% of infections [92]. As such, empiric therapy should generally cover these microbes along with those common in the healthcare facility. When possible, indwelling catheters should be removed immediately. For those that require extended catheterization, intermittent catheterization lowers the rate of bacteriuria and UTI when compared to long term indwelling catheters [93].

Community-acquired infections

While infections in the hospital setting are increasingly common and lethal, the vast majority still occur in community. Between adult and pediatric populations, there are hundreds of

pathogens that cause illness. Between vaccination, supportive care, and occasional hospital treatment, the vast majority of community-acquired infections are mild [94]. However, community-acquired infections such as bacterial pneumonia account for the largest share of infectious-related deaths in the United States [95]. Community-acquired pneumonia (CAP) provides an illustrative example of the evolving dynamics between host and pathogen outside of the hospital.

CAP is one of the most common medical conditions encountered in outpatient clinical practice [96]. It accounts for 4.5 million visits annually in the United States, corresponding to 0.5% of all encounters [97]. Of those that develop CAP, 1.5 million will require hospitalization annually, with nearly 10% requiring rehospitalization within the same year for a second episode [98]. Like nosocomial infections, older age and chronic comorbidities significantly increase the risk for CAP. In particular, a diagnosis of COPD increases the risk for CAP by nearly 10-fold [98, 99].

The most common microorganism causing CAP is *Streptococcus pneumoniae*, although respiratory viruses, and several other bacterial species are known causes [100]. CAP is an illustrative case of infection in the community for two reasons: vaccination against *S. pneumoniae* has been effective in diminishing the incidence of CAP due to the organism, but the rise of antimicrobial resistance in *S. pneumoniae* threatens to mitigate those advances [101-103]. Another notable, albeit rare, cause of CAP is methicillin-resistant *S. aureus* (MRSA). Community-acquired MRSA (also known as CA-MRSA) disproportionately affects young and healthy persons without traditional risk factors for CAP [104]. CA-MRSA causes necrotizing pneumonia, septic shock, and respiratory failure. As a larger proportion of *S. aureus* isolates contain resistance elements, such as the SCCmec type IV secretion pump found in CA-MRSA isolates, the risk from these infections will increase [105].

Pseudomonas aeruginosa

The Gram-negative bacteria *Pseudomonas aeruginosa* poses one of the most prevalent and urgent microbial threats in modern healthcare. *P. aeruginosa* is ubiquitous in the environment and can survive in a multitude of media, including in distilled water [106, 107]. This allows the bacteria to thrive and spread through water supplies, especially in hospitals. Currently, *P. aeruginosa* is estimated to be the sixth most common cause of nosocomial infection [92]. It is the second and third most commonly identified organism in HAP and catheter associated UTIs, respectively. The prevalence of *P. aeruginosa* infection is increasing and will likely continue to do so due to growing antimicrobial resistance. Multidrug-resistant *P. aeruginosa* alone caused over 30,000 infections in hospitalized patients, leading to 2,700 deaths [108].

Specific high-risk patient populations are particularly susceptible to *P. aeruginosa* infection. Patients with cystic fibrosis are predisposed to pulmonary infections due to ineffective mucous clearance. *P. aeruginosa* is the most common pathogen isolated from adult cystic fibrosis patients [109]. Furthermore, *P. aeruginosa* infection is the most common precipitant of respiratory failure and death in adult cystic fibrosis patients [110]. Additionally, patients with neutropenia, defined as an absolute neutrophil count below 1500/ μ L of blood, due to chemotherapy, genetic disease, or myelodysplastic conditions, are extremely vulnerable to bacterial infection. For several decades, *P. aeruginosa* was one of the most common pathogens identified in the bloodstream of febrile neutropenic patients. The discovery of carbapenem antibiotics greatly diminished the impact of *P. aeruginosa* in this patient population, but as carbapenem resistance increases, this may soon change [111]. In patients that require inpatient care for burn wounds, *P. aeruginosa* is the most common species of bacteria isolated from infected wounds, accounting for 57% of infections [112].

The ubiquity of *P. aeruginosa* is due in part to its large genome and extensive repertoire of response and regulatory mechanisms [113, 114]. *P. aeruginosa* gene expression varies

significantly depending on the metabolites available in the environment. This allows the bacteria to utilize a variety of carbon sources, including glucose, glutamate, and amino acids for primary metabolism. Additionally, *P. aeruginosa* can metabolize itaconate, which is a derivative of the citric acid cycle that is upregulated in host immune cells during infection [115]. A high concentration of itaconate initially induces membrane stress in *P. aeruginosa* but sensing of itaconate leads to transcription of an operon containing itaconate-clearing genes. Additionally, *P. aeruginosa* are one of several bacterial species able to switch from a highly virulent and mobile phenotype to a quiescent and protective phenotype by forming a biofilm. Large colonies of *P. aeruginosa* will form cellular aggregates and embed themselves within an extracellular polymeric substance to provide protection from patrolling immune cells, oxidative stress, and antimicrobial agents [116].

P. aeruginosa also have several intrinsic resistance mechanisms to several antibiotics. This stems from the wide repertoire of genetic products found in the *P. aeruginosa* genome, as well as naturally protective properties of Gram-negative bacteria [107]. *P. aeruginosa* possess four multidrug efflux pumps that are often present in clinical strains and provide protection against β -lactams, fluoroquinolones, and aminoglycosides [117, 118]. Interestingly, the gene sets encoding these four pumps are under different regulator factors, meaning that they are specialized to respond to distinct types of cellular stress, potentially widening the number of antibiotics that will activate the pumps [117]. Genes that serve a physiologic function for the bacteria in other contexts can also be upregulated in mutant strains after exposure to antibiotics that can be metabolized by the translated proteins [119].

Hydrophilic antibiotics are able to enter the cell by way of porin proteins or membrane channels [120]. However, *P. aeruginosa* can limit entry of these antibiotics by decreasing expression of the porin channels in subsequent generations and replacing them with more specific channels. In some cases, clinical strains of *P. aeruginosa* are isolated that completely lack the OprD porin, which is utilized by imipenem to enter the cell [121]. This is the most

common mechanism that *P. aeruginosa* uses to develop resistance to carbapenems and cephalosporins, two categories of drugs that are heavily relied on to treat infections with the bacteria [122].

In summary, *P. aeruginosa* infection is a deadly and growing microbial threat, both within the healthcare setting and in the community. *P. aeruginosa* contains several antimicrobial resistance mechanisms, and the bacteria may continue to accumulate more through horizontal gene transfer. Therefore, novel strategies are needed to prevent and treat these infections. However, one must understand the host response to infection in order to develop rational targets for therapy.

Innate Immunity against Infection

Overview

The common feature between all infections affecting high risk or low risk patients, occurring in the hospital or community setting, and containing resistance elements or not, is that the pathogens successfully evade or overcome the first line of defense for the host: the innate immune system. Innate immunity comprises physical barriers, preprogrammed and rapidly acting cellular systems, and mediators of inflammation that attempt to immediately detect and contain an invading microbe. Because innate immune responses are rapid and nonspecific, they have traditionally been considered impossible to modulate. However, this is now known to be false. Before introducing these dynamic changes, which serve as the foundation for this thesis, an understanding of the key cellular and molecular elements of innate immunity is required.

Physical defenses

Fundamentally, infections arise when a pathogenic microorganism enters through a compromised barrier meant to protect the host. This can be a physical barrier, such as the skin or outward-facing epithelial tissue in the pulmonary system. Additionally, tissue-resident immune cells are stationed in essentially all organ systems to detect and clear pathogens that bypass

the physical barriers. In many cases, pathogenic bacteria, fungi, or viruses have developed clever mechanisms to subvert – or even utilize – the defensive barriers to gain entry. Otherwise, physical injury, both accidental and intentional, provides direct access to the body for the pathogens.

The skin is the first line of defense against the environment and may be breached inadvertently due to injury or purposefully in medical procedures. Minor injuries are rapidly blocked off from the vasculature and internal organs by activation of the clotting cascade and subsequent hemostasis. This initial response is followed by an inflammatory response that promotes fibrosis, closure of the wound, and eventual wound healing [123]. Injuries involving a larger surface area of the skin barrier, such as burns, take longer to seal off and are much more likely to involve acute pathogen exposure and often require immediate medical care to prevent devastating infection [124].

The pulmonary tract constantly comes into contact with microorganisms present in inhaled air but has two primary mechanisms to prevent their translocation into host tissue. The first line of defense is the cough reflex. The upper respiratory tract is highly sensitive to foreign objects, detection of which will trigger an immediate neurological response that results in a cough to clear the object [125]. As mentioned above, the mucocilliary escalator is the process by which ciliated cells in the upper respiratory tract beat synchronously to move mucous produced from goblet cells to the oropharynx where it is transferred to the gastrointestinal tract [126]. Pathogens trapped in this mucous are therefore expelled from the pulmonary tract before translocation can occur. This second-line defense protects the lower respiratory tract.

Cellular and molecular defenses

If a pathogen successfully crosses a tissue barrier in the host, the next defense mechanism is the detection of the microbe by the cellular arm of innate immunity. Macrophages are specialized cells that patrol the body looking for molecular signals of microbial colonization or tissue damage. Macrophages detect these pathogen-associated molecular patterns (PAMPs)

and damage-associated molecular patterns (DAMPs) with a diverse array of surface receptors, known as pattern recognition receptors (PRRs), that detect common motifs across microbes and tissues. The binding of PRRs on the surface of macrophages triggers a complex array of intracellular signaling that results in a coordinated response designed to neutralize the threat and initiate the healing process. Activated macrophages secrete cytokines and chemokines to recruit additional innate leukocytes and trigger a body-wide inflammatory response. Macrophages also possess the capacity to internalize the microbe by phagocytosis. Phagosomes containing microbes are sent to fuse with lysosomes containing proteases, reactive oxygen species (ROS), and a reduced pH to lyse and kill the pathogen.

Secreted cytokines enter the circulation and trigger the recruitment of additional macrophages, as well as neutrophils and lymphocytes. Together, macrophages and neutrophils constitute the bulk of the innate response to infection. Neutrophils are rapidly attracted after detection of the chemokine IL-8 in humans, or the homologous CXCL1 and CXCL2 in mice, by the surface receptors CXCR1 and CXCR2 [127, 128]. Recruited neutrophils secrete ROS and neutrophil extracellular traps (NETs) to neutralize detected microorganisms. Like macrophages, they also possess the ability to phagocytose and kill pathogens internally. To recruit circulating monocytes to the infection, macrophages secrete the chemokines CCL2, CCL3, CCL5, and CCL8 [129].

To activate the adaptive arm of the immune system, dendritic cells and macrophages capture PAMPs from the environment, return from the site of the infection to a tertiary lymphoid organ, such as a lymph node, and present the PAMPs to T cells [130]. However, the adaptive immune response is slow, and can take between 7 and 14 days to initiate pathogen clearance [131]. Thus, the innate leukocytes play an essential role in the early containment and clearance of invading pathogens. To date, strategies to fight infection have focused solely on bactericidal antimicrobials and inducing long-term adaptive protection with vaccines. Given the need for novel strategies, it is worthwhile to consider targeting innate immunity.

Targeting innate immunity to fight infection

While targeting the innate immune system has not been rigorously approached as a clinical solution for infection, there has been evidence that innate immunity could be enhanced for several decades. In 1956, Landy and Pillemer demonstrated that treating mice with low doses of lipopolysaccharide (LPS) isolated from Gram-negative bacteria cell wall protected those mice from subsequent Gram-negative infection in a timeframe too short for adaptive immunity to play a significant role [132]. While early human trials with LPS administration demonstrated a potent anti-tumor effect, the work was quickly abandoned due to the toxicity of LPS to humans [133]. This was followed by discovery that the most abundant component of the fungal cell wall, β -glucan, also exhibited the same protective properties, even when the mice were challenged with bacterial infections where the pathogen contained no β -glucans (discussed below). Understanding how β -glucan treatment provides broad protection against subsequent heterologous infections will inform how β -glucan treatment may be used to combat infections in the modern clinical setting.

Structure and Biological Activity of β -glucans

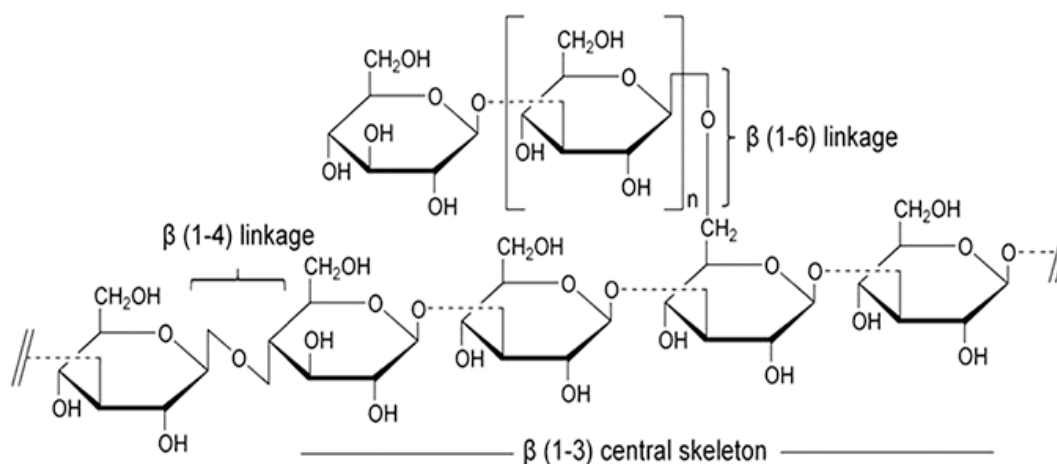


Figure 1. Chemical structure of β -glucans.

Beta-linkages between carbon 1 and 3 (β (1-3)), carbon 1 and 4 (β (1-4)), and carbon 1 and 6 (β (1-6)). Reproduced from Oliva-Neto *et al.* 2016 [134].

Overview of β -glucan biology

Glucans are a family of glucose polysaccharides that occur in various form in nature. These glucose polymers are the most abundant carbohydrates on the planet [135]. Glucans differ in their structure and arrangement significantly. Glucans are subdivided primarily by the glycosidic linkage in their backbone into α - and β -glucans. Alpha-glucans are composed of glucose moieties linked by several types of α -glycosidic bonds between carbons, including 1,4; 1,6; and 1,3 bonds. Because of this diversity, α -glucans are found in soluble fractions of cells or in insoluble fractions. Glycogen, an essential energy storage material in eukaryotic cells, is a soluble-fraction α -glucan [136]. Insoluble α -glucans are found in conjunction with β -glucans in cell walls of plants, bacteria, and fungi [137].

Typically, β -glucans are comprised of a linear backbone of glucose molecules linked in 1-3- β -glycosidic bonds with a variety of lengths, number of side-chains, and side-chain complexities (Figure 1) [137]. Branched β -glucans are the predominant component of fungal cell walls, where they are covalently bound to proteins, lipids, and other carbohydrates [138]. Because of their ability to form large, microfibrillar structures, β -glucans are essential to the integrity and rigidity of fungal cell walls [135, 139]. Indeed, lysis of cell wall β -glucans is sufficient to induce total cellular lysis in fungi [140]. Additionally, more recent evidence suggests that β -glucans contribute more than just a structural role: they are essential for fungal septation, sporulation and maturation, as the location of β -glucan synthase correlates with septation sites in *Saccharomyces pombe* cells and deletion of different subunits of the synthase enzyme disrupts these processes [141].

Medical interest in β -glucans dates back several thousand years as mushrooms were used for their medicinal qualities in traditional medicine. Over the course of the twentieth century, the components of fungi responsible for observed immunologic properties, such as

activation of the alternative complement pathway, were refined into molecular units [142]. Work by Pillemer and Ecker in 1941 found that chemically fractionating yeast isolated the biologically active component in a water-insoluble fraction [143]. Zymosan, which is a mixture of glucans, mannans, proteins, and lipids, was isolated as the active component of yeast and used extensively in the characterization of immune functions such as inflammation and phagocytosis. Eventually, through a combination of biochemical and immunologic techniques, β -glucan was identified as the biologically active component [144]. Beta-glucans derived from sources other than fungi, such as oat grains, have been studied for their potential cholesterol-lowering properties, but do not retain the immunogenic properties of yeast β -glucan [145]. This is due to the fact that yeast-derived β -glucan is highly branched with long side chains and a relatively large molecular weight compared to β -glucans from other species [146, 147].

Beta-glucan in fungal infection

Humans come into contact with fungi constantly, as they are present in the air and soil. The vast majority of these interactions are benign. Fungi also compose part of the normal human skin microbiome, although their exact role in that environment is debated [148]. However, certain isolated fungi can cause disease when inhalation of a significant number of spores occurs. In the United States, these infections include histoplasmosis, blastomycosis, and coccidioidomycosis, all of which follow a specific geographic distribution based on the ideal growth conditions for the causative fungi [149]. These primary fungal infections cause isolated pneumonia that is highly responsive to therapy. In contrast, patients who suffer from immunosuppression are at risk for several opportunistic fungal infections that may become systemic and affect multiple organ systems.

While exposed on the surface of yeast cells or as a large polymer in circulation, β -glucan is recognized by PRRs on innate leukocytes (Figure 2). The large whole glucan particles (WGPs) are slowly phagocytosed by these innate cells and processed into smaller fragments

that are either destroyed or re-released into circulation. Both WGPs and smaller soluble β -glucans activate a range of PRRs on innate leukocytes including dectin-1, complement receptor 3 (CR3), TLR2, lactosylceramide, and scavenger receptors [150-152].

The type II transmembrane receptor dectin-1 is the primary PRR for WGP β -glucan in mice and humans [153, 154]. It is expressed primarily on macrophages, neutrophils, and dendritic cells [155]. Dectin-1 is a member of the C-type lectin receptor superfamily and contains a single extracellular carbohydrate-recognition domain that mediates β -glucan recognition [156]. Downstream signaling from dectin-1 requires phosphorylation of an internal immunoreceptor tyrosine-based activating motif (ITAM). Unlike other C-type lectin family members, activation of downstream signaling from Dectin-1 does not require dimerization [156]. Dectin-1 signaling is activated only by WGP and not smaller soluble β -glucans. Larger, more complex WGPs form three-dimensionally complex interactions with dectin-1, CD45 and CD148 that permit intracellular signaling. Phosphorylated ITAM regions recruit Spleen Tyrosine Kinase (Syk) to the cell surface. In turn, phosphorylated Syk activates a wide variety of signaling cascades including protein kinase C delta (PKC- δ), protein kinase B (Akt), and pathways leading to inflammatory gene production (Figure 2). During a fungal infection, recognition of β -glucan by dectin-1 is essential for activation of innate immune defenses, as individuals with dectin-1 signaling are at risk for recurrent mucocutaneous fungal infections [157].

CR3, which is a dimer of the surface receptors CD11b and CD18, is primarily found on natural killer cells, neutrophils, and lymphocytes [158-160]. After WGPs are processed and released as smaller fragments, CR3 binds soluble β -glucans via a lectin domain. Beta-glucan binding primes CR3 for recognition of inactivated complement protein 3b (iC3b) on target cells, which tags them for lysis [161]. Beta-glucan administration enhances anti-tumor activity of leukocytes by this CR3 priming ability. Whether β -glucans induce intracellular signaling following CR3 ligation is an area of active research [162]. Human embryonic kidney cells transfected with

CR3 show downstream protein phosphorylation following β -glucan treatment, but this has not been replicated in functioning leukocytes [163]. CR3 may also contribute to signaling through p38 MAPK pathway after cells bind WGs that are too large to be properly phagocytosed [154]. As these WGs spend more time adhered to the cell surface, activation of CR3, along with dectin-1, enhances cytokine secretion in a process known as “frustrated phagocytosis” [164]. While complete phagocytosis of *C. albicans* is required for an inflammatory response in phagocytes, impaired or frustrated phagocytosis of WGs enhances inflammation [165]. This difference highlights the importance of structural diversity and particle size on signaling and ultimate immune response.

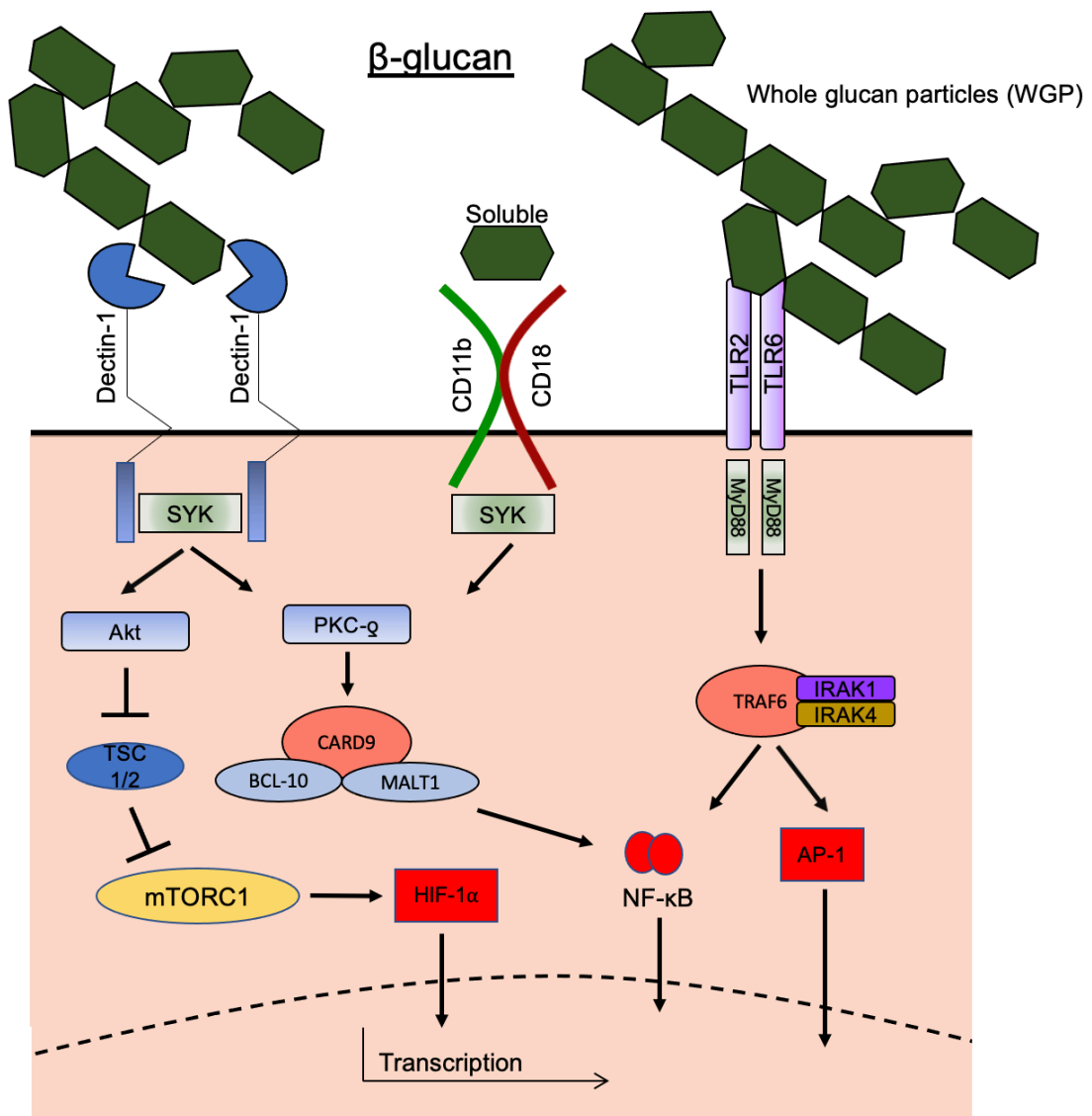


Figure 2. Overview of β -glucan signaling

Schematic of key signaling cascades downstream of particulate (whole glucan particles) and soluble β -glucan. Inspired by Brown. *Nat. Rev. Immunol.* 2006 [156].

Trained Innate Immunity

Historical perspective

For over 100 years, the traditional wisdom of innate immunology was that innate responses were genetically pre-encoded, rapid, and non-specific. Importantly, innate immune cells were not thought to possess the capacity for long-term modulation to their antimicrobial ability. It is now understood that organisms with the most rudimentary innate immune system, such as plants and simple invertebrate animals, still display persistent memory characteristics. Even in the complete absence of B- and T-cells, plants that survive an infection develop “systemic acquired resistance” against reinfection in the future [166]. This work was extended in *Drosophila melanogaster* flies infected with a sublethal dose of *S. pneumoniae* [167]. After the initial infection, the flies were protected for the rest of their lives. Importantly, it was also shown that this phenomenon is dependent on innate phagocytes and the Toll pathway, providing evidence that innate leukocytes might display memory in more complex species.

As investigators began to seriously consider the possibility of memory in innate immune cells, discoveries made by Carl Naeslund in 1931 were re-evaluated. Dr. Naeslund showed that after neonates received the bacillus Calmette-Guerin (BCG) vaccine against *M. tuberculosis*, neonatal mortality dropped significantly in his province – far more than should be expected from reducing the incidence of tuberculosis alone [168]. Because neonates lack a developed adaptive immune system, the protection afforded by the BCG vaccine represented initial evidence for innate immune memory in people. Two additional studies in mice showed that BCG vaccination protected athymic nude mice from a subsequent lethal infection with *C. albicans* by augmenting the killing capacity of macrophages [169, 170].

Over the same period of time, β -glucan was also shown to induce protection against subsequent infections in mice [171]. As mentioned above, β -glucan’s immunostimulatory properties on isolated leukocytes had been described in the 1940s and 1950s. For *in vivo*

studies, it was initially shown that administration of β -glucan to mice several days prior to infection with the same fungus provided homologous protection [171]. Similarly to BCG vaccine, β -glucan was then shown to protect against a heterologous challenge. Intraperitoneal administration of β -glucan 4 days prior to intravenous infection with *S. aureus* provided a significant survival benefit in mice along with increased innate immune activity [172, 173]. After these initial studies, β -glucan was also tested in experimental models of murine viral hepatitis and *E. coli*; administration of β -glucan was protective in both cases [174, 175]. While these studies prompted enthusiasm for β -glucan as a potential therapeutic, it would be several decades before induction of memory in the innate immune system was considered as the mechanism for its protective effects.

Discovery of β -glucan-induced trained innate immunity

In 2012, Mihai Netea and colleagues provided the first evidence that the mechanism of BCG vaccine's non-specific protection and β -glucan's innate leukocyte-driven protection were one in the same. Infecting mice with low dose *C. albicans* induced protection against subsequent lethal *C. albicans* infection, as observed before [176]. To further refine the mechanism, they utilized *Ccr2*-deficient mice, which are defective in monocyte recruitment from the bloodstream to tissues [177]. Mice lacking monocyte recruitment were not protected by *C. albicans* pre-infection. In contrast, mice with defective T- or B-cell activity or depleted NK cells still displayed a survival benefit. They also utilized an *in vitro* model of monocyte treatment and differentiation to demonstrate that monocytes treated with a primary stimulus had an altered inflammatory response to a secondary stimulus 7 days later. Monocytes treated with *C. albicans* or β -glucan isolated from *C. albicans* displayed enhanced proinflammatory cytokine production upon secondary stimulation while untreated controls did not. Interestingly, other PAMPs that activated TLR4 (LPS), TLR2 (Pam3CSK4), TLR3 (Poly I:C), and TLR9 (CpG ODN) did not

enhance the proinflammatory cytokine response upon secondary stimulus, while β -glucan did [178].

This work led to a pair of landmark studies from the same research group that uncovered the unique mechanism that underpins β -glucan-induced training. Because innate immune cells lack the machinery that produces a memory response in adaptive immunity, the authors investigated whether epigenetic modifications, and subsequent alterations to gene expression, after β -glucan treatment drove the long-term changes. Differentiation of human monocytes to macrophages dramatically shifted the epigenetic landscape that ultimately remodeled access to metabolic enzyme genes and attenuated access to inflammatory pathways [179]. Treatment with β -glucan shifted the epigenetic response; approximately 3,000 genes had new methylation markers at the 4th lysine residue of the H3 histone protein (H3K4me1) and acetylation of the 27th lysine of the H3 histone protein (H3K27ac), indicating activation of these promoters and enhancers [180-182]. In contrast, monocytes treated with LPS prior to differentiation uniquely altered H3K4me1 and H3K27ac sites at approximately 500 genes. Assessment of transcription factors associated with regions of high epigenetic plasticity found increased expression of specific transcription factor families differed between untreated, β -glucan-treated, and LPS-treated macrophages. This added further evidence that treatment with β -glucan induced specific, long-term changes in macrophage function by altering the transcriptional profile for at least 7 days after stimulation.

Concurrently, Dr. Netea and his colleagues examined the cellular metabolism of monocytes as they differentiated into macrophages, with or without initial exposure to β -glucan. After β -glucan treatment and 7 days of differentiation, macrophages had elevated glucose consumption, lactate production, and NAD⁺/NADH ratio, all suggesting that the rate of glycolysis was increased in these cells [183]. Despite normal levels of oxygen in the cell culture, the macrophages also demonstrated decreased oxygen consumption, both at rest and after

pushing the cells toward maximum oxygen consumption by uncoupling the mitochondrial electron transport chain from ATP synthesis. Previously, the phenotype of increased glucose consumption without oxygen deficit had been observed in cancer cells and dubbed aerobic glycolysis. The shift to this type of metabolism is known as the Warburg effect, named after Dr. Otto Warburg, who first observed the phenomenon in cancer cells in the 1920s [184]. Finally, they found that the mechanistic/mammalian target of rapamycin (mTOR) signaling pathway led to the stabilization of hypoxia-inducible factor (HIF)-1 α , which was essential for the metabolic shift. This set of studies established β -glucan as the canonical inducer of trained innate immunity, setting the stage for several studies probing the mechanistic underpinnings of β -glucan's activity over the last 7 years.

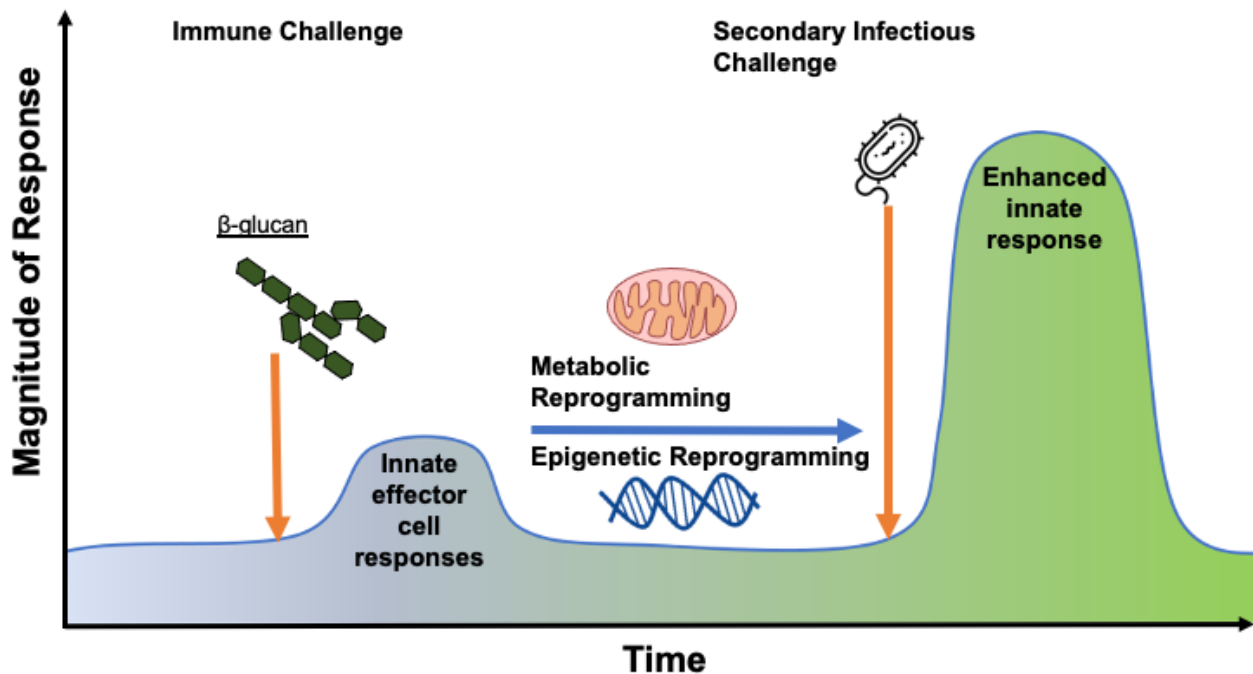


Figure 3. Overview of the concept of trained immunity

Schematic of innate immune response over time to primary and secondary immune challenge.

Modified with permission from McBride *et al.* 2020 [185].

Cellular metabolism of β -glucan trained immunity

Since the initial characterization of trained immunity, significant strides have been made in detailing the modulations in cellular function that occur after BCG or β -glucan treatment. The discovery of Warburg-like metabolism in trained monocytes has prompted further studies on the metabolic underpinnings of trained immunity. It is now appreciated that several metabolic pathways contribute to trained immunity. While increased ATP is important to meet the energy demands for trained immunity, the metabolic intermediates in these pathways also serve as cofactors for other essential processes, such as histone methylation, membrane lipid generation, and bacterial killing [186-188].

Upregulation of glycolysis is essential for proper activation of macrophages after stimulation with PAMPs [189]. As mentioned above, trained monocytes sustain this upregulation for several days following stimulation. Glycolytic enzymes are rapidly inducible, meaning that cells can quickly increase ATP production. While oxidative phosphorylation is more efficient at ATP generation, more time is needed to ramp up this process. Beta-glucan increases lactate production in monocytes, indicating that the cells are utilizing glycolysis for ATP [190]. Additionally, several metabolites from glycolysis are cofactors for histone methyltransferases, meaning that augmented metabolism also likely plays a role in epigenetic modifications after β -glucan. Interestingly, genes of the glycolytic enzymes hexokinase and pyruvate kinase are a target for epigenetic modification after β -glucan training, suggesting a positive feedback loop [179].

Trained immunity also leads to enrichment of TCA cycle intermediates in monocytes. Indeed, treatment with fumarate alone was shown to induce part of the trained phenotype. Succinate also acts independently of its metabolic role by stabilizing HIF-1 α and promoting IL-1 β production [191]. Enrichment is achieved by increasing pyruvate flux into the TCA cycle from glycolysis and by increased uptake of glutamine, which can feed into an anaplerotic reaction to

undergo conversion to α -ketoglutarate. Itaconate, which is derived from a TCA cycle intermediate, modulates mitochondrial metabolism and prevents monocyte training with β -glucan [192]. Finally, trained immunity ultimately enhances oxidative phosphorylation, leading to an increase in ATP production and mitochondrial ROS, which can serve both metabolic and antimicrobial functions [193, 194]. Increased glucose uptake, as well as flux into the TCA cycle, fuels the production of NADH and FADH₂, which are utilized by the complexes of the electron transport chain in the mitochondrion to power ATP synthase, and thus, generate ATP.

In monocytes, the activation of these myriad metabolic pathways fuels the epigenetic and functional changes that define innate immunity. In particular, monocyte trained immunity is associated with an enhanced production of IL-1 β , IL-6 and TNF- α after restimulation [195]. Whether β -glucan-induced metabolic reprogramming induces these functional changes in other innate leukocytes has not been studied. However, the systemic administration of metabolic inhibitors such as rapamycin (mTOR pathway), 2-deoxyglucose (glycolysis), or metformin (glycolysis and oxidative phosphorylation) blunt the trained phenotype [196]. In summary, studies of β -glucan in monocytes have clearly defined essential metabolic alterations underpinning trained immunity. However, further work is needed to generate broad principles across different cell types.

Gaps in Knowledge and Goals of this Thesis

Harnessing trained innate immunity is a promising strategy to combat infection. Boosting innate immunity is broadly protective given the nonspecific nature of the response. Importantly, trained immunity enhances protection without relying on antibiotics, which are increasingly ineffective. Since the discovery of trained immunity, β -glucan has been the prototypical training reagent and many of the key facets of training stem from those studies. However, significant questions remain regarding the cellular and molecular mechanisms underlying β -glucan training.

Circulating monocytes are short lived and cannot fully explain the protective effects of innate immune memory, yet the majority of studies exploring the mechanism of β -glucan training have utilized these cells. One hypothesis is that alterations to the bone marrow hematopoietic precursor cells (HSPCs) account for long-term training. Seven days following β -glucan treatment, monocyte-precursor HSPC numbers are elevated in the bone marrow of mice [197]. When these mice were challenged with mycobacterium tuberculosis, β -glucan treatment significantly improved survival 28 days later. Interestingly, there was no increase in resident alveolar macrophages or recruited monocytes in the lungs of β -glucan-treated infected mice. How, then, were these mice protected? Another explanation is that macrophages already present at sites of infection, which can survive for weeks to months, are trained by β -glucan and promote the protective phenotype. In this thesis, I test this hypothesis by treating differentiated macrophages, rather than monocytes, with β -glucan. As I will show, after β -glucan training, these macrophages provide protection when adoptively transferred into untreated mice.

Another gap in the understanding of trained immunity is the lack of functional characterization of innate leukocytes following treatment with β -glucan. A significant body of work has been published intricately detailing the metabolic and epigenetic transformation of monocytes as they differentiate into macrophages following β -glucan treatment. However, subsequent analysis of the macrophages is often limited to cytokine production. While cytokines play an essential role in the host response to infection, previous data from the Sherwood lab suggests that the capacity of a training ligand to enhance cytokine production does not correlate with its ability to protect from infection *in vivo* [198]. Furthermore, the capacity of isolated leukocytes to produce cytokines *in vitro* does not predict the cytokine levels in the infected host. Thus, there is a need to expand the scope of analysis of innate leukocytes. In this thesis, I utilize a variety of techniques to more fully explore the ability of β -glucan to enhance antimicrobial functions such as phagocytosis, respiratory burst, and microbial clearance.

My overarching goal in this thesis is to build upon the foundations of trained immunity to explore the clinical efficacy of β -glucan treatment. To accomplish this, I combine translational models of murine infection with studies of macrophage stimulation and function. I use a model of intraabdominal *P. aeruginosa* inoculation to demonstrate the potential of β -glucan as an immunoprophylactic treatment against infection that functions independently of antibiotics and could be used in vulnerable populations. Additionally, I provide evidence that long-lived differentiated macrophages are an essential component of trained immunity. By profiling the transcriptomic, metabolic, and antimicrobial phenotype of trained macrophages, I expand the concept of trained immunity beyond monocytes to include differentiated leukocytes. Finally, I utilize mice deficient in the canonical β -glucan receptors Dectin-1 and TLR2 to provide the first in-depth study of the signaling mechanisms underpinning trained immunity. Together, these studies advance our understanding of innate immunology and innate leukocyte biology and support the translational and clinical development of β -glucan trained immunity.

CHAPTER 2. Beta-glucan Augments Innate Immunity against *Pseudomonas aeruginosa*

Introduction

P. aeruginosa is an opportunistic pathogen, often targeting individuals with defective immune function [109]. This is particularly true in the hospital setting where *P. aeruginosa* is the 6th most common cause of nosocomial infection. Worldwide, it is the third most common causes of intraabdominal infections [199]. These infections arise secondary to disruption of the gastrointestinal tract barrier, inflammation in the peritoneal cavity or puncture injury that introduces bacteria from the environment. *P. aeruginosa* infection of the abdominal cavity is common in individuals who have had recent or frequent healthcare exposures, especially if they are immunocompromised [200]. In the case of inadequate or ineffective therapy, the infection will spread to the bloodstream, triggering a global inflammatory response. Continuing unabated, sepsis can develop. Sepsis is a condition of life-threatening organ dysfunction caused by the dysregulated and unchecked inflammation that comes from untreated infection [201]. Sepsis is the leading cause of death in patients with infection. Once a patient develops sepsis, the mortality rate approaches 40%. According to the CDC, one out of every three patient fatalities in the hospital is due to sepsis [202]. Currently, there are no approved therapies for sepsis, therefore adequately controlling the infection prior to dysregulated inflammation is essential to improve mortality in *P. aeruginosa* infections. The standard of care for *P. aeruginosa* intraabdominal infection is source control, drainage, and antibiotics [203]. Unfortunately, *P. aeruginosa* is rapidly developing antimicrobial resistance.

Recently, Ciarlo and colleagues demonstrated that treatment with zymosan several days prior to infection improved survival in mice after *P. aeruginosa* pneumonia was induced by intranasal injection [204]. Zymosan is a crude mixture of β -glucan with proteins. Zymosan can contain from 0% to 60% β -glucan based on the preparation. While these studies don't

definitively demonstrate that β -glucan protects against *P. aeruginosa* infection, it provides compelling preliminary data to investigate further. Furthermore, previous research in the Sherwood lab has demonstrated that treating mice with the lipopolysaccharide (LPS) derivative monophosphoryl lipid A (MPLA) protects the mice from subsequent *P. aeruginosa* [205]. The ability of purified β -glucan to protect against *P. aeruginosa* remains unknown. In this section, I show that β -glucan treatment as early as seven days prior to infection protects mice from *P. aeruginosa* by improving innate immune recruitment and bacterial clearance at the site of infection. Improved clearance reduced systemic inflammation and allowed the mice to remain physiologic stability. Additionally, I utilize a model of bone marrow-derived macrophages (BMDM), treated with β -glucan and adoptively transferred into mice, to demonstrate that differentiated macrophages partially recapitulate protection seen with β -glucan training.

Methods

Mice

Wildtype male C57BL/6 mice, age 10-12 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments and procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Intraperitoneal infection model

Pseudomonas aeruginosa was purchased from American Type Culture and Collection (Manassas, VA; ATCC 19660). Bacterial cultures were grown in tryptic soy broth for 22 hours at 37°C, washed, and diluted in sterile saline. Mice were inoculated i.p. with 1×10^8 colony-forming units (CFUs) *P. aeruginosa* in 0.5 mL saline. Six hours after inoculation, body temperatures were recorded by rectal thermometer and mice were anesthetized. Whole blood was collected by carotid artery laceration under isoflurane anesthesia into heparinized microcentrifuge tubes, centrifuged at $2000 \times g$ for 15 minutes at 4°C and plasma was collected and stored at -80°C for

storage until subsequent cytokine analysis. Following cervical dislocation, the peritoneal cavity was lavaged with 5 mL of cold sterile phosphate buffered saline (PBS). A portion of lavage fluid was diluted and plated on tryptic soy agar overnight and bacterial colonies were counted to determine CFUs/mL recovered. The remaining peritoneal lavage fluid was centrifuged at $300 \times g$ for 6 minutes at 4°C and diluted appropriately for flow cytometric analyses.

Flow cytometry

Cells collected by peritoneal lavage were resuspended in PBS at a concentration of 1×10^7 cells/mL and incubated with $1 \mu\text{g/mL}$ anti-mouse CD16/32 (eBioscience, San Diego, CA) prior to addition of fluorochrome-conjugated antibodies ($0.5 \mu\text{g}/10^6$ cells) and incubation for 15 minutes at room temperature. Antibodies used to differentiate peritoneal leukocytes included anti-F4/80-FITC (clone BM8; eBioscience), anti-Ly6G-PE (clone 1A8; BD Biosciences, San Jose, CA), anti-Ly6C-PE Cy5.5 (clone HK1.4; eBioscience) alongside respective isotype controls. Monocytes were identified as $\text{F4/80}^+\text{Ly6C}^+$, macrophages as $\text{F4/80}^+\text{Ly6C}^-$, and neutrophils as $\text{Ly6G}^+\text{F4/80}^-$. Data were collected using an Accuri C6 flow cytometer and analyzed using Accuri C6 software (BD Biosciences).

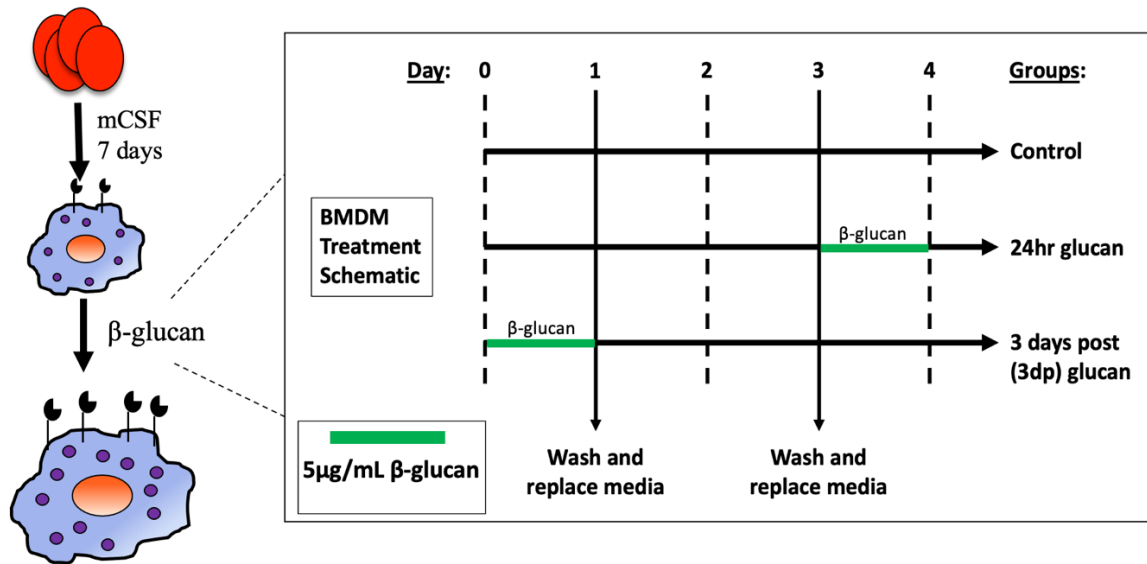


Figure 4. Schematic of differentiation and treatment of bone marrow-derived macrophages.

Macrophages are differentiated from bone marrow precursors in the presence of macrophage colony stimulating factor (mCSF) for 7 days. Media is changed and macrophages are treated with β -glucan and assayed at the indicated times.

Bone marrow-derived macrophages (BMDM)

Femurs were harvested from mice and flushed with RPMI 1640 containing 2 mM glutamine and 25 mM HEPES (Gibco, Grand Island, New York) supplemented with 10% certified performance plus fetal bovine serum (FBS, Gibco), 1% antibiotic-antimycotic (Gibco), and 10 ng/mL mouse recombinant macrophage-colony stimulating factor (M-CSF, R&D Systems), henceforth referred to complete media. Bone marrow cell suspensions were centrifuged at $300 \times g$ for 6 minutes at 4°C and plated at a concentration of 5×10^4 cells/mL in complete media. After 7 days of differentiation, BMDM received fresh media and were treated with $5 \mu\text{g/mL}$ β -glucan or vehicle as unstimulated controls for 24 hours. Macrophages were washed and allowed to rest in complete media for 3 days to generate the trained phenotype (3 days-post group; 3dp). Separately, BMDM were maintained in complete media and stimulated

with 5 $\mu\text{g}/\text{mL}$ β -glucan or vehicle for 4 or 24 hours prior to assessment (4h and 24h groups). Differentiation and treatment represented in Figure 4.

BMDM adoptive transfer

Trained BMDM were prepared as above, harvested, and resuspended at a concentration of 5×10^6 cells/mL in PBS. One day prior to infection, mice received 1×10^6 control or trained BMDM by i.p. injection. Twenty-four hours later, mice were inoculated i.p. with 1×10^8 CFUs of *P. aeruginosa*. Core body temperature, whole blood collection, peritoneal lavage, flow cytometry, and cytokine analysis were performed as described above.

Statistics

Data were analyzed using GraphPad Prism (La Jolla, CA) software unless otherwise noted. Data are expressed as mean \pm SEM or median where noted. Data from experiments containing multiple groups were compared using one-way ANOVA followed by Tukey's post hoc multiple comparison test. Body temperature and bacterial counts were compared using the Mann-Whitney test when comparing two groups or Kruskal-Wallis test followed by Dunn's post hoc multiple comparison test when comparing more than two groups.

Results

Beta-glucan trained immunity protects against intraabdominal *P. aeruginosa*

First, I sought to investigate the innate immune response to *P. aeruginosa* intraabdominal infection after β -glucan training. To accomplish this, mice were treated intraperitoneally (i.p.) with 1 mg β -glucan at several time points prior to i.p. infection with *P. aeruginosa* (Figure 5A). Because trained immunity has been shown to persist for at least a week, I treated mice at 7 and 8 days prior to infection (7d). Additionally, mice treated 3 and 4 days prior to infection (3d) and 1 and 2 days prior to infection (1d) were included to determine if the strength of β -glucan waned over time. Six hours after infection, core body temperature was assessed with a rectal probe and the mice were sacrificed. Vehicle-treated control mice became

hypothermic while β -glucan-primed mice maintained normothermia (Figure 5B). Mice treated with β -glucan at 7d, 3d, and 1d showed a similar body temperature after infection. After flushing the peritoneal cavity, bacterial burden, leukocyte recruitment, and cytokine secretion were quantified. Beta-glucan-treated mice showed significantly lower *P. aeruginosa* CFUs in peritoneal lavage as compared to vehicle-treated mice at all time points (Figure 5C). Interestingly, mice treated 7d before infection showed the most efficacious clearance response, with 3d and 1d showing decreased clearance. Cell recruitment to the peritoneal cavity was determined by flow cytometry (Figure 5D). The numbers of monocytes (Figure 5E), neutrophils (Figure 5F), and macrophages (Figure 5G) at the site of infection were significantly higher in β -glucan-treated mice compared to controls. Notably, β -glucan treatment alone did not induce leukocyte recruitment to the peritoneal cavity (Figure 5E-G). Mice treated at 1d showed significantly higher monocyte recruitment than those at 7d. In contrast, 7d and 3d displayed the most robust neutrophil recruit, as compared to 1d treated and untreated controls. Finally, macrophage recruitment peaked at 3d. Both 1d and 7d treated mice recruited macrophages than untreated controls, however.

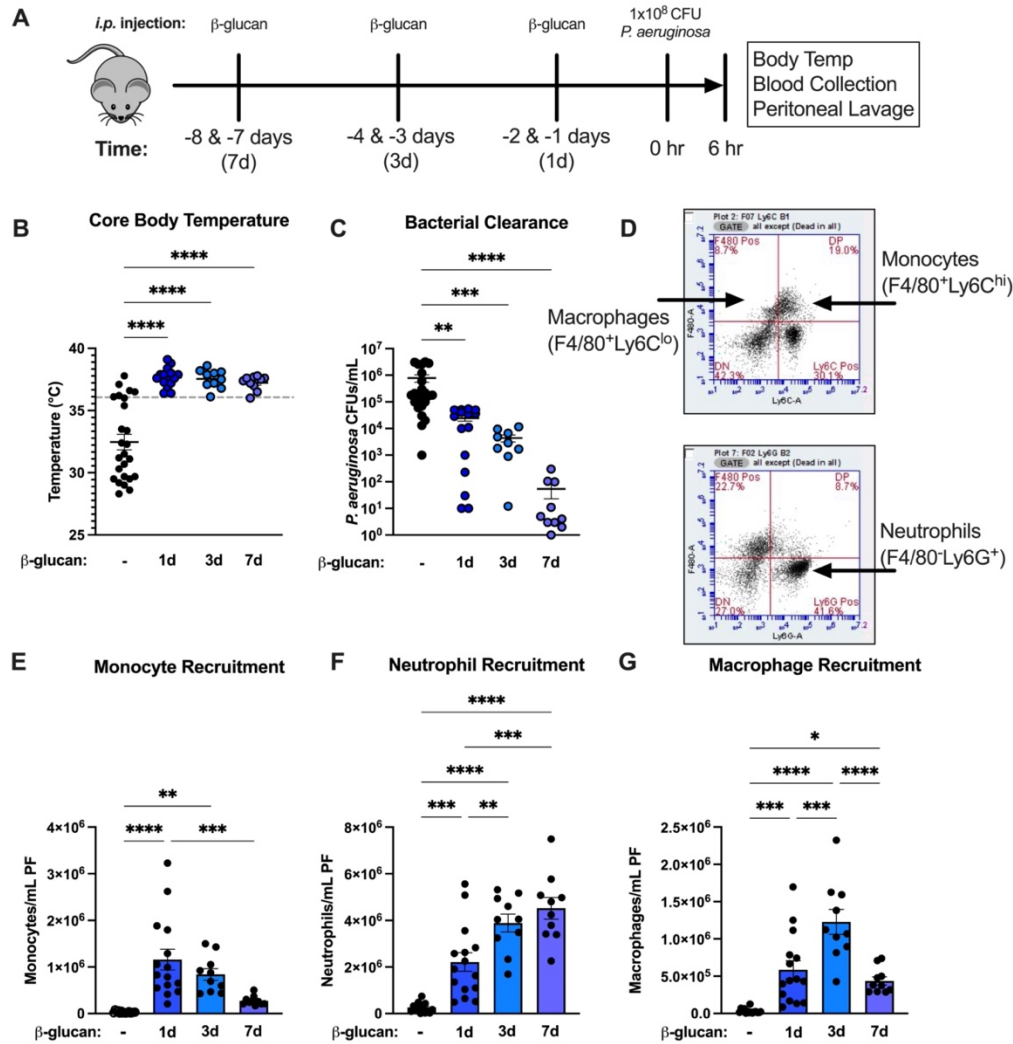


Figure 5. Beta-glucan augments innate immune defense against *P. aeruginosa* in mice.

(A) C57Bl/6 mice were injected i.p. with β -glucan (1 mg) or vehicle at several time points prior to i.p. inoculation with 10^8 CFU/mL *P. aeruginosa* with subsequent harvest of plasma and peritoneal lavage fluid 6 hours later. (B) Core (rectal) body temperature in vehicle- or β -glucan-treated mice 6 hours after i.p. *P. aeruginosa*. (C) CFUs of *P. aeruginosa* per mL of peritoneal fluid. (D) Representative flow gating schematic. DN = double negative; DP = double positive. Gates determined using single-fluorophore stains. All cells were gated on forward side scatter (FSC) and side side scatter (SSC) for cell viability prior to gating. Monocytes were defined as $F4/80^+Ly6C^{hi}$; neutrophils as $F4/80-Ly6G^+$; and macrophages as $F4/80^+Ly6C^{lo}$. (E-G) Number of monocytes (E), neutrophils (F), or macrophages (G) in uninfected β -glucan-treated mice ($n=3$), or infected vehicle- or β -glucan-treated mice. $N=15$. Data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Mann-Whitney U test (B, C) or ANOVA with Tukey's post-hoc multiple comparison test (E-G).

Adoptive-transfer of β -glucan-trained macrophages protects against *P. aeruginosa* infection

Because β -glucan protected mice from *P. aeruginosa* for at least a week following administration, I sought to determine the cell population that contributed to the sustained effect. While macrophages, monocytes, and neutrophils were all recruited in greater numbers to the site of infection after β -glucan treatment, only differentiated macrophages survive beyond a few days *in vivo*. Therefore, the importance of differentiated macrophages for the protective phenotype of β -glucan was determined. Bone marrow-derived macrophages (BMDM) were isolated from mice, differentiated for 7 days in the presence of macrophage-colony stimulating factor, treated with β -glucan for 24 hours, and allowed to rest for 3 days (Figure 4). Trained or control BMDM were adoptively transferred i.p. 24 hours prior to infection with *P. aeruginosa* (Figure 6A). Mice treated with vehicle (PBS) or control BMDM developed hypothermia 6 hours after infection, while mice that received β -glucan-treated BMDM maintained normothermia (Figure 6B). Similar to *in vivo* systemic training with β -glucan, mice that received trained BMDM had lower bacterial counts in the peritoneal cavity than mice treated with vehicle or control BMDM (Figure 6C). Mice treated with β -glucan-trained BMDM recruited significantly more monocytes (Figure 6D) and neutrophils (Figure 6E) to the site of infection compared to mice receiving PBS or control BMDM, however the number of macrophages present in the peritoneal cavity did not differ significantly between groups (Figure 6F). These data demonstrate that adoptive transfer of trained macrophages contribute to the protective benefit of β -glucan treatment, suggesting a role for these cells in β -glucan-induced trained immunity and resistance to infection.

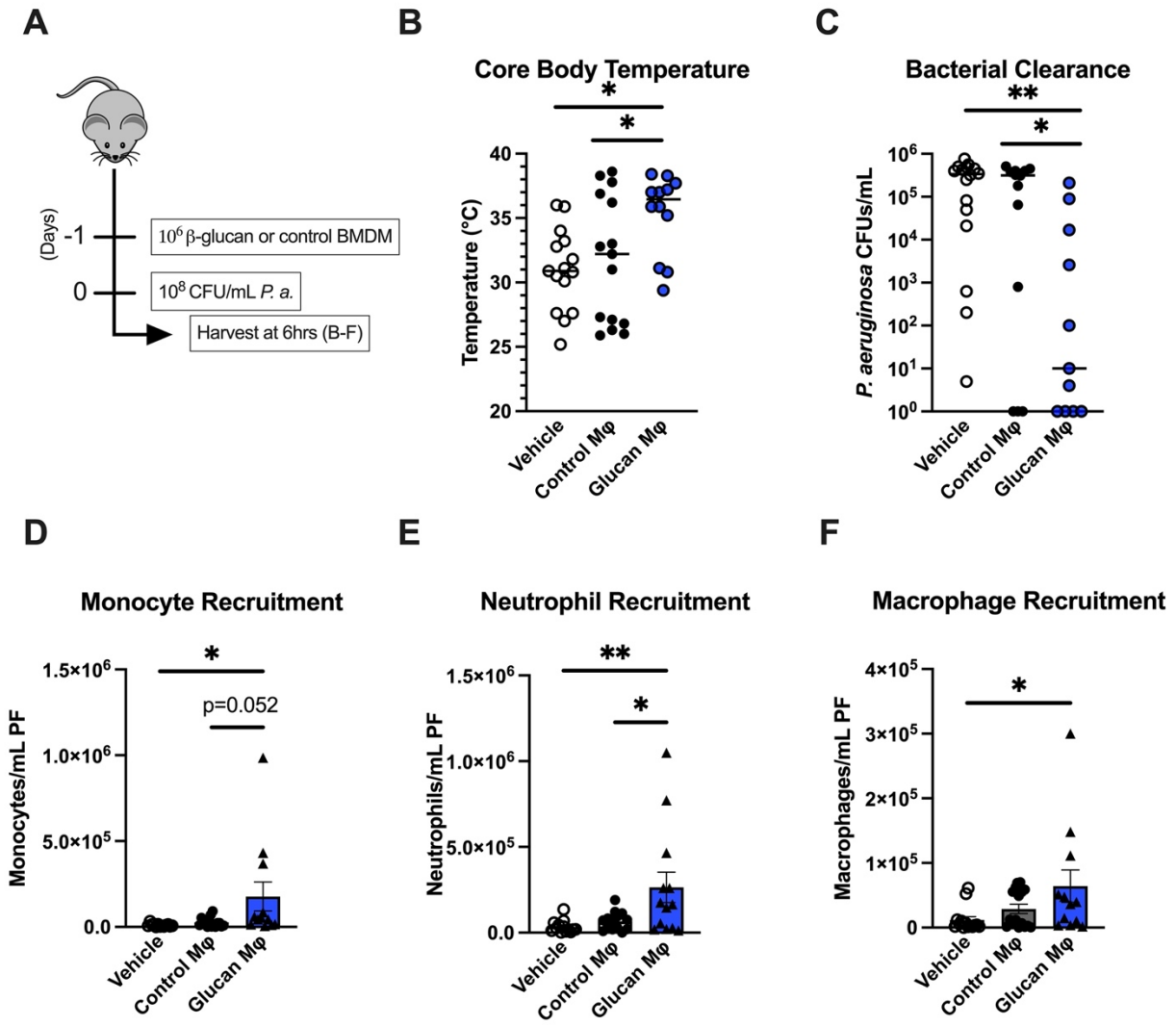


Figure 6. Adoptive transfer of β -glucan-trained macrophages protects against *P. aeruginosa* infection.

(A) BMDM were treated with β -glucan or vehicle and allowed to rest for 3 days. C57Bl/6 mice were injected i.p. with PBS, control BMDM, or β -glucan-treated BMDM prior to i.p. inoculation with 10^8 CFU *P. aeruginosa* with subsequent harvest of plasma and peritoneal lavage fluid 6 hours later. (B) Core (rectal) body temperature in vehicle, control-BMDM, or β -glucan-BMDM-treated mice 6 hours after i.p. *P. aeruginosa*. (C) CFU of *P. aeruginosa* per mL of peritoneal fluid. (D-F) Number of monocytes (D), neutrophils (E), or macrophages (F) in vehicle, control- or β -glucan-BMDM-treated mice. N=15. Body temperature and clearance data shown with median. All other data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ by Kruskal Wallis test with Dunn's post-hoc multiple comparison test (B, C) or ANOVA with Tukey's post-hoc multiple comparison test (D-F).

Discussion

These data demonstrate that β -glucan is an effective prophylactic agent against *P. aeruginosa* infection in mice. Treatment with β -glucan as early as 7 days prior to infection is sufficient to rewire innate immunity, enabling innate leukocytes to rapidly respond to subsequent infection. Intraperitoneal treatment with β -glucan alone does not induce significant recruitment of innate leukocytes to the peritoneal cavity. This is important because an alternative explanation for these data is that pre-recruited macrophages and neutrophils coincidentally respond to subsequent infection in the same compartment, i.e., the peritoneal cavity. Previous studies have demonstrated that peritoneal administration of β -glucan is protective against a bloodstream infection. However, those studies did little to shed light on the cellular and molecular underpinnings of this effect. In this case, my studies identify that β -glucan treatment alone is not significantly proinflammatory. Instead, mice treated with β -glucan are poised to respond to subsequent infection due to some other underlying mechanism.

Beta-glucan has long been described as an immunomodulatory agent with the capacity to augment the host response against bacterial, viral, and fungal pathogens [173, 176, 206, 207]. More recent studies show that β -glucan-mediated innate immunomodulation lasts for several weeks [208]. This raises the question of what mechanisms support this innate immune memory phenotype. One possibility is that β -glucan induces long-term changes to bone marrow progenitor cells that can serve to sustain the trained phenotype. Indeed, β -glucan promotes hematopoietic stem cell precursor (HSPC) expansion and myelopoiesis, which confers a survival benefit in mice repeatedly treated with the myeloablative drug 5-fluorouracil [209]. Additionally, HSPCs treated with depleted zymosan, a β -glucan enriched for Dectin-1 signaling, and adoptively transferred into Dectin-1 deficient mice secreted higher levels of IL-6 and TNF- α when reisolated and challenged *ex vivo* with the synthetic triacylated lipopeptide Pam₃CSK₄ [210]. Mice trained with β -glucan and subsequently infected with *Mycobacterium tuberculosis*

(Mtb) undergo HSPC expansion after training and ultimately demonstrate improved survival after pulmonary infection [197]. Notably, after 14 days of Mtb infection, there were no differences in the number of alveolar resident macrophages or recruited myeloid cells in the lungs. This supports the notion that enhanced local antimicrobial activity, and not recruitment of β -glucan-trained myeloid cells, enhanced protection against Mtb. Taken together, the evidence that β -glucan-trained HPSCs mediate protection against an acute severe infection alone is unclear, especially as tissue-resident and differentiated macrophages are among the essential first responders to infection [211].

My studies show that macrophages trained with β -glucan partially confer resistance to infection after adoptive transfer, which supports the notion that β -glucan trains macrophages that are poised to immediately respond to infection. To my knowledge, very little is known about β -glucan-induced innate immune memory in differentiated macrophages, although there is some evidence to suggest that short-term treatment with β -glucan immediately before LPS influences BMDM activation [212]. Macrophages represent a population of leukocytes that are well suited to sustain the trained phenotype due to their central role as regulators of the innate immune response to infection and a life span that is compatible with preserving the trained phenotype for weeks [213]. However, these data also provide robust evidence that β -glucan-induced neutrophil recruitment is also contributing to infection. As I will discuss in Chapter 5, future studies exploring the potential for adoptively transferred β -glucan-trained neutrophils to contribute to infection are an important future direction for this work.

Chapter 3. Beta-glucan Training Enhances Macrophage Antimicrobial Function and Metabolism

Introduction

Macrophages are essential first responders to infection across the body. They are responsible for detection of, and early antimicrobial activity against, microorganisms that have gained entry beyond a physical barrier. Detection of microbial PAMPs by PRRs on macrophage cell membranes initiates an inflammatory response ultimately aimed at recruiting additional leukocytes to the area by cytokine and chemokine secretion. In this way, macrophages orchestrate the innate response to infection [211]. Additionally, resident and recruited macrophages persist at the site of infection in order to phagocytose and kill pathogens [214]. Despite their central role in antimicrobial immunity, the contribution of differentiated macrophages to trained immunity has not been robustly examined.

In chapter 2, I demonstrated that macrophages treated *ex vivo* with β -glucan confer protection against *P. aeruginosa* infection upon intraperitoneal adoptive transfer into mice. Adoptively transferred macrophages enhanced recruitment of neutrophils to the peritoneal cavity following infection. In turn, these mice maintained normothermia and cleared bacteria more efficiently than mice receiving untreated macrophages. These results lead to the question of how β -glucan augments macrophage function.

To answer this question, I begin by performing an unbiased transcriptomic analysis of macrophages after various β -glucan treatment times. Given the enhanced microbial clearance observed in chapter 2, I postulated that β -glucan-treated macrophages would display enhanced antimicrobial activity. To test this, I characterize macrophage morphology, phagocytosis, and respiratory burst following β -glucan. In monocytes, trained immunity is defined by an enhanced cytokine response upon restimulation [215]. Despite this, in this chapter I demonstrate that β -

glucan does not significantly alter macrophage proinflammatory cytokine secretion. Finally, because altered cellular metabolism is a hallmark of trained immunity, I hypothesized that trained macrophages would display persistently elevated metabolism. Interestingly, I show that β -glucan induces a distinct metabolic phenotype in differentiated macrophages as compared to monocytes and that this phenotype is not significantly altered upon LPS restimulation.

Methods

RNA sequencing

Total RNA was isolated under an RNase-free environment, using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and treated with DNase (Qiagen). Total RNA quality and concentration were verified with a Thermo Scientific NanoDrop 2000 spectrophotometer. Purified RNA was assessed with Qubit and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and RNA integrity number (RIN) was determined for each sample [216, 217]. All samples had a RIN score of 7 or greater. mRNA Libraries were prepared using NEBNext Poly(A) selection (New England Biolabs). Sequencing was performed at paired-end 150 bp on an Illumina NovaSeq 6000 with at least 50 million reads per sample by Vanderbilt Technologies for Advanced Genomics. RNASeq data were analyzed with Basepair (www.basepairtech.com). Reads were aligned to the mm10 genome using STAR after trimming and undergoing quality control with QC30 [218]. Read counts were measured using featureCounts [219]. Differentially expressed genes were identified using DESeq2 [220]. Transcripts with log2foldchange of at least 1.0 and p-adjusted <0.05 were considered significant for individual gene analysis. Gene Set Enrichment Analysis (GSEA) was used to define significantly altered biological processes sorted with Gene Ontology [221-223].

Reactive oxygen species detection

BMDM were assessed with the Respiratory Burst Assay Kit (Cayman Chemical, Ann Arbor, MI). BMDM were incubated with dihydrorhodamine-123, which is converted into

rhodamine-123 that fluoresces in the presence of reactive oxygen species, for 1 hour at 37°C. BMDM were washed with warm PBS and rhodamine-123 fluorescence was determined by flow cytometry. Living cells were gated using forward-side scatter and side-side scatter. Rhoadamine-123 staining was determined by mean fluorescent intensity (MFI)

Phagocytosis assay

Staphylococcus aureus particles (Invitrogen) labelled with pHrodo-red dye were suspended in phenol red-free RPMI 1640 and sonicated for 10 minutes. pHrodo particles were added to BMDM cultures and placed in a Synergy H1 plate reader at 37°C (BioTek, Winooski, VT). pHrodo fluorescence was measured every 15 minutes for the indicated time. pHrodo MFI was determined at each time point.

Lipopolysaccharide (LPS) stimulation

BMDM were treated with β -glucan as described above. Fresh media containing 100 ng/mL LPS (ultrapure, Invitrogen) derived from *Escherichia coli* 0111:B4 was added to the cell culture. Four hours after incubation with LPS, cellular lysates were harvested for RNA as above. Separately, conditioned cell culture media was collected six hours after incubation. After 6 and 24 hours of LPS stimulation, the Seahorse extracellular flux assay as was performed as below.

Seahorse extracellular flux analysis

One day prior to the assay, BMDM were plated at 5×10^4 cells/well in a 96-well Seahorse assay plate. All measurements were performed on a Seahorse XF⁹⁶ Extracellular Flux Analyzer (Agilent). The glycolysis and mitochondrial stress tests were performed using the manufacturer's protocol. Briefly, extracellular acidification rate was measured at baseline and after the addition of 10 mM glucose (Sigma-Aldrich), 1 μ M oligomycin (Agilent), and 50 mM 2-deoxyglucose (2-DG; Sigma-Aldrich). Oxygen consumption rate was measured at baseline and after the addition of 1 μ M oligomycin, 1 μ M FCCP (Agilent), and 0.5 μ M antimycin A and rotenone (Agilent).

MitoTracker and Tetramethylrhodamine, methyl ester (TMRM) staining

BMDM were plated at 2.4×10^5 cells/well in a 24-well plate one day prior to the assay. 50 mM MitoTracker Green dye (Invitrogen, Carlsbad, CA) or 100 nM TMRM dye (Invitrogen) was added for 30 minutes at 37°C to stain total and active mitochondria, respectively. BMDM were washed and assessed by flow cytometry using channel FL1 (green) for MitoTracker and FL3 (red) for TMRM.

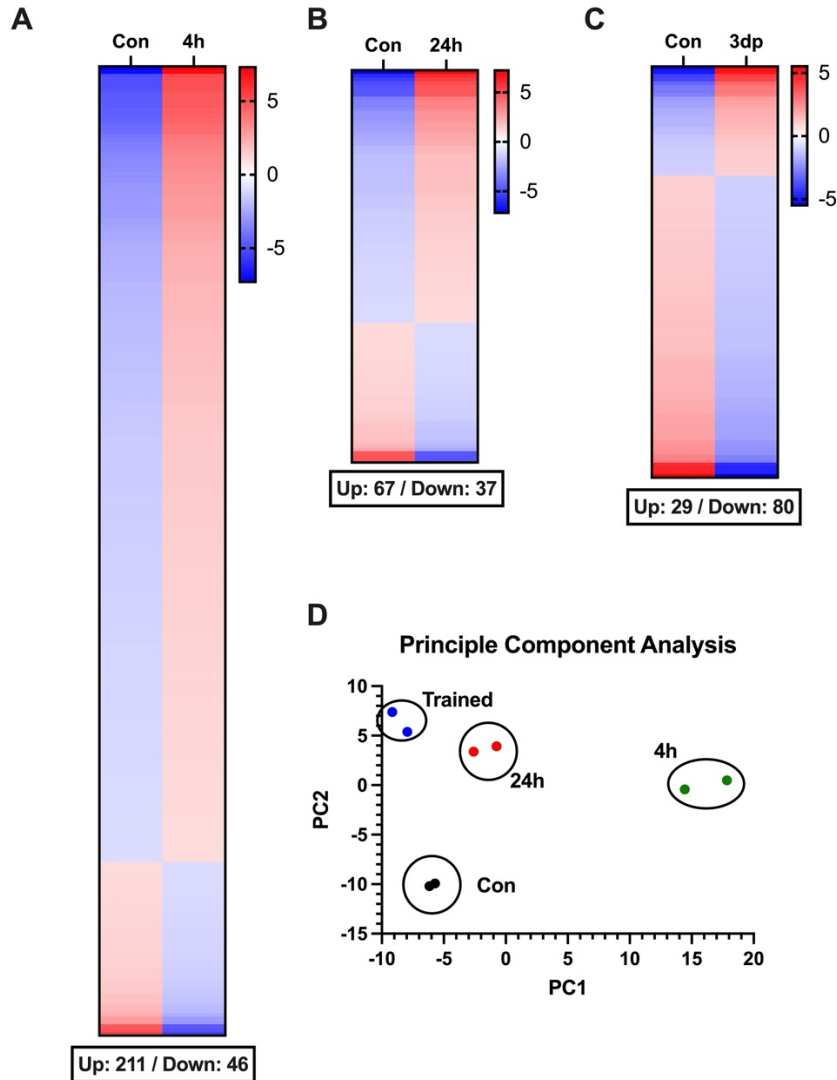


Figure 7. Beta-glucan induces a distinct transcriptomic profile in macrophages.

BMDM were treated with 5 $\mu\text{g}/\text{mL}$ β -glucan for 4 hours, 24 hours, or for 24 hours followed by 3 days of rest (3dp). At each time point, RNA was harvested for RNA Sequencing. (A) Heatmap displaying genes significantly differentially expressed in 4h β -glucan vs. control BMDM. (B) Heatmap displaying genes significantly differentially expressed in 24h β -glucan vs. control BMDM. (C) Heatmap displaying genes significantly differentially expressed in trained vs. control BMDM. (D) Principal component analysis displaying two-dimensional reduction in variance between each group. Colors in (A-C) distinguish increased (red) and decreased (blue) genes. Colors in (D) used to distinguish treatment groups. RNA quantification performed in duplicate. Up/Down genes had a $\log_2(\text{fold change})$ of at least ± 1.0 and a $p < 0.05$.

Results

RNA-Sequencing Reveals Dynamic Transcriptional Changes Following β -glucan

Treatment

Given that adoptive transfer of β -glucan-trained BMDM protected against *P. aeruginosa* infection *in vivo*, I explored the transcriptional response of BMDM after acute β -glucan treatment and after induction of the trained phenotype over time. To accomplish this, I performed an unbiased transcriptomic analysis of macrophages treated with β -glucan for 4 or 24 hours or after 24 hours of treatment and three days of rest (“trained”). After 4 hours of β -glucan stimulation, the expression of 257 (211 up/ 46 down) genes were altered compared to vehicle-treated controls (Figure 7A). Twenty-four hours after stimulation, BMDM were less transcriptionally active than after acute 4hr exposure, with 104 (67 up/ 37 down) transcripts significantly altered (Figure 7B). After completion of the trained immunity protocol, 109 transcripts were altered, with most being downregulated rather than upregulated (29 up/ 80 down) (Figure 7C). To better understand the relationship between these distinct states, the transcriptomic analysis for each treatment group was visualized using a two-dimensional principal component analysis (Figure 7D). This representation demonstrates that acute stimulation (4hr) with β -glucan caused the largest variance, with 24h and trained BMDM more closely resembling control BMDM in PC1, although these groups sustained distinct differences in PC2.

Evaluation of GO terms sorted by enrichment score (ES) showed that macrophages treated with β -glucan for 4 hours triggered pathways consistent with acute inflammation and activation of the innate immune system (Table 1). At 24 hours and in the trained BMDMs, gene pathways associated with housekeeping and cellular differentiation predominated (Tables 2 and 3). These results point toward early alterations in transcription that underpin the antimicrobial phenotypic changes seen in the trained BMDM.

4hr β-glucan	
Pathway	ES
GO REGULATION OF NEUTROPHIL CHEMOTAXIS	0.77735853
GO POSITIVE REGULATION OF B CELL MEDIATED IMMUNITY	0.7699145
GO REGULATION OF INFLAMMATORY RESPONSE TO ANTIGENIC STIMULUS	0.7695317
GO PHENOL CONTAINING COMPOUND BIOSYNTHETIC PROCESS	0.7530623
GO REFLEX	0.74588317
GO REGULATION OF HETEROTYPIC CELL CELL ADHESION	0.73833513
GO ORGAN OR TISSUE SPECIFIC IMMUNE RESPONSE	0.73464817
GO REGULATION OF GRANULOCYTE CHEMOTAXIS	0.72888786
GO CHEMOKINE ACTIVITY	0.72237235
GO BASAL LAMINA	0.7180193
GO REGULATION OF TYROSINE PHOSPHORYLATION OF STAT5 PROTEIN	0.7093345
GO T CELL DIFFERENTIATION INVOLVED IN IMMUNE RESPONSE	0.70857376
GO CD4 POSITIVE ALPHA BETA T CELL ACTIVATION	0.70825434
GO POSITIVE REGULATION OF T HELPER CELL DIFFERENTIATION	0.7073817
GO NEGATIVE REGULATION OF LIPID STORAGE	0.7029887
GO MAST CELL MEDIATED IMMUNITY	0.70048547
GO REGULATION OF TOLL LIKE RECEPTOR 4 SIGNALING PATHWAY	0.6990434
GO T HELPER 1 TYPE IMMUNE RESPONSE	0.6952899
GO CHEMOKINE RECEPTOR BINDING	0.6937222

Table 1. Top 20 induced Gene Ontology Biological Process Pathways in BMDM treated with β -glucan for 4 hours.

GO Pathways ranked by enrichment score (ES) and derived from two independent RNASeq experiments.

24hr β-glucan	
Pathway	ES
GO NEUROTRANSMITTER TRANSPORTER ACTIVITY	0.7493079
GO MELANOCYTE DIFFERENTIATION	0.74889207
GO ACTIVIN RECEPTOR SIGNALING PATHWAY	0.7464345
GO SYNAPTIC VESICLE ENDOCYTOSIS	0.72780377
GO SODIUM ION HOMEOSTASIS	0.72518533
GO RIBOSOMAL LARGE SUBUNIT ASSEMBLY	0.71612716
GO NEGATIVE REGULATION OF OXIDOREDUCTASE ACTIVITY	0.7136146
GO REGULATION OF MACROPHAGE DIFFERENTIATION	0.7121472
GO POSITIVE REGULATION OF CHONDROCYTE DIFFERENTIATION	0.711167
GO POSITIVE REGULATION OF NEUROLOGICAL SYSTEM PROCESS	0.7024139
GO PIGMENT CELL DIFFERENTIATION	0.70051867
GO PROTEASOME ACCESSORY COMPLEX	0.6890811
GO SYNAPTIC VESICLE RECYCLING	0.6876776
GO POSITIVE REGULATION OF FATTY ACID METABOLIC PROCESS	0.68446004
GO ATPASE COMPLEX	0.6805824
GO SUPEROXIDE METABOLIC PROCESS	0.67747295
GO HINDLIMB MORPHOGENESIS	0.67212015
GO DENDRITIC SPINE ORGANIZATION	0.6691955
GO PRERIBOSOME LARGE SUBUNIT PRECURSOR	0.66447765

Table 2. Top 20 induced Gene Ontology Biological Process Pathways in BMDM treated with β -glucan for 24 hours.

GO Pathways ranked by enrichment score (ES) and derived from two independent RNASeq experiments.

3dp β-glucan (trained)	
Pathway	ES
GO CHROMATIN SILENCING AT RDNA	0.7271427
GO DNA REPLICATION DEPENDENT NUCLEOSOME ORGANIZATION	0.7208158
GO RNA POLYMERASE II TRANSCRIPTION COREPRESSOR ACTIVITY	0.708858
GO PROTEIN HETEROTETRAMERIZATION	0.70509976
GO NEGATIVE REGULATION OF MEGAKARYOCYTE DIFFERENTIATION	0.70374286
GO PRERIBOSOME LARGE SUBUNIT PRECURSOR	0.6980801
GO APOPTOTIC PROCESS INVOLVED IN DEVELOPMENT	0.69346565
GO SMALL NUCLEOLAR RIBONUCLEOPROTEIN COMPLEX	0.6904768
GO PHOSPHOTYROSINE BINDING	0.68822813
GO PARAXIAL MESODERM DEVELOPMENT	0.68543035
GO REGULATION OF CGMP BIOSYNTHETIC PROCESS	0.68375486
GO TELOMERE CAPPING	0.6811334
GO TRNA SPECIFIC RIBONUCLEASE ACTIVITY	0.67511857
GO WNT ACTIVATED RECEPTOR ACTIVITY	0.6681536
GO REGULATION OF T CELL RECEPTOR SIGNALING PATHWAY	0.65795386
GO RIBOSOMAL LARGE SUBUNIT ASSEMBLY	0.6559397
GO RIBOSOMAL LARGE SUBUNIT BIOGENESIS	0.65314704
GO 90S PRERIBOSOME	0.6516961
GO_REGULATION_OF_ALPHA_AMINO_3_HYDROXY_5_METHYL_4_ISOXAZOLE_PROPIONATE SELECTIVE GLUTAMATE RECEPTOR ACTIVITY	0.64946026
GO REGULATION OF TELOMERASE RNA LOCALIZATION TO CAJAL BODY	0.64740914

Table 3. Top 20 induced Gene Ontology Biological Process Pathways in BMDM treated with β -glucan for 24 hours. And allowed to rest for 3 days (3dp, trained).

GO Pathways ranked by enrichment score (ES) and derived from two independent RNASeq experiments.

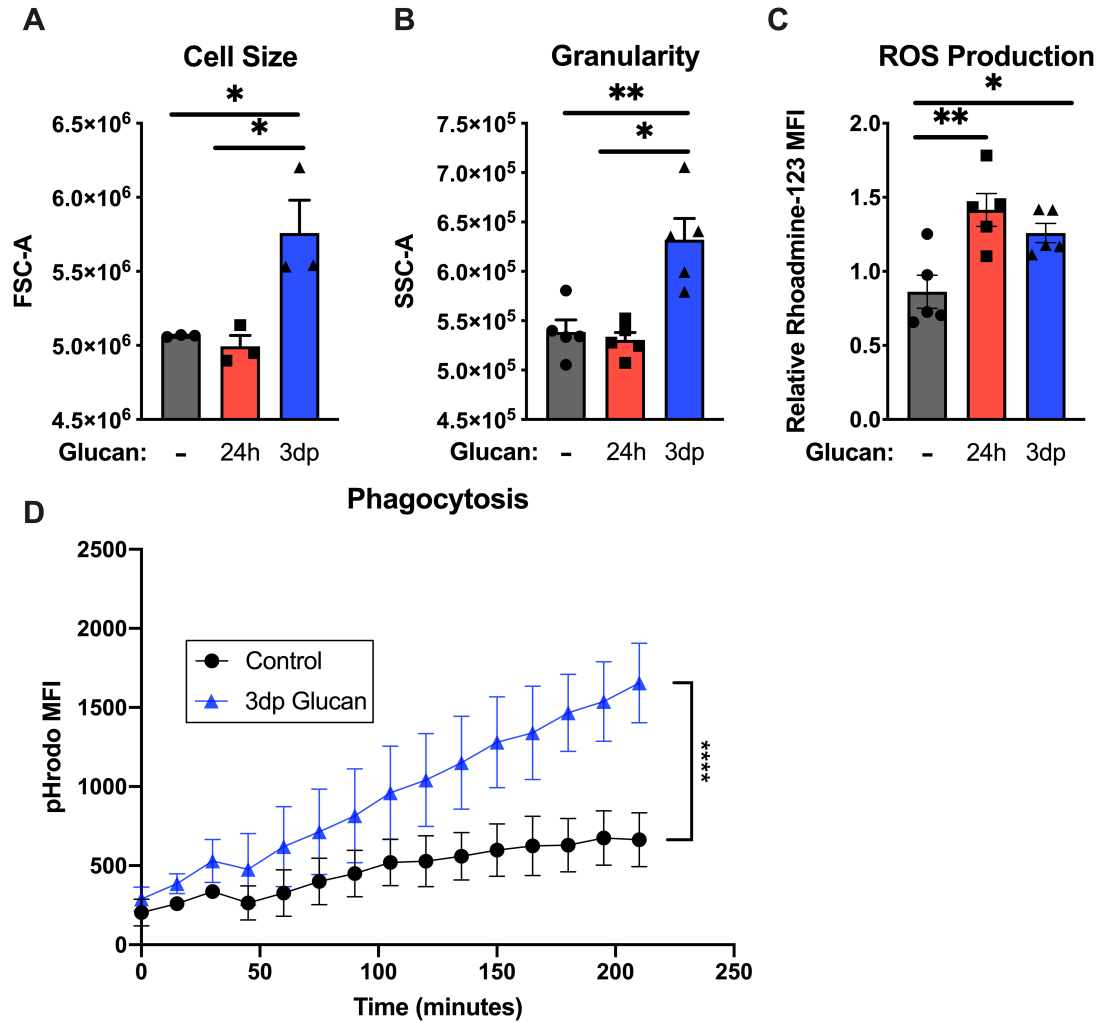


Figure 8. Beta-glucan-trained macrophages display a robust antimicrobial phenotype.

(A) Cell size was measured by forward scatter area in each group. (B) Cell granularity as measured by side scatter area in each group. (C) Rhodamine-123 fluorescence was measured after a 15-minute incubation period by flow cytometry. (D) Control or trained BMDM were incubated with pHrodo *S. aureus* particles. pHrodo MFI was measured every 15 minutes for 5 hours. Data shown as mean \pm SEM. Experiments were performed with 3-5 biological replicates. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by ANOVA with Tukey's post-hoc multiple comparison test (A-C) or repeated two-way ANOVA (D).

Beta-glucan-trained macrophages display a robust antimicrobial phenotype

Based on my *in vivo* data, I hypothesized that β -glucan enhances macrophage antimicrobial capacity, which I assessed by several approaches. Macrophages trained with β -glucan (3dp) were significantly larger (Figure 8A) and more granular (Figure 8B) than control and 24h BMDM, as measured by forward and side scatter using flow cytometry. Rhodamine-123 staining showed increased reactive oxygen species (ROS) production in 24h and 3dp BMDM as compared to the control BMDM (Figure 8C). Trained BMDM phagocytosed significantly more pHrodo-labelled bacterial particles over time than control BMDM (Figure 8D).

Transcriptomic data revealed that 4-hour stimulation of BMDM with β -glucan induced transcription of numerous proinflammatory cytokines and reduced transcription of others, such as the Th2 cytokine IL-4, subunits of transforming growth factor- β and IL-17 (Figure 9A). In the 24hr-treated and trained BMDM, transcription of pro-inflammatory cytokines returned to near baseline or below baseline levels. Because β -glucan training *in vivo* leads to increased leukocyte recruitment, we next sought to determine if trained BMDM had enhanced capacity to produce and secrete chemokines. Transcripts of several cytokines that facilitate neutrophil and monocyte recruitment were upregulated 4 hours after β -glucan stimulation in BMDM (Figure 9B).

Because transfer of β -glucan-trained BMDM afforded protection against *P. aeruginosa in vivo*, I wondered how trained macrophages would transcriptionally respond in the context of infection. To mimic this scenario, control and trained BMDM were stimulated with 100 ng/mL LPS for 4 hours and global gene expression was measured. Profiling of the transcriptional changes for cytokines and chemokines after LPS indicated similar expression in trained BMDM as compared to control BMDM (Figure 9C). To confirm this observation at the protein level, IL-6 (Figure 9D), TNF- α (9E), CXCL1 (9F), and CXCL2 (9G) protein were measured 6 hours after LPS stimulation in culture supernatants from control and trained BMDM. Control and trained

BMDM secreted similar amounts of both cytokines in response to LPS. Thus, β -glucan induces sustained chemokine production and direct bactericidal activity but only transient production of proinflammatory cytokines, which are dependent on β -glucan-induced augmentation of metabolism. Furthermore, the cytokine response to LPS stimulation does not significantly differ between trained and control BMDM.

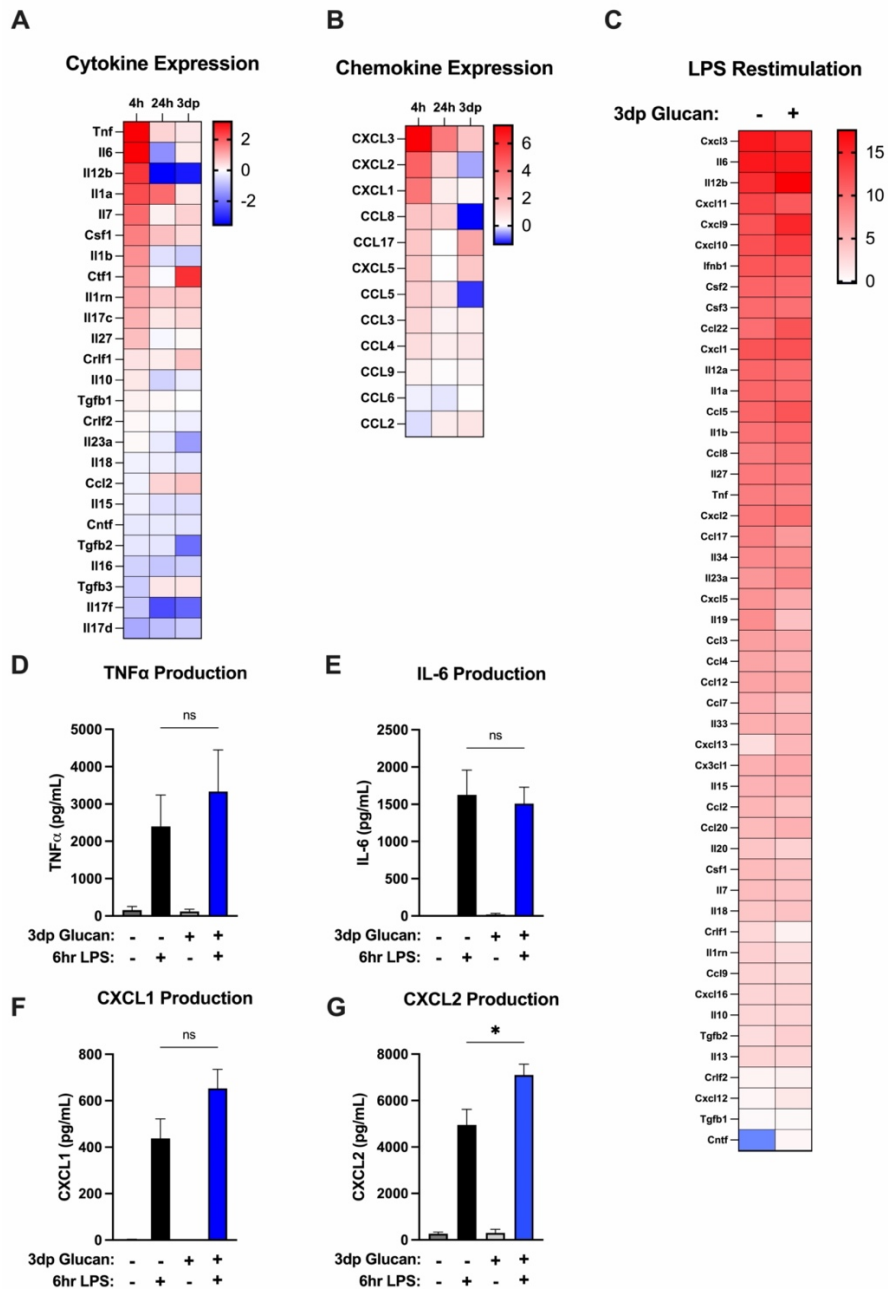


Figure 9. Beta-glucan alters macrophage cytokine and chemokine production.

(A-B) Heatmap of cytokine (A) and chemokine (B) expression in 4h, 24h, or trained BMDM relative to control as determined by RNASeq. (C) Heatmap showing cytokines in BMDM stimulated with 100 ng/mL LPS for 4 hours with or without β -glucan training. (D-G). TNF- α (D), IL-6 (E), CXCL1 (F), and CXCL2 (G) secretion in control or trained BMDM with or without 100 ng/mL LPS stimulation for 6 hours. RNASeq experiments were performed in duplicate. All other experiments were performed with 3 biological replicates. Cytokine data shown as mean \pm SEM and analyzed by ANOVA with Tukey's post-hoc multiple comparison test. * $p < 0.05$.

Beta-glucan training augments glycolytic and oxidative metabolism in macrophages

I next sought to evaluate the metabolic phenotype of macrophages trained with β -glucan. BMDM treated with β -glucan for 24 hours had elevated baseline and maximal extracellular acidification rate (ECAR), an indirect measure of lactate production and glycolysis. Trained BMDM displayed significantly higher ECAR levels than those treated for 24 hours (Figure 10A-C). Both 24h and trained (3dp) BMDM displayed elevated baseline and maximal oxygen consumption rate (OCR) compared to control, indicating a sustained increase in oxidative phosphorylation (Figure 10D-F).

Because β -glucan increased oxidative metabolism in macrophages, I assessed mitochondrial content and activity. First, I asked whether trained BMDM increased the available mitochondrial pool. Trained BMDM had significantly higher mitochondrial content as compared to control or 24h BMDM as demonstrated by increased MitoTracker Green staining (Figure 11A). In concert with this finding, trained BMDM displayed increases in transcriptomic signatures associated with oxidative phosphorylation (GO:0033108, NES = 1.344, Figure 11B). These changes were mirrored by increased mitochondrial membrane potential, as measured by tetramethylrhodamine (TMRM) staining, and an increase in transcription related to ATP biosynthesis (GO: 0042775, NES = 1.18) and (Figure 11C-D). Thus, β -glucan treatment induces a unique metabolic phenotype in differentiated macrophages characterized by elevated ECAR and OCR in association with increases in mitochondrial content and function.

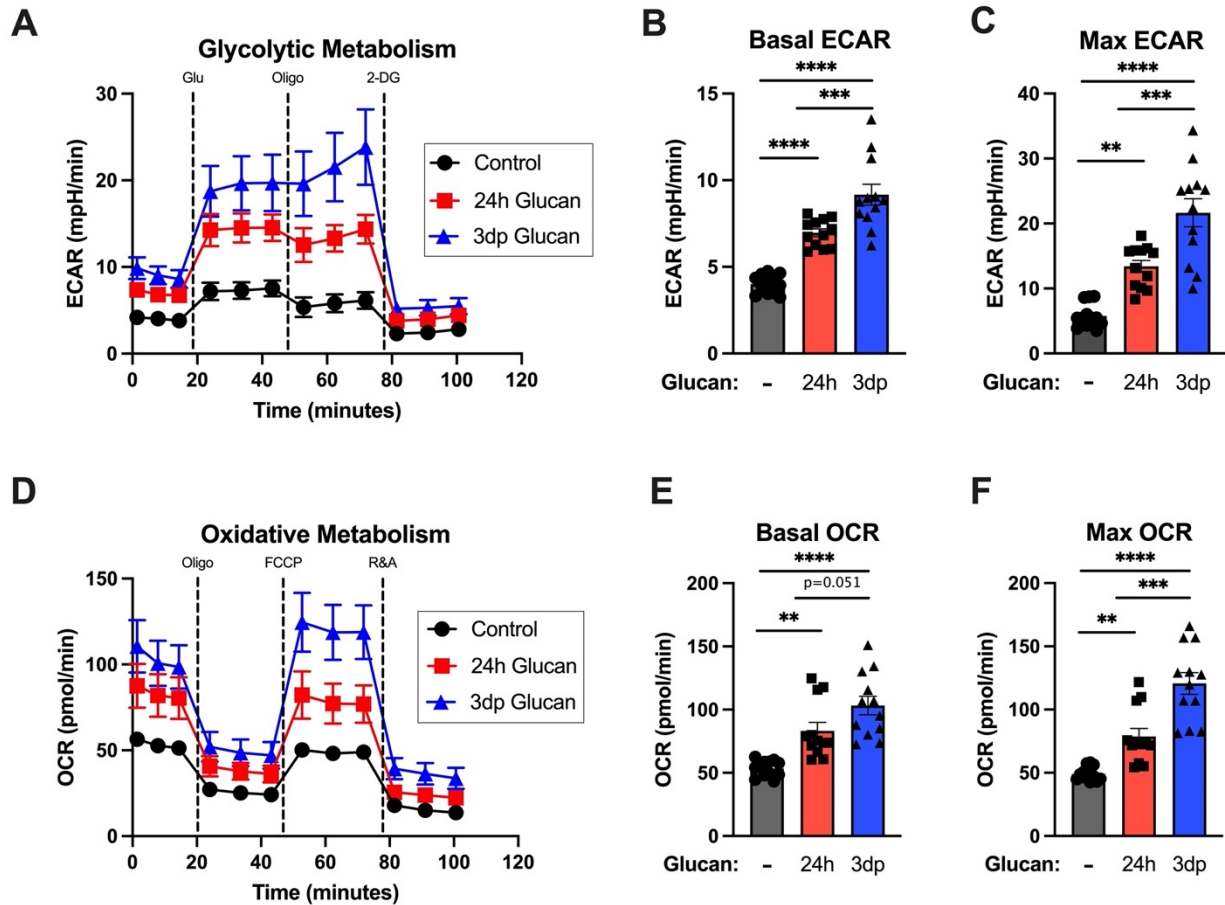


Figure 10. Beta-glucan induces persistent alterations in glycolytic and oxidative metabolism in macrophages.

(A) Glycolysis stress test of control, 24h, and trained BMDM on the Seahorse Xfe96. Extracellular acidification rate was measured over time at baseline and after glucose, oligomycin, and 2-deoxyglucose administration. (B) Summation of the first three measurements of ECAR in each group. (C) Summation of the three measurements following oligomycin in each group. (D) Oxidative stress test of control, 24h, and trained BMDM on the Seahorse Xfe96. Oxygen consumption rate was measured over time at baseline and after oligomycin, FCCP, and R&A administration. (E) Summation of the first three measurements of OCR in each group. (F) Summation of the three measurements following FCCP in each group.

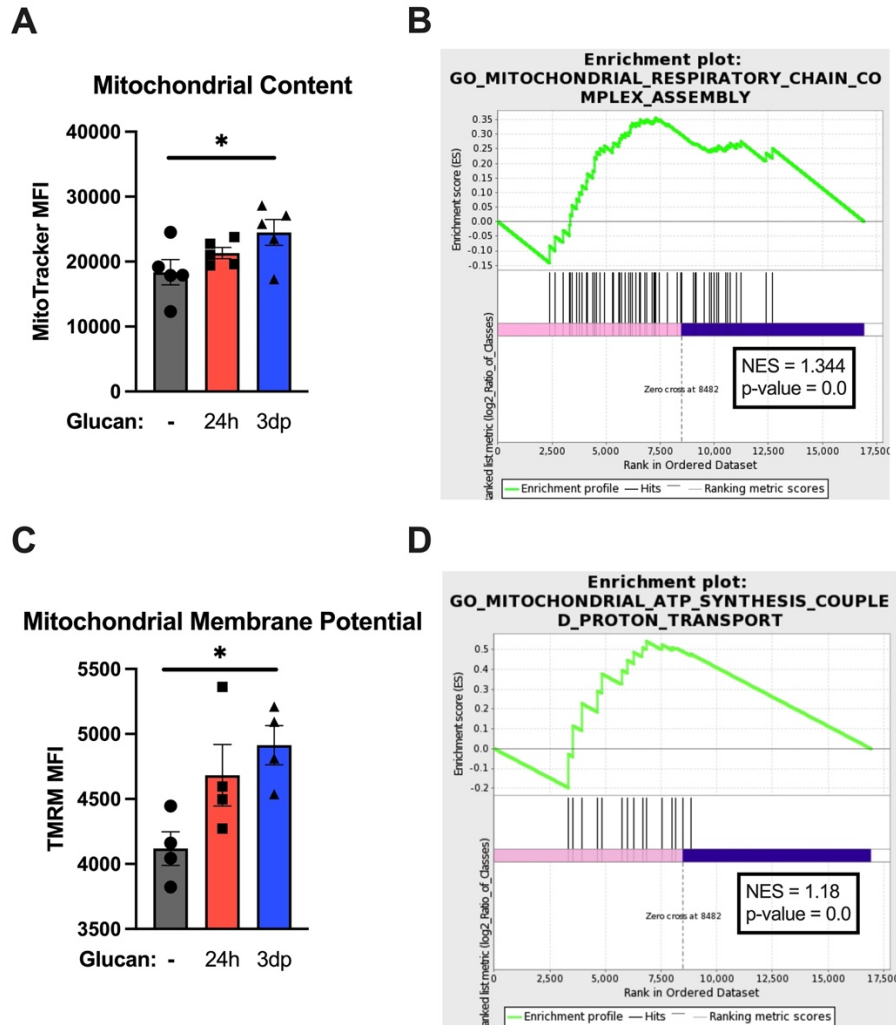


Figure 11. Beta-glucan increases mitochondrial content and membrane potential.

(A) MitoTracker Green MFI was measured in BMDM by flow cytometry after 30 minutes incubation. (B) Enrichment plot for Gene Ontology (GO) term “Mitochondrial Respiratory Chain Complex Assembly” for control vs. trained BMDM. Normalized enrichment score (NES) determined by gene set enrichment analysis. (C) TMRM MFI was measured in BMDM by flow cytometry after 30 minutes incubation (D) Enrichment plot for GO term “Mitochondrial ATP Synthesis-Coupled Proton Transport” for control vs. trained BMDM. NES determined by gene set enrichment analysis. * $p < 0.05$. $N = 4$ for MitoTracker and TMRM staining. $N = 2$ for GO analysis following RNASeq.

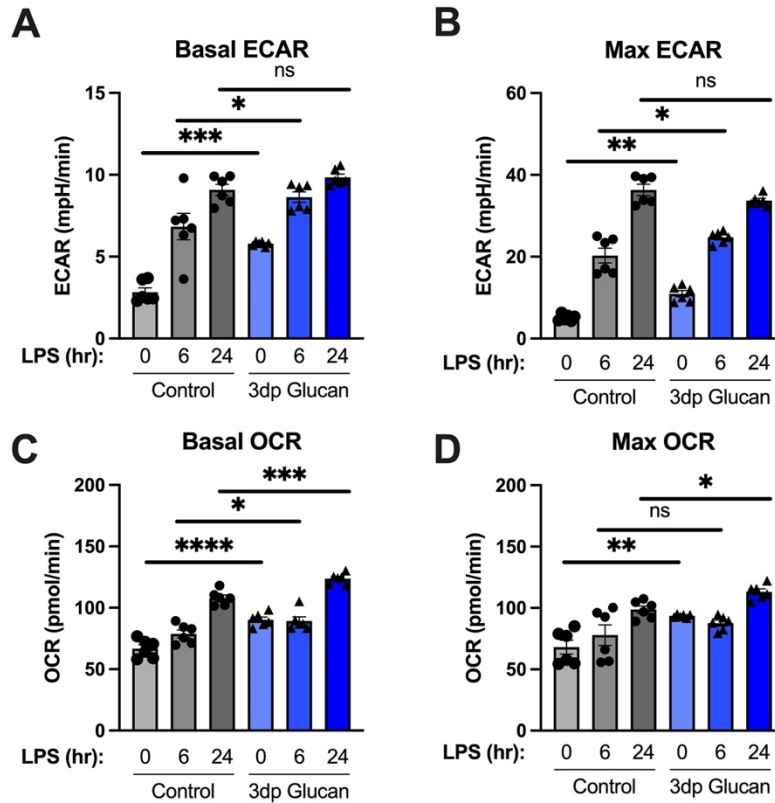


Figure 12. Comparison of control or β -glucan-treated macrophage metabolism after stimulation with LPS.

(A-B) Basal (A) and maximal (B) ECAR as determined by Seahorse Xfe96 in control and trained BMDM stimulated with vehicle or 100 ng/mL LPS for 4hr and 24hr. (C-D) Basal (C) and maximal (D) OCR as determined by Seahorse Xfe96 in control and trained BMDM stimulated with vehicle or 100 ng/mL LPS for 4hr and 24hr. Data shown as mean \pm SEM. Experiments were performed with 3-5 biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by ANOVA with Tukey's post-hoc multiple comparison test.

LPS restimulation alters both control and β -glucan-treated macrophage metabolism

Next, I sought to determine the effect of β -glucan training on the macrophage metabolic response to LPS challenge. Control and β -glucan-trained BMDM were stimulated with 100 ng/mL LPS for 6 and 24 hours and ECAR and OCR were measured. Trained BMDM displayed a significant increase in basal and maximal glycolytic rate at baseline and after 6 hours of LPS stimulation as compared to control BMDM (Figure 12A-B). After 24 hours of LPS stimulation, both control and trained BMDM showed similar glycolytic rates. Trained BMDM showed increased basal and maximal OCR at baseline compared to control BMDM. Both control and trained BMDM showed no or modest increases in basal and maximal OCR, respectively, at 6 hours after LPS challenge (Figure 12C-D). However, trained BMDM showed more robust increases in basal and maximal OCR in response to 24 hours of LPS stimulation compared to control BMDM.

Discussion

Taken together, these data demonstrate a broad rewiring of macrophage transcription, antimicrobial activity, and metabolism following treatment with β -glucan. Macrophages mount a mild proinflammatory response to β -glucan immediately after administration. In contrast, after the macrophages rest for 3 days, the β -glucan-trained phenotype is characterized by altered morphology, enhanced direct antimicrobial activity, and chemokine transcription. Remarkably, trained macrophages display an essential identical response to LPS stimulation as untrained macrophages, suggesting that proinflammatory cytokine release is independent of the trained phenotype. Additionally, I show that macrophages adopt persistently elevated levels of glycolysis and mitochondrial metabolism following β -glucan training. This was driven, in part, by increased transcription of genes related to mitochondrial energy production, increases in mitochondrial mass, and enhanced mitochondrial membrane potential. Finally, β -glucan induced mild alterations in cellular metabolism following LPS stimulation.

As early as four hours following β -glucan treatment, macrophages undergo changes in gene transcription. At this early time point, the most enriched pathways include antimicrobial functions such as promotion of neutrophil chemotaxis and tissue-specific immune responses. Interestingly, macrophages trained with β -glucan three days prior to assessment do not show enrichment in these pathways yet are able to mediate enhanced neutrophil recruitment and antimicrobial immunity both *in vivo* and *in vitro*. Thus, β -glucan functionally reprograms macrophages without maintaining them in a proinflammatory state, like classically activated macrophages [224]. The presence of proinflammatory macrophages in tissues for a week or longer would trigger prolonged tissue damage through ROS, reactive nitrogen species, protease release, and cytokine production [225]. Instead, β -glucan drives macrophages to grow, become more granular, and retain enhanced phagocytic capacity. These distinct adaptations, along with the observation that plants and insects lacking adaptive immunity display a type of innate immune memory, suggest that trained immunity is not merely a drug-induced phenotype, but a fundamental mode of macrophage biology [226].

The studies presented here show that this poised phenotype is driven by a sustained increase in glycolytic and oxidative metabolism. Enhanced glycolysis can enrich the pentose phosphate pathway (PPP), which is linked to ROS generation in macrophages [227]. Koo and colleagues noted that increased glucose uptake and PPP activity did not lead to ROS release from macrophages at baseline, but after microbial challenge, the PPP was essential for ROS production. Additionally, glycolysis supports phagocytosis through the HIF-1 α signaling pathway [228]. While glycolysis is typically associated with acute, proinflammatory macrophage activity, sustained elevations after β -glucan treatment are consistent with enhancement in these antimicrobial functions in macrophages after transcriptional and proinflammatory changes subside.

Increased oxidative metabolism is the most efficient way to generate ATP. In the intervening time between β -glucan treatment and microbial challenge, a significant amount of protein translation is likely required to support the alteration in macrophage function. Notably, protein synthesis is one of the most energy-demanding processes in the cell [229]. Also, as discussed in Chapter 1, intermediates of the TCA cycle along with subunits of the electron transport chain serve dual roles in leukocytes as both metabolic mediators and immune regulators [191, 230]. Thus, it is likely that enhanced metabolism is directly linked to the transcriptional changes seen after β -glucan treatment, although more work is needed to concretely establish this connection.

Interestingly, β -glucan-treated and control macrophages displayed a highly similar transcriptional response to LPS stimulation. While macrophages from both groups changed several thousand genes in response to LPS, fewer than 50 genes differed between the two (data not shown). Previously, the Sherwood lab has demonstrated treatment of macrophages with different PAMPs can either potentiate or decrease the proinflammatory response to LPS [198]. However, I show here for the first time that β -glucan training in differentiated macrophages is uncoupled from the magnitude of their response to LPS. It is perplexing that mice trained with β -glucan *in vivo* or receiving β -glucan-trained macrophages recruit significantly more neutrophils to the site of infection, yet β -glucan training does not meaningfully enhance macrophage secretion of CXCL1 and CXCL2 after LPS stimulation. Importantly, future studies should probe the effect of β -glucan training on neutrophils directly, including antimicrobial activity and metabolism. In chapter 5, I will discuss strategies to potentially investigate this phenomenon.

Chapter 4. Neither Dectin-1 nor TLR2 are required for beta-glucan training

Introduction

Translation of murine and *in vitro* studies of β -glucan trained immunity into the development of clinically applicable pharmaceuticals will require a detailed understanding of the cellular signaling that occurs downstream of β -glucan binding on macrophages. In the context of fungal infection, PRRs for β -glucan have studied extensively. In the context of biochemical characterization of β -glucan signaling, studies have been complicated by preparations of glucans that contain varying amounts of β -glucans, proteins, and other carbohydrates [231]. With that being said, genetic knockouts and receptor-blocking studies have demonstrated that macrophages detect β -glucan by the pattern recognition receptors Dectin-1 and Toll-like receptor (TLR)-2 [142, 232, 233]. Genetic deficiency of Dectin-1 leads to recurrent infections with *C. albicans* in humans [157]. Only intact, insoluble β -glucan particles elicit signaling through Dectin-1. In dendritic cells and macrophages, co-stimulation of Dectin-1 and TLR2 leads to enhanced production of TNF- α , IL-10, and IL-12 [234, 235]. Downstream signaling from Dectin-1 and TLR2 converge on caspase recruitment domain containing protein 9 (CARD9) through Syk and MyD88 signaling in macrophages [236].

However, some macrophage responses to β -glucan are independent of Dectin-1 and TLR2 [237]. For example, it appears that Syk phosphorylation is essential for β -glucan phagocytosis in dendritic cells, but not macrophages [238]. Furthermore, the importance of Dectin-1 and TLR for inducing trained immunity in macrophages has not been studied. While complement receptor 3 (CR3) is also an important PRR for β -glucan, it solely recognizes solubilized and smaller fragments, not the whole polysaccharide, and thus likely does not contribute to recognition of the β -glucan training reagent, which is a colloidal suspension of whole glucan particles (WGPs) [239]. Therefore, I hypothesized that Dectin-1 and TLR2

mediated the protective effects of β -glucan training against *P. aeruginosa* infection.

Furthermore, I investigated the importance of Dectin-1 and TLR2 for the macrophage response to β -glucan. Surprisingly, I show that neither Dectin-1 nor TLR2 are required for protection from infection in mice or augmentation of macrophage function. Therefore, these studies provide important preliminary evidence that the biochemical mechanism of β -glucan trained immunity is unique. Understanding the signaling cascades that contribute to protective phenotype is important for optimizing the translational potential of β -glucan immunotherapy.

Methods

Mice

Clec7a^{-/-} (Dectin-1 KO) and *Tlr2*^{-/-} (TLR2 KO) mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Clec7a*^{-/-}*Tlr2*^{-/-} mice (DKO) were generated at Vanderbilt University. All mouse genotypes were confirmed by routine genotyping. Male and female mice aged 8-12 weeks were used in experiments. Male and female mice were statistically evaluated separately when appropriate. No differences between genders were found unless noted. All experiments and procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Western blots

Cellular lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MI) containing cOmplete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Lysate protein concentrations were quantified using the bicinchoninic acid assay for normalization (Pierce, ThermoFisher, Waltham, MA). Samples were separated by gel electrophoresis on Mini-Protean 4-20% Tris-glycine gels (Bio-Rad). Sample proteins were transferred onto nitrocellulose membranes overnight (PerkinsElmer, Boston, MA). Membranes were blocked with 5% fraction V BSA (RPI, Mount

Prospect, IL) and incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. Membranes were washed and then placed in HRP-conjugated secondary antibodies (1:2000 dilution) for two hours at room temperature followed by ECL reagent (Bio-Rad). Protein bands were detected by film exposure.

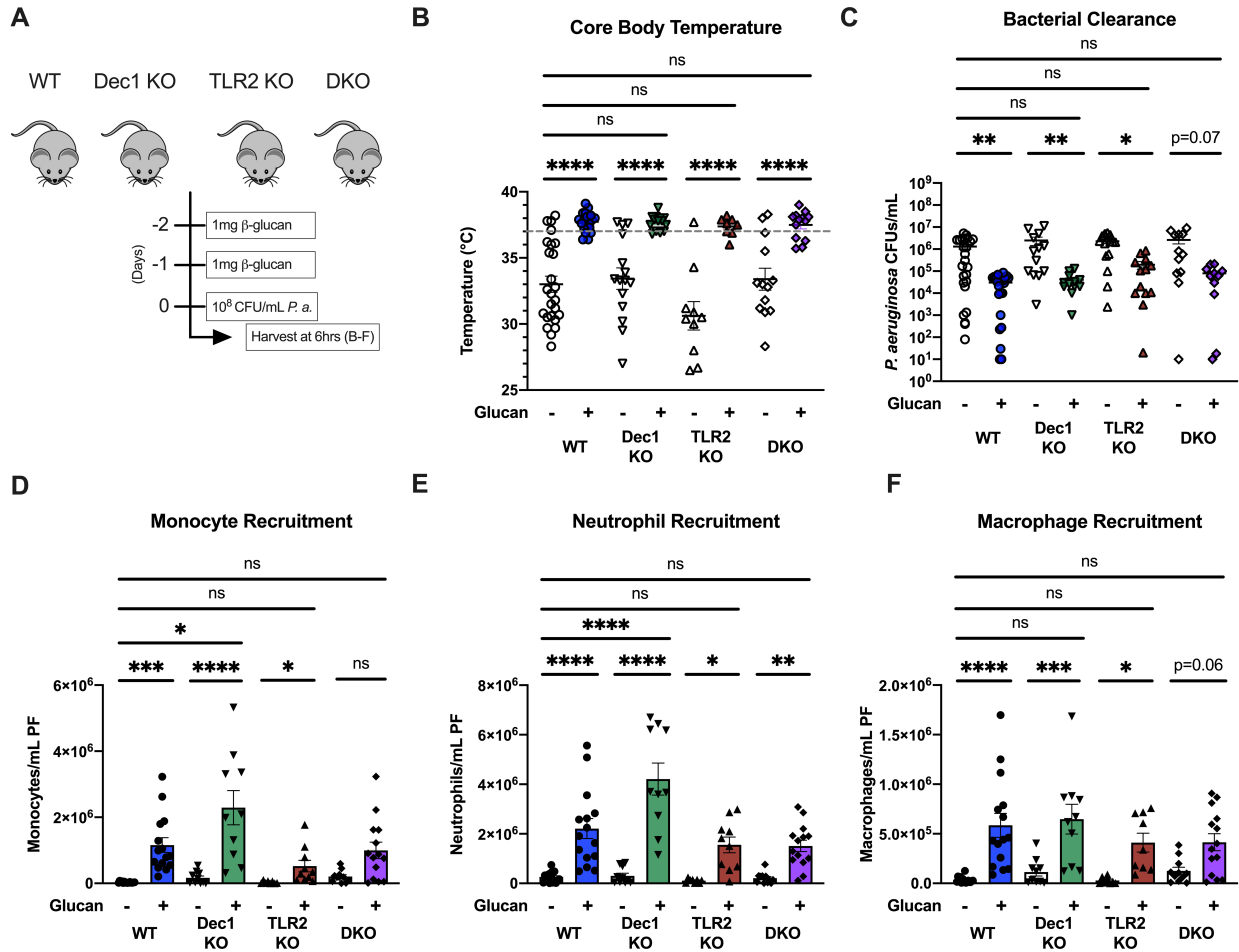


Figure 13. Beta-glucan-induced protection against *P. aeruginosa* is independent of Dectin-1 and Toll-like receptor (TLR)-2.

(A) Wildtype, Dectin-1 knockout, TLR-2, or Dectin-1/TLR2 double knockout C57Bl/6 mice were injected i.p. with β -glucan (1 mg) or vehicle 48 and 24 hours prior to i.p. inoculation with 10^8 CFU *P. aeruginosa* with subsequent harvest of plasma and peritoneal lavage fluid 6 hours later. (B) Core (rectal) body temperature in vehicle- or β -glucan-treated mice 6 hours after i.p. *P. aeruginosa*. (C) CFUs of *P. aeruginosa* per mL of peritoneal fluid. (D-F) Number of monocytes (D), neutrophils (E), or macrophages (F) in infected vehicle- or β -glucan-treated mice. N=10-15 per group. Body temperature and clearance data shown with median. All other data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Kruskal-Wallis test followed by Dunn's post hoc multiple comparison test (B, C) or ANOVA with Tukey's post-hoc multiple comparison test (D-F).

Results

Beta-glucan-induced protection against *P. aeruginosa* is independent of Dectin-1 and TLR2

We aimed to define the pattern recognition receptor (PRR) pathways necessary for β -glucan-induced protection from infection. Because Dectin-1 and Toll-like receptor (TLR)-2 are the two major surface receptors for β -glucan on innate leukocytes, we hypothesized that deficiency of these receptors would ablate β -glucan-induced trained immunity. To test this hypothesis, we treated wildtype, Dectin-1 (Dec1 KO), TLR-2 (TLR2 KO), and Dectin-1/TLR-2 double (DKO) knockout mice with β -glucan 48h and 24h prior to i.p. inoculation with *P. aeruginosa* (Figure 13A). In all genotypes, vehicle-treated controls developed hypothermia, but β -glucan-treated mice maintained normothermia (Figure 13B). There were no statistically significant differences observed in core body temperature when comparing β -glucan-treated mice across genotypes. Beta-glucan-treated mice of all genotypes showed lower *P. aeruginosa* CFUs in peritoneal lavage than vehicle-treated controls (Figure 13C). There were no differences in *P. aeruginosa* CFUs between genotypes in the β -glucan-treated mice. Beta-glucan treatment increased the numbers of monocytes (Figure 13D), neutrophils (Figure 13E), and macrophages (Figure 13F) in the peritoneal lavage after infection. Dec1 KO mice had significantly higher numbers of monocytes and neutrophils in the lavage than wild type mice. No other differences were seen between genotypes in the β -glucan-treated groups.

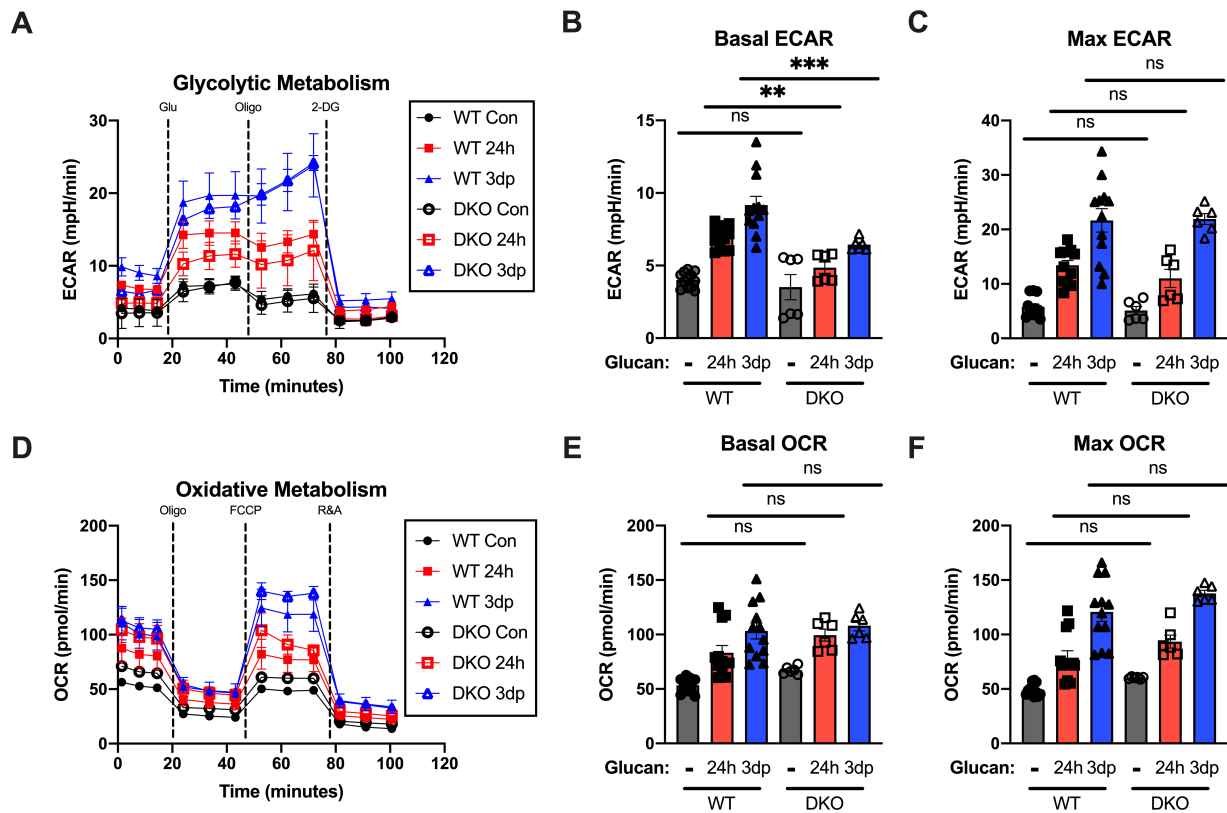


Figure 14. Metabolic alterations in β -glucan-trained macrophages are independent of Dectin-1 and Toll-like receptor 2.

(A) Glycolysis stress test of control, 24h, and trained BMDM on the Seahorse Xfe96 in wildtype and DKO mice. Extracellular acidification rate was measured over time at baseline and after glucose, oligomycin, and 2-deoxyglucose administration. (B) Summation of the first three measurements of ECAR in each group. (C) Summation of the three measurements following oligomycin in each group. (D) Oxidative stress test of control, 24h, and trained BMDM on the Seahorse Xfe96. Oxygen consumption rate was measured over time at baseline and after oligomycin, FCCP, and R&A administration. (E) Summation of the first three measurements of OCR in each group. (F) Summation of the three measurements following FCCP in each group. Data shown as mean \pm SEM. Experiments were performed with 3 biological replicates. ** $p < 0.01$, *** $p < 0.001$ by ANOVA with Tukey's post-hoc multiple comparison test.

Beta-glucan-induced metabolic alterations in macrophages are independent of Dectin-1 and TLR2

Additionally, I determined whether loss of Dectin-1 and TLR-2 impacted β -glucan-induced metabolic alterations in BMDM by performing extracellular flux analysis. Wild type and DKO BMDM were treated with β -glucan for 24 hours and assessed immediately or allowed to rest for 3 days to induce trained immunity (i.e., 3dp). DKO BMDM had reduced basal ECAR levels after both 24hr and 3dp β -glucan, however no differences were seen in max ECAR levels (Figure 14A-C). WT and DKO BMDM showed no significant differences in basal and max OCR levels after 24h β -glucan and in the trained group (Figure 14D-F).

Purified β -glucan is a weak inducer of Dectin-1 and TLR2 signaling in differentiated macrophages

To directly measure activation of the Dectin-1 and TLR-2 signaling pathways by the β -glucan training reagent, I treated BMDM with 5 μ g/mL β -glucan (two separate batches) for 0.5, 1, and 2 hours and measured phosphorylation of inhibitor of κ B kinase (I κ K) and spleen tyrosine kinase (Syk) by Western blot. Concurrently, BMDM were treated with 5 μ g/mL linear β -glucan for 0.5, 1, and 2 hours or with 100 ng/mL LPS for 1 hour as positive controls. LPS and linear glucan, induced strong I κ K phosphorylation and moderate Syk phosphorylation, while neither batch of β -glucan training reagent induced phosphorylation of either protein as compared to unstimulated BMDM (Figure 15).

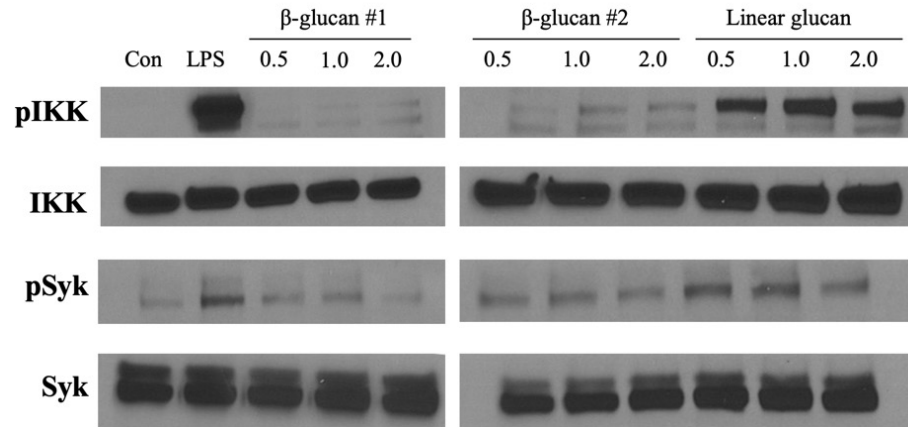


Figure 15. The β -glucan training agent induces weak dectin-1 and toll-like receptor 2 activation.

Protein was isolated from BMDM treated with β -glucan for 0.5, 1.0, or 2.0 hours. BMDM treated with 100 ng/mL LPS for 1 hour or linear glucan for 0.5, 1.0, or 2.0 hours served as positive controls. Western blot of phosphorylated I κ B kinase (pI κ K), total I κ K (I κ K), phosphorylated spleen tyrosine kinase (pSyk), and total Syk (Syk). Blots are representative of three repeated experiments.

Discussion

In this chapter, I showed that mice lacking dectin-1 and TLR2 retain augmented innate immunity against intraabdominal *P. aeruginosa* infection. Dectin-1 and TLR2 are the primary PRRs for β -glucan on macrophages. One possibility for this discrepancy is that other innate leukocytes, such as neutrophils, rely on another PRR to respond to β -glucan and that the macrophage response is dispensable for protection. Indeed, neutrophils express higher levels of complement receptor 3 (CR3), which is another known PRR for β -glucan [161]. However, as I discussed in Chapter 1, CR3 is only able to bind smaller β -glucan fragments and not the larger particles used in these studies. Additionally, in Chapter 2, I demonstrated that treatment of macrophages *ex vivo* protected mice from *P. aeruginosa* after adoptive transfer. While β -glucan-trained macrophages did not entirely recapitulate the protective phenotype of *in vivo* β -glucan treatment, these studies confirmed activity of β -glucan on macrophages.

The fact that a linear preparation of β -glucan, which has not been shown to induce trained innate immunity, induces more robust Dectin-1 and TLR2 signaling is fascinating. Little is known about purely linear preparations of β -glucan given the difficulty associated with purifying them. However, studies by Zheng *et al.* isolate linear glucan within >99% purity, as confirmed by mass spectrometry and ^{13}C nuclear magnetic resonance [240]. Similar to the findings I report here, they show that linear glucan is a potent stimulant of I κ B phosphorylation. They do not investigate Syk phosphorylation because dectin-1 has diminished expression on RAW264.7 cells, which they utilized in this study [241]. I hypothesize that linear glucan will induce protection similarly to branched β -glucan used in this dissertation and other work. While β -glucan and BCG vaccine are widely studied inducers of trained immunity, an array of studies are emerging suggesting that the vast majority of PAMPs or microbes induce trained innate immunity [242-244]. While the specifics of effector cells, tissue location, and dynamics of protection differ slightly, these various ligands point to a unified mechanism whereby innate leukocytes utilize frequent, low-dose exposures to PAMPs to modulate host defenses, at least in the short-medium term. Indeed, as I have referenced throughout this thesis, the Sherwood lab has shown that a variety of ligands including β -glucan, LPS, MPLA, bacterial DNA, and synthetic lipoproteins not only induce the trained phenotype in innate leukocytes, but, importantly, protect in preclinical models of infection [198, 245].

While the data in this chapter raises many questions, I have shown, using a combination of murine, metabolic, and biochemical studies, that further work is needed to understand the signaling mechanisms underpinning β -glucan trained immunity. In the next chapter, I will discuss several strategies available to probe libraries of surface receptors and signaling cascades to attempt to narrow down the target candidates.

Chapter 5. Conclusions and Future Directions

Conclusions

Preface

Infections, especially those occurring in the hospital setting or caused by antimicrobial resistant microbes, are a significant threat for the 21st century. Targeting the innate immune response to infection is an old idea with fresh life due to the discovery and characterization of trained innate immunity in the last decade. Training innate immunity is a promising antimicrobial strategy because it targets the underlying pathophysiology that drives infection mortality: immunosuppression, failure to contain a local infection, and the development of sepsis. Beta-glucan is the canonical innate training agent. Despite significant advances in β -glucan biology, there has been a dearth of studies developing the translational potential of β -glucan. Herein, I used a combination of the preclinical models developed and validated in the Sherwood laboratory in combination with studies on differentiated macrophages to advance the understanding of trained innate immunity.

The major finding of this thesis is that β -glucan elicits a trained phenotype in differentiated macrophages that confers protection from infection with *P. aeruginosa*. I found that β -glucan training augments the host response to *P. aeruginosa* infection by facilitating recruitment of innate leukocytes to the site of infection, which is associated with enhanced bacterial clearance. Adoptively transferring macrophages treated *ex vivo* with β -glucan into naïve mice recapitulates the protective phenotype. Furthermore, β -glucan induces significant alterations in macrophage gene transcription and metabolism. These changes underpin a robust enhancement of macrophage antimicrobial functions, including phagocytosis, ROS generation, chemokine production, and leukocyte recruitment. This study is the first to show that differentiated macrophages contribute to β -glucan-induced trained immunity and to explore the mechanism of training in these effector cells. My findings suggest that differentiated

macrophages, which survive for weeks to months *in situ*, likely contribute to the protective phenotype conferred by treatment with β -glucan. In addition, β -glucan-induced protection is preserved after loss of Dectin-1 and TLR-2, which suggests an alternative receptor drives the protective phenotype.

In this chapter, I discuss the implications of our research, as well as the limitations of the studies. All in all, the field of trained immunity is poised to make significant contributions to the fight against infection. Future studies must be grounded in fundamental immunology, as these principles will allow investigators to predict and overcome hurdles on the course to clinical implementation. Below, I will discuss the importance of appropriate preclinical models in trained immunity studies, the impact of developing our understanding of β -glucan's effect on differentiated macrophages, and how our findings that β -glucan activity is independent of Dectin-1 and TLR2 implicates future studies. Finally, I discuss the exciting future directions raised by this work.

The importance of preclinical models in trained immunity

Since the discovery of the microbiological causes of infections, significant improvements have been made in the caring of an infected patient that did not require a nuanced understanding of the host immune response. The widespread implementation of hand hygiene among medical professionals has had an unquantifiable impact on surgical, obstetric, and wound infections. More recently, sterilization of entry sites for intravenous and arterial punctures has decreased the incidence of intravenous infections. For patients that present emergently with infection, adoption of strict standards for the timing of antimicrobial treatments have decreased the development of sepsis [246].

Given these large strides, it is surprising to observe that targeting the host immune system to prevent acute bacterial infection or sepsis has been largely unsuccessful outside of development of specific vaccines. Granulocyte-colony stimulating factor (G-CSF) is approved by

the FDA to treat patients receiving myelosuppressive anti-cancer drugs and has been successful in reducing the incidences of febrile neutropenia in these patients [247]. Otherwise, modulation of the immune system against bacterial infection has been limited to preliminary studies, although a few candidates have advanced to clinical trials [248]. In fact, nearly every clinical trial for immunomodulation in sepsis has failed [249].

What accounts for these failures? One significant contributor is the lack of fidelity between preclinical models that inform treatments and the targets that ultimately receive them in clinical trials [250]. The use of low fidelity murine infection models, or a complete lack of *in vivo* models, generates conflicting and potentially harmful data conclusions. It is worth noting that standards for design and reporting of animal studies have been proposed in other diseases such as pulmonary fibrosis or heart failure but have only recently been suggested for sepsis [250-252]. Furthermore, the extrapolation of data from *in vitro* to *in vivo* systems must occur carefully.

With this in mind, the studies presented in this thesis use the combination of a clinically relevant infection model with *in vitro* studies to provide rigor. A key tenet of β -glucan trained immunity is augmented cytokine production in response to infection or LPS challenge and its purported ability to reverse endotoxin tolerance in monocytes previously exposed to LPS [215]. Results of the present study show that macrophages trained with β -glucan sustain the ability to secrete cytokines in response to LPS. However, the Sherwood lab has previously disputed the association between a training agent's ability to increase or decrease cytokine production in macrophages with its ability to protect against infection [198, 216]. This interpretation has also been corroborated by work in human monocytes [253, 254].

Instead, I propose that careful examination of direct antimicrobial functions, such as phagocytosis, ROS production, microbial killing, and leukocyte recruitment, rather than reliance on cytokine production alone, is a more accurate metric of the ability of an agent to confer

trained immunity. Here, I show that macrophages trained with β -glucan secrete comparable levels of IL-6 and TNF- α after stimulation with LPS compared to untrained controls. Interestingly, β -glucan also attenuates cytokine production after LPS stimulation in microglia, which are closely related to macrophages [255]. Furthermore, transcriptomic profiling of macrophages after LPS challenge revealed similarity between β -glucan-trained and control macrophages in cytokine and chemokine transcripts. Trained macrophages instead showed enhanced phagocytosis and ROS generation indicating augmentation of direct antimicrobial functions. Taken together, these findings indicate that β -glucan induces a macrophage phenotype that is more effective at recruiting leukocytes to the site of infection and mediating direct microbial clearance. As I discuss below, the work in this dissertation paves the way for future clinical studies that capitalize on the translational relevance in the presented experiments.

The distinction between trained macrophages and monocytes

My characterization of innate immune memory in macrophages reveals interesting distinctions when compared to the canonical monocyte profile. Trained monocytes activate hypoxia-inducible factor-1 α to shift energy production primarily toward glycolysis and away from oxidative metabolism, even in oxygen-rich environments, a phenomenon known as aerobic glycolysis [256]. In contrast, training of macrophages with β -glucan augments both glycolytic and oxidative metabolism. Here, I show that enhancement of oxidative metabolism seems to be achieved by expansion of the functional mitochondria pool and increased mitochondrial membrane potential. This suggests that augmentation of broad metabolic pathways is critical for the antimicrobial effects of β -glucan training.

Previous studies in the Sherwood lab using chemokine (C-C motif) receptor 2 (CCR2)-deficient mice, which have impaired circulating monocyte recruitment to sites of infection, demonstrate the monocytes are dispensable for protection mediated by immunomodulation [216]. These results, combined with the observation that adoptive transfer of β -glucan-treated

differentiated macrophages provide protection against subsequent infection suggest that focusing on murine monocytes or human peripheral blood mononuclear cells may fail to fully elucidate the protective effects of β -glucan. Instead, future studies should aim to correlate *in vitro* findings, from macrophages or otherwise, with studies in appropriate infection models to ensure that cellular and molecular studies appropriately inform translational studies.

Beta-glucan activity outside of Dectin-1 or TLR2

Dectin-1 and TLR-2 are both well-characterized PRRs for β -glucan [257, 258]. Dectin-1 deficiency diminishes cellular response to β -glucan *in vitro*, but its role in response to fungal infection has been disputed [259, 260]. Similarly, the role of TLR-2 has not been fully elucidated *in vivo* [261]. Here, I show that neither Dectin-1 nor TLR-2 are required for β -glucan-induced protection against *P. aeruginosa* infection or β -glucan-induced metabolic reprogramming in macrophages. Given that different fungi containing β -glucan trigger different surface receptors on leukocytes, this phenomenon should be confirmed in infections with other pathogens. Interestingly, β -glucan has been shown to stimulate macrophages independently of Dectin-1 and TLR-2, adding further evidence to possibility that β -glucan trained immunity occurs independently of either receptor [237, 262]. These varying results may be due to the fact that β -glucans with varying structural motifs can be derived from wide ranging species of fungi [263, 264]. Our finding that a linear β -glucan derived from *C. albicans* induces Syk and IKK phosphorylation whereas the branched β -glucan training reagent does not, supports this contention. Further research is needed to further identify β -glucan structure, receptors and signaling pathways (discussed below).

Limitations of These Studies

There are limitations to these studies that should be discussed to inform critical interpretation of the results and to direct future studies. Chief among them is the feasibility of translating intraperitoneal β -glucan treatments into the clinical realm. On average, C57BL/6J

mice aged 10-12 weeks weigh around 25g [Jackson Laboratory]. These mice receive two *i.p.* 1mg β -glucan injections. Given that the average American male is 90kg and female 77kg, that translates to a dose of between 3.08kg and 3.6kg for patients needing infection prophylaxis [265]. With that being said, my studies provide important comparisons to others investigating the mechanism of trained immunity, which typically use the same dose [173, 183, 266]. By confirming these previous studies in a translational infection model, this work bolsters the evidence that clinical evaluation should be pursued. Pharmacologic studies begin by identifying a lead compound and follow with refining the target until it is appropriately formulated to be administered as a drug [267]. Furthermore, the biochemical studies presented in this thesis demonstrating that β -glucan-induced innate immune training is independent of Dectin-1 and TLR2 provide a critical filter to focus pharmacologic development of β -glucan.

As mentioned above, the translation from mouse to human studies is complicated, perhaps even more so in models of infection and sepsis [249]. The murine inflammatory response to bacterial PAMPs differs significantly from humans [268]. However, previous work in trained immunity utilizing the Bacillus Calmette-Guerin (BCG) vaccine provides a framework for translation to human studies [168, 269]. Peripheral blood mononuclear cells (PBMCs) isolated from individuals treated with the BCG vaccine have demonstrated a trained immunity phenotype. These cells can be differentiated into macrophages similarly to murine bone marrow progenitors, although they represent a monocytic rather than tissue macrophage origin. With more effort, human macrophages could be derived from bone marrow. Given that β -glucan is a common oral supplement, a more plausible route is to develop the molecular pharmacology of β -glucan to a point where direct administration to human subjects can be performed [270]. Interestingly, particulate β -glucan has been proposed as an oral vaccine delivery platform due to its avoidance of oral tolerance and ability to survive the intestinal environment, suggesting that oral administration of a future candidate could be possible [271].

Finally, while bone marrow-derived macrophages (BMDM) have been shown to be representative of differentiated macrophages in model systems, limitations still exist surrounding the use of BMDM in monoculture. In the context of infection, such as the intraperitoneal *P. aeruginosa* model I utilized in Chapter 2, macrophages are immersed in a complex milieu containing other innate leukocytes, parenchymal cells, and microbes [272]. Nevertheless, my studies utilizing BMDM treated *in vitro* prior to adoptive transfer to mice demonstrate that β -glucan acting on macrophages alone is sufficient to bolster the host response to infection. Given that the response to β -glucan-treated BMDM was not entirely equivalent to *in vivo* treatment, future work is warranted to determine how leukocytes and parenchymal cells contribute to the trained phenotype. Indeed, evidence suggest that mesenchymal stromal cells, epithelial cells, and fibroblasts may contribute to trained immunity [273].

Future Directions

Based on the key findings of this thesis, along with the limitations of the studies, several future experiments are warranted. The efficacy of β -glucan to induce neutrophil recruitment to sites of infection up to 7 days after priming raises the question of whether neutrophils themselves respond to β -glucan training. A recent study by Kalafati and colleagues demonstrated that treatment of mice with β -glucan boosted anti-tumor immunity in a neutrophil-dependent manner [274]. Adoptive transfer of neutrophils from β -glucan-treated mice into untreated recipients was sufficient to deter tumor growth. These results are reminiscent of my findings in Chapter 2 where adoptive transfer of β -glucan-treated macrophages protected recipient mice from infection. There is significant overlap in the innate immune response to infection and cancer [275]. Thus, an exciting next step for this project would be to determine if adoptively transferred β -glucan-trained neutrophils display a protective phenotype against *P. aeruginosa*. Previous work in the Sherwood has shown that treatment with the LPS derivative monophosphoryl lipid A (MPLA) induces neutrophil recruitment and secretion of the neutrophil-

promoting cytokine granulocyte-colony stimulating factor, which in turn provide protection in a burn wound infection. MPLA induced expansion of neutrophil precursors in the bone marrow [276]. Similar studies should be performed in β -glucan-treated mice to determine if a similar mechanism is present.

The discovery that neither dectin-1 nor TLR2 are required for β -glucan-induced protection against infection raises the tantalizing question of what surface receptors mediate the protection. Because these studies were pursued using a genetic knockout approach, compensatory alterations in the expression of other receptors cannot be ruled out. To tackle this question, dectin-1 and TLR2 blocking antibodies could be administered prior to injection of β -glucan to determine if acute receptor blockade is sufficient to ablate protection. These studies are ongoing in the lab currently. As discussed in Chapter 1, complement receptor 3 (CR3) possess a carbohydrate-binding domain and initiates signaling after detection of small soluble β -glucan particles [161]. Experiments testing the importance of CR3 for murine infection have been limited due to the fact that deletion of the CD11b or CD18 subunits of CR3 independently hinder neutrophil transmigration across the endothelium [277]. Identification of a pharmacologic inhibitor of CR3 that could be administered prior to β -glucan, but cleared from the body prior to infection, would allow for assessment of the receptor in training alone.

Another possibility is the presence of a previously unidentified β -glucan receptor. To approach this hypothesis, one could utilize clustered regularly interspaced short palindromic repeats (CRISPR) screening technology. Genome-scale libraries of guide RNA are now widely available. Preliminary work to develop a Cas9-expressing cell line that demonstrates binding activity with β -glucan would need to be completed. A recently described protocol utilizes such a cell line to generate a CRISPR knockout cell library combined with a fluorescently labeled target protein to exclude non-binding mutants by flow cytometry [278, 279]. Finally, a phospho-proteomic screen could be utilized to identify key signaling hubs downstream of β -glucan

treatment in macrophages. Identification of signaling nodes would allow for upstream investigation of surface receptors to determine if any likely have β -glucan binding capability.

The ultimate goal of this project is to develop an immunomodulator available for prophylaxis in those deemed at risk for infection. While many obstacles remain before that goal, the current studies have established a robust preclinical model to test such a drug once it is available. In collaboration with Dr. David Williams at East Tennessee State University, the Sherwood lab intends to contribute to search for such a drug. Dr. Williams is currently developing small β -glucan-like synthetic ligands that activate the mTOR signaling pathway, which is associated with β -glucan-induced protection (data not shown) [183]. After the identification of a protective compound, studies in small and large animal models would be undertaken to identify toxicity and efficacy. Finally, the drug would enter clinical trials.

Closing Summary

There is an urgent need for novel strategies to combat increasingly deadly infections. This dissertation identifies the potential of prophylactic training of innate immunity to achieve this goal. Furthermore, this work advances our understanding of macrophage antimicrobial immunity and metabolism. The paradigms of innate immunity are evolving. This dissertation contributes a strong biological and preclinical foundation for future studies that will harness the mechanisms of trained innate immunity to reduce the burden of infectious diseases in the United States and beyond.

Appendix A: Exploring the role of mechanistic target of rapamycin (mTOR) signaling in endothelial cells during infection

To identify additional targets for intervention during the pathophysiology of infection, I began a side project exploring the importance of cellular metabolism for the endothelial cell response to infection. I provide preliminary evidence to support mechanistic target of rapamycin (mTOR) as a signaling hub linking immunity and metabolism in microvascular endothelial cells.

Introduction

A circulatory system designed for delivery of oxygen and nutrients to, and removal of waste from, organs is common among all vertebrate animals [280]. Oxygenated blood is delivered to the body via large muscular arteries that branch into increasingly smaller vessels as they travel closer to organs to be perfused. Smaller vessels within organs, known as the microvasculature, specialize in nutrient delivery, barrier function, and organ-specific functions. The primary cellular constituents of the microvasculature are endothelial cells, which line the inner surface of all blood vessels. Along with their primary role in maintaining vascular homeostasis, endothelial cells have indispensable functions in several other processes including immunity [281].

Blood vessels are the conduits by which circulating leukocytes are recruited to sites of infection. Tissue-resident antigen-presenting cells, such as macrophages, detect invading pathogens and secrete chemokines to attract additional cells to initiate the innate immune response to infection [282]. However, endothelial cells do not merely play a passive role in the immune response to infection. Endothelial cells express pattern recognition receptors on their luminal surface, allowing them to detect microbial products themselves [283]. Furthermore, endothelial cells secrete cytokines and chemokines to amplify the inflammatory response [284]. Finally, endothelial cells relax cell-cell tight junctions during infection to facilitate the delivery of cellular and soluble components of the immune system to the site of infection [285].

While the endothelial cell response to infection is an essential component of antimicrobial defense, it can also be pathologic. Endothelial dysfunction is an essential component of the pathophysiology of sepsis, which is defined as a dysregulated host response to infection [201]. Excess permeability due to prolonged endothelial dysfunction leads to loss of intravascular fluid and starves tissues of oxygen and nutrients. Eventually, if the circulatory system is extensively damaged, septic shock will develop whereby a patient will need immediate cardiopulmonary support or face substantially increased risk of mortality [286]. The steps of this cascading feedback loop are well understood, yet there are few interventions that specifically target endothelial dysfunction during infection and sepsis.

Given the profound metabolic dysfunction that occurs secondary to excess inflammation and sepsis, one potential target for intervention is the endothelial metabolic response to infection [287]. Angiogenesis, the sprouting of new blood vessels, occurs when blood flow is needed in a relatively hypoxic environment [288]. Thus, despite their role in oxygen-delivery, endothelial cells primarily rely on glycolysis to meet energy demands [289, 290]. The mechanistic target of rapamycin (mTOR) signaling pathway is a central node that regulates a broad array of cellular processes and is essential for endothelial cell proliferation and assembly [291]. Additionally, extensive work demonstrates a key role for mTOR signaling in leukocytes for appropriate metabolic adaptations to microbial stimulation [183, 292-294]. Interestingly, hypoxia-inducible factor (HIF)-1 α , a transcription factor activated downstream of mTOR signaling, mediates a shift to glycolysis in activated leukocytes, even in the presence of oxygen, that augments antimicrobial responses [256]. Thus, I hypothesized that mTOR signaling modulated the endothelial response to infection.

Methods

Endothelial cell culture

Primary pulmonary human microvascular endothelial cells (HMVECs) were obtained from Lonza (CC-2527). Donors were verified as non-smoking, below 60 years of age, and without vascular comorbidities. Prior to seeding, T-75 flasks (Corning) were coated with 0.3% gelatin solution (Sigma) for 20 minutes at 37°C. The gelatin solution was removed and 15 mL of microvascular endothelial cell growth media (EC media) containing human epidermal growth factor, vascular endothelial growth factor, R3-insulin-like growth factor-1, ascorbic acid, hydrocortisone, human fibroblast growth factor-beta, fetal bovine serum, and gentamicin/amphotericin-B was added to the flask. HMVEC cryovials were quickly thawed at 37°C and seeded at a density of 5,000 cells/cm². EC media was refreshed after 24 hours and then every 48 hours until cells reached 70%-85% confluency. HMVECs were subcultured using TrypLE (Gibco) until sufficient quantities were obtained for functional assays (final seeding and vessels listed below).

Western blots

Cellular lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing cOmplete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche). Lysate protein concentrations were quantified using the bicinchoninic acid assay for normalization (Pierce, ThermoFisher). Samples were separated by gel electrophoresis on Mini-Protean 4-20% Tris-glycine gels (Bio-Rad). Sample proteins were transferred onto nitrocellulose membranes overnight (PerkinElmer). Membranes were blocked with 5% fraction V BSA (RPI) and incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. Membranes were washed and then placed in HRP-conjugated secondary antibodies (1:2000 dilution) for two hours at room temperature followed by ECL reagent (Bio-Rad). Protein bands were detected by film exposure.

Quantification of HMVEC permeability

Twenty-four-well plates with 0.3 μ M transwell inserts were coated with Matrigel (Corning) diluted 2:1 with EC media lacking growth factors. The Matrigel was immediately removed. Transwell plates were placed at 37°C for 30 minutes. HMVEC were harvested as above and seeded onto transwells at a final concentration of 1×10^5 cells/well. 300 μ L and 1mL of EC media with growth factors was added to the upper chamber and lower chamber of the transwell, respectively. After 24 hours, HMVECs were stimulated with 100 ng/mL lipopolysaccharide (LPS, Sigma) overnight. LPS was removed and replaced with 500 μ L of fresh EC media with growth factors. FITC-labeled 70kD dextran (Sigma) was added to the upper chamber at a final concentration of 250 μ g/mL. Every 30 minutes, 20 μ L of media was removed from the lower chamber and placed into a 96-well plate. After 180 minutes, all aliquots were diluted with 80 μ L of H₂O. Fluorescence intensity was measured using a plate reader (excitation: 485nm, emission: 520nm).

Seahorse extracellular flux analysis

One day prior to the assay, HMVEC were plated at 2×10^4 cells/well in a 96-well Seahorse assay plate coated with 0.3% gelatin for 30 minutes prior to seeding. All measurements were performed on a Seahorse XF⁹⁶ Extracellular Flux Analyzer (Agilent). The glycolysis and mitochondrial stress tests were performed using the manufacturer's protocol. Briefly, extracellular acidification rate was measured at baseline and after the addition of 10 mM glucose (Sigma-Aldrich), 1 μ M oligomycin (Agilent), and 50 mM 2-deoxyglucose (2-DG; Sigma-Aldrich). Oxygen consumption rate was measured at baseline and after the addition of 1 μ M oligomycin, 1 μ M FCCP (Agilent), and 0.5 μ M antimycin A and rotenone (Agilent).

Quantification of cytotoxicity by lactate dehydrogenase (LDH) release

EC cytotoxicity following treatment with rapamycin and LPS was determined using the CyQUANT LDH cytotoxicity assay kit (Invitrogen). HMVECs were seeded on a 96-well plate coated with 0.3% gelatin at 2×10^4 cells/mL and treated as indicated. Following treatments, 50 μ L of culture media was removed and added to 50 μ L of reaction mixture containing tetrazolium salt (INT) and NADH, prepared according to manufacturer instructions. Maximum LDH activity was determined using media from HMVECs treated with lysis buffer to release intracellular LDH. Samples were incubated with reaction mixture at room temperature protected from light for 30 minutes. Absorbance at 490nm was measured on a Synergy H1 plate reader. Percent cytotoxicity was determined by dividing the sample LDH activity by maximum LDH activity and multiplying the result by 100.

Results

Endothelial cells activate mTOR following LPS stimulation

First, to determine whether endothelial cells activated mTOR signaling after microbial challenge, I stimulated HMVECs with 100 ng/mL LPS for 1 hour and measured phosphorylation of ribosomal protein S6 kinase (S6K). Concurrently HMVECs were pretreated with the mTOR inhibitor rapamycin (100 nM) for 2 hours prior to LPS stimulation. HMVEC grown in growth factor-rich media served as a positive control. LPS induced phosphorylation of S6K, which was inhibited by pretreatment with rapamycin (Figure 16). Beta-actin served as a loading control.

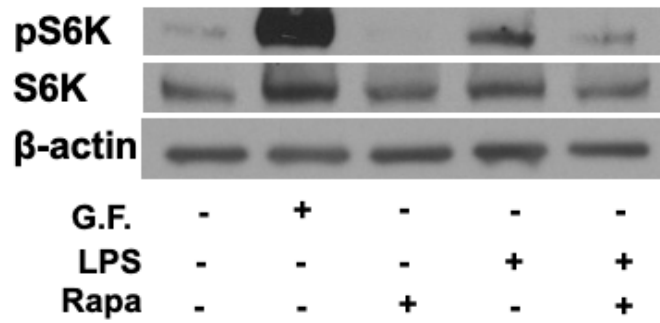


Figure 16. LPS activates mTOR signaling in endothelia cells.

HMVEC were grown in growth factor (G.F.)-free conditions for 24 hours. Subsequently, protein was isolated from HMVEC following treatment with 100 ng/mL LPS for 1 hour with or without pretreatment with 100 nM rapamycin (Rapa). Western blot of phosphorylated ribosomal protein S6 kinase (pS6K), total ribosomal protein S6 kinase (S6K) and β-actin.

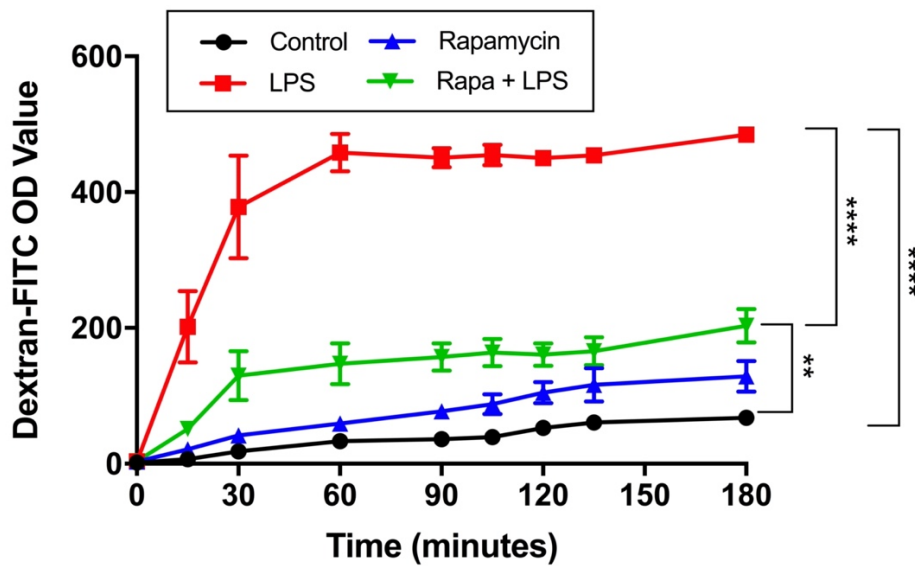


Figure 17. mTOR blockade prevents LPS-induced vascular barrier permeability.

HMVECs were plated on Matrigel-coated transwells with 0.3μm pores. FITC-labeled 70kD dextran was added to upper chamber after treatment with 100 ng/mL LPS for 24 hours with or without 100 nM rapamycin pretreatment for 2 hours. Media from lower chamber was removed every 30 minutes and fluorescence was measured. n=6. Data shown as mean ± SEM. Groups compared by repeated measure two-way ANOVA. ** p<0.01 **** p<0.0001.

mTOR blockade prevents LPS-induced vascular inflammation

Because LPS stimulation induced mTOR activation in HMVEC, I next investigated whether mTOR signaling was required for vascular inflammatory responses, including increased permeability and cytokine secretion. First, I investigated the importance of mTOR signaling for alterations in endothelial cell permeability following challenge with LPS. HMVEC were seeded to confluence on Matrigel-coated transwell inserts to simulate the endovascular permeability barrier. Endothelial cells stimulated with LPS for 24 hours displayed loss of barrier function, as measured by translocation of FITC-dextran across the transwell (Figure 17). In contrast, HMVEC that were pretreated with 100 nM rapamycin for 2 hours prior to stimulation with LPS showed significant reduction in permeability as compared to HMVEC treated with LPS alone, although both groups demonstrated significantly higher permeability than control HMVEC. Interestingly, rapamycin treatment in the absence of LPS significantly increased permeability as compared to control HMVEC.

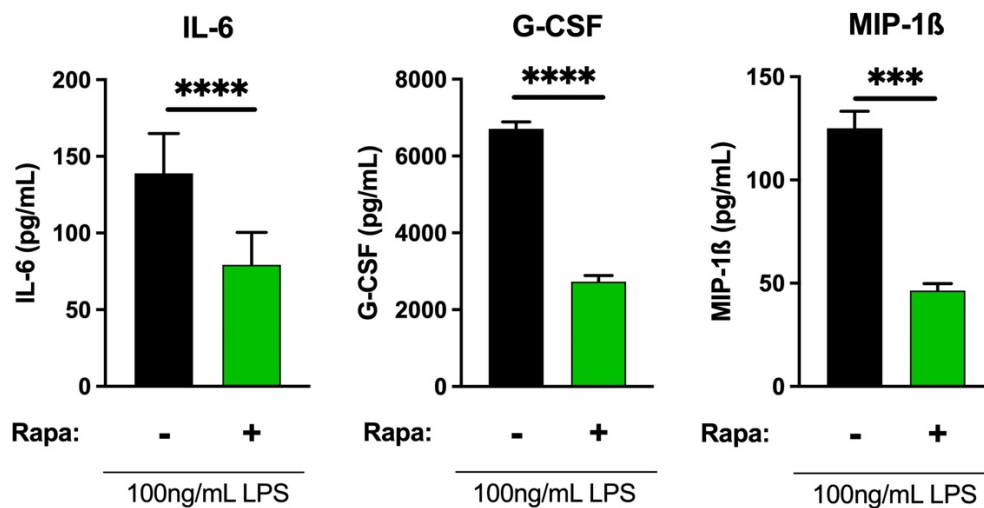


Figure 18. mTOR blockade diminishes HMVEC cytokine secretion after LPS.

HMVECs were treated with 100 nM rapamycin or vehicle for 2 hours followed by 100 ng/mL LPS for 6 hours. Conditioned media was analyzed by ELISA. Data shown as mean \pm SEM. $n=6$.
*** $p<0.0005$ **** $p<0.0001$ by t-test.

To determine whether endothelial mTOR signaling contributes to the vascular inflammatory response, I treated HMVEC with 100 nM rapamycin for 2 hours and quantified secretion of endothelial cytokines (IL-6) and chemokines (G-CSF, MIP-1 β) after 6 hours of LPS stimulation by ELISA (Figure 18). Treatment with rapamycin significantly blunted the secretion of all three measured cytokines. HMVEC treated with vehicle or rapamycin alone did not secrete detectable amounts of IL-6, G-CSF, nor MIP-1 β (data not shown). Taken together, these data suggest that mTOR signaling the HMVEC inflammatory response.

LPS and rapamycin drive distinct metabolic changes in endothelial cells

Finally, because metabolic reprogramming is essential for the leukocyte response to infection, I questioned whether endothelial cells alter cellular metabolism following stimulation with LPS. Furthermore, given the link between mTOR signaling and enhanced glycolysis, I determined whether mTOR blockade abrogated these effects. HMVEC were stimulated with 100 ng/mL LPS for 1 hour and metabolism was determined using Seahorse extracellular flux analysis. LPS treatment alone significantly increased glycolytic metabolism, as determined by extracellular acidification rate (Figure 19A) but did not significantly increase oxidative metabolism as measured by the oxygen consumption rate (Figure 19B). Pretreatment of HMVEC with 100 nM rapamycin for 2 hours significantly blunted glycolytic and oxidative metabolism after LPS stimulation but had no affect alone. Finally, to determine whether treatment with rapamycin, LPS, or both was cytotoxic to HMVEC, cells were treated as above and extracellular LDH release was quantified by spectrophotometer. No significant changes in cytotoxicity were noted with any of the treatment combinations (Figure 19C).

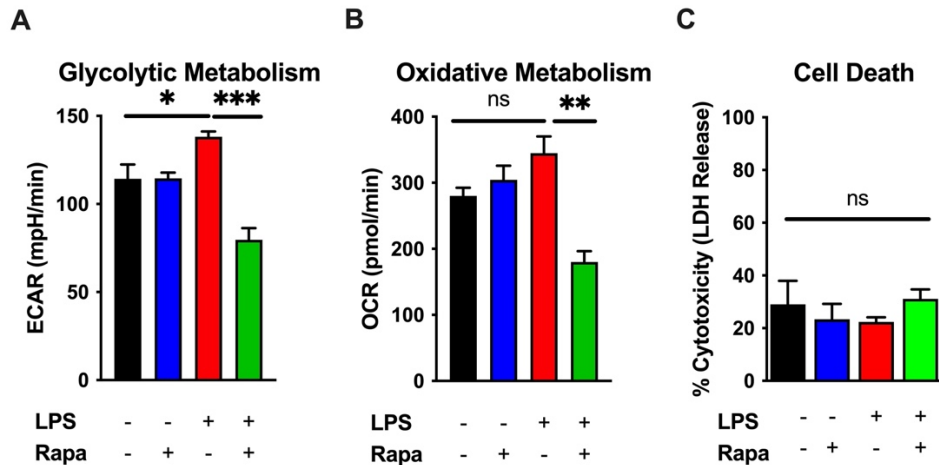


Figure 19. Rapamycin decreases cellular metabolism following LPS stimulation but does not cause cell death.

(A-B) HMVECs were treated with 100 nM rapamycin for 2 hours prior to addition of 100 ng/mL LPS for 1 hour. Cells equilibrated in a non-CO₂ incubator for 1 hour in Seahorse media. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured. (C) HMVEC were treated with rapamycin and LPS similarly and percent cell death was determined by quantifying LDH release into culture media. Groups were compared using one-way ANOVA with Tukey's post-hoc test. Data shown as mean ± SEM. n=4. * p<0.05 ** p<0.01 *** p<0.001.

Summary

The data presented in this Appendix suggest that mTOR signaling is an important component of the endothelial immune response to infection. Relaxation of the vascular permeability barrier is an essential early step in inflammation [295]. While endothelial cells are not the primary source of proinflammatory cytokines and chemokines during infection, a previous study by Boettcher and colleagues demonstrated that endothelial-specific defects in LPS detection worsened survival during *in vivo* infection due to lack of G-CSF-mediated neutrophil recruitment [284]. Thus, future studies elaborating on the contribution of mTOR signaling to these processes is warranted. While preliminary, the metabolic data shown here points toward a role for augmented endothelial cell glycolysis during infection. Additional experiments specifically targeting glycolysis and HIF-1 α , which regulates glycolysis downstream of mTOR activation should be performed to further characterize this mechanism.

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