IMMUNOMODULATORY STRATEGIES FOR THE PREVENTION OF NOSOCOMIAL INFECTION

By

Benjamin Ari Fensterheim

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Approved:

Edward Sherwood, M.D., Ph.D., Advisor

Amy Major, Ph.D., Chair

Mark Boothby, M.D., Ph.D.

Luc Van Kaer, Ph.D.

Owen McGuinness, Ph.D.

DEDICATION

To my grandparents, Rose, Nancy, Alvin, and Norman

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CHAPTER I

INTRODUCTION

Overview

Nosocomial infection, also known as hospital-acquired infection, is one of the most complex and threatening conditions in modern healthcare. The high incidence of nosocomial infection in hospitals is driven by a combination of pathogen exposure and patient immunosuppression. Unfortunately, the efficacy of antibiotics used to treat nosocomial infection is decreasing due to antimicrobial resistance of common nosocomial pathogens. Therefore, new strategies to decrease the incidence and severity of nosocomial infection are needed. Elimination of pathogenic microbes from the hospital environment and restoration of immune competency in patients are attractive options.

Strategies that destroy pathogenic microbes before they cause infection, such as antibiotics and decontamination, are the mainstays of current nosocomial infection prophylaxis. As there are no established ways to determine the infection risk of an individual patient, nearly all patients that undergo invasive procedures are given prophylactic antibiotics. As a result, antibiotic overuse has driven pathogens to rapidly develop resistance to antibiotics used for prophylaxis and treatment. In order to reduce the incidence of nosocomial infection, it is essential that we 1) develop ways to assess infection risk in hospitalized patients and 2) develop non-antibiotic strategies that can restore immune competency in hospitalized patients.

Discoveries by our group and others have repeatedly shown that immunostimulatory molecules such as Toll-like receptor (TLR) ligands can reduce the risk of bacterial infection in mice if given as a prophylactic. These findings demonstrate

that TLR ligands modulate immunity in a manner that can change infection risk. Thus, by studying the impact of TLR ligands on immunity, one can uncover the factors that precipitate infection risk as well as develop novel therapeutics that change infection risk. In this dissertation, I leverage the ability of TLR ligands to modulate innate immune processes in order to achieve these goals.

To provide context for my experimental findings, **Chapter I** explores the history and epidemiology of nosocomial infection in the United States, with a particular emphasis on the currently available strategies to prevent nosocomial infection. Additionally, I detail the molecular and immunologic mechanisms that macrophages use to control invading pathogens and prevent infection. In **Chapter II**, I demonstrate that a method commonly used to assess innate immune competency, the magnitude of the pro-inflammatory cytokine response to lipopolysaccharide (LPS), does not predict host susceptibility to infection because it does not correlate with antimicrobial capacity. In **Chapter III**, I demonstrate that a clinically available TLR4 agonist, monophosphoryl lipid A (MPLA), induces persistent resistance to *S. aureus* infection by augmenting the antimicrobial capacity of tissue macrophages and neutrophils. In **Chapter IV**, I demonstrate that MPLA induces a novel and persistent metabolic phenotype in macrophages, and in **Chapter V**, I demonstrate how this metabolic phenotype supports persistent macrophage antimicrobial activities. In **Chapter VI**, I discuss the implications of my findings, limitations of the studies, as well as future directions. Overall, this work paves the way for more successful risk assessments of hospitalized patients, and outlines novel molecular strategies to improve resistance to infection.

Nosocomial Infection in the United States

Since the origin of hospitals in the United States, physicians have recognized that infections arise while patients are receiving care. Even prior to the introduction of germ theory, Dr. Ignaz Semmelweis, an Austrian obstetrician, noted in 1847 that the infection rate of women giving birth in the hospital was higher if the baby was delivered by a medical student rather than by a midwife[1]. Moreover, Dr. Semmelweis noted that a pathologist's hands, which could become contaminated during a morning autopsy, could infect a living patient undergoing a surgical procedure later in the day. Since the proliferation of hospital-based care, Dr. Semmelweis's observations have proven remarkably prescient. In the United States today, there are approximately 1.75 million nosocomial infections each year, occurring in about 1 out of every 20 hospitalized patients[2]. These infections go on to cause nearly 100,000 deaths as well as significant and largely unquantifiable morbidity, at an estimated cost of around $17-20 billion dollars annually[3,4].

Nosocomial infections arise due to the interaction between a patient and pathogenic microorganisms in the hospital environment. While Dr. Semmelweis's early observations demonstrated that exposure to pathogenic microorganisms is an important driver of infection, they also illustrated that simple exposure to pathogens fails to explain the epidemiology of nosocomial infection. Hospitalized patients are much more susceptible to pathogenic microorganisms than the hospital workforce, even though both groups are exposed to pathogenic microorganisms. The explanation for this discrepancy is that patients have a compromised barrier and immune functions and are thus unable to prevent pathogens from colonizing[5].

Unsurprisingly, the most common form of immune-compromise in the hospital is an iatrogenic breach of a normal tissue barrier, which provides easier access of microorganisms into a sterile space or cavity. Disruption of tissue barriers is often required for modern hospital care, and occurs routinely following catheter placement or surgical incision. In addition to a physical breach, standard medical care can also lead to immunological compromise. For example, patients undergoing organ transplantation require immunosuppressive drugs to maintain their transplant, and cancer patients often receive chemotherapeutic agents that cause appreciable immune dysfunction[6,7]. Beyond iatrogenic causes, some immunosuppression arises from the admitting condition itself. This can be due to skin breach, such as a wound or burn, or occur due to severe immune dysfunction experienced during a disease, such as human immunodeficiency virus (HIV) or sepsis[8,9]. Patients at the ends of the age spectrum, neonates and the elderly, are also susceptible to infections due to inadequately developed immune systems and the onset of immunoscenscence, respectively[10]. Regardless of the etiology, patients that develop nosocomial infection have a gross inability to prevent colonization and dissemination of pathogenic microorganisms. This immune compromise not only predisposes patients to infection, but also decreases their ability to contain infections, which could lead to dissemination, sepsis, and death.

To understand how to treat and prevent nosocomial infection, one must examine the ways in which these infections arise. Given the significant diversity of predisposing conditions that patients endure, nosocomial infections must first be categorized by their initiating location. The subtypes of nosocomial infection that occur most commonly

include surgical site infections, urinary tract infections, catheter associated bloodstream infections, and pneumonia (Figure I-1).

Surgical Site Infection

Surgical site infections (SSIs) are infections of the surgical wound that occur in around 2-5% of all surgeries in the United States, leaving between 300,000 and 500,000 patients with nosocomial infection each year[11]. Among all hospital patients, SSIs account for 18-20% of all nosocomial infections[12]. Once infected, SSIs drive significant patient morbidity and increase the cost and length of stay of hospitalized surgical patients[13]. SSIs are stratified by whether they occur within an organ or at the site of skin incision[14]. Skin incisional SSIs can be further categorized as superficial or deep. As SSIs are, by definition, caused by surgical intervention, they are the most prototypical iatrogenic nosocomial infection.

Arguably the greatest advancement in surgical care is the development of sterile practices during surgery (discussed below). Eliminating microbes from the operating room by patient decontamination, surgical staff decontamination, and changes in operating room airflow have been critical in permitting more invasive and longer surgeries[14]. However, despite strict decontamination practices, SSIs persist[15]. Two of the three most common surgeries that result in SSIs are small and large bowel surgeries, suggesting that the gut provides a difficult-to-control source of microbes for infection[16]. However, even in procedures involving sterile organs, such as coronary artery bypass graft, the rate of SSI is still significant[17].

In addition to microbe exposure, patients can be predisposed to develop SSI due to comorbidities that develop well prior to surgery or during the peri-operative period. In

2002, Malone et al. identified that patients with diabetes, low hematocrit, and ascites are at an increased the risk of SSIs compared to patients without these conditions[18]. Additionally, patients that receive a blood transfusion during surgery are much more likely to develop SSI[19]. This association is not thought to be an epiphenomenon, but due to the immunosuppressive effects of RBC transfusion[20]. Finally, surgery itself can induce profound changes to immune function that can predispose patients to infection[21].

Urinary Tract Infection

Urinary tract infections (UTIs) are infections of the bladder and urethra and are the most common bacterial infection[22]. Thus, it is no surprise that UTIs are the most common type of nosocomial infection, accounting for over 35% of all hospital acquired infections. UTIs occur in more than 1 million hospitalized patients in the U.S. each year, resulting in greater costs and hospital length of stay[23,24]. Nosocomial UTIs are almost always associated with the presence of an indwelling urinary catheter, which demonstrates the significant iatrogenic component of infection pathogenesis[25]. The urinary tract is a normally sterile site, suggesting that causative organisms must be inoculated from sites outside the urinary tract.

When a urinary catheter is inserted, perineal microbes on the patient or microbes on the hands of the health care worker can inoculate the catheter. These microbes colonize the extraluminal surface of the catheter and inoculate the surrounding urethral tissue, offering microbes a home within close proximity to the bladder[26]. Not all patients with a urinary catheter develop a UTI, and two of the largest predisposing factors are length of time with a catheter, as well as comorbidities such as diabetes and

malnutrition[27,28]. Comorbid severe immunodeficiencies also predispose patients to UTI, and patients with HIV/AIDS or multiple sclerosis have increased incidence of UTI[22].

Central Line-Associated Bloodstream Infection

Patients receiving hospital care frequently require insertion of an indwelling bloodstream catheter, also called a central line. Given the frequency with which central lines are inserted, central line-associated bloodstream infections cause around 10% of all nosocomial infections in the U.S., leaving over 250,000 patients with a life-threatening infection each year[29,30]. If a patient acquires a bloodstream infection, length of hospital stay can be extended between 7 and 25 days, which is associated with significant cost and morbidity to the patient[31]. Infections of a bloodstream catheter are almost always associated with bacteremia, which encourages the development of sepsis. The crude mortality rate from nosocomial bloodstream infections is around 27%[30].

Central lines are inserted through the skin, which is a major colonization site for *S. aureus* as well as other pathogens that can inoculate the portion of the catheter exposed to the bloodstream[32]. Strategies that limit the amount of bacteria that colonize the catheter, such as antimicrobial impregnated catheters and improved barrier precautions are helpful in reducing the incidence of infection[33], but infections persist despite these measures. The incidence of infection ranges from approximately 1% of ICU patients to 36% in patients receiving a bone marrow transplant[29,34]. As bone marrow recipients are grossly immunosuppressed, this finding again illustrates the impact of immunosuppression on the development of infection.

Ventilator Associated Pneumonia

Nosocomial pneumonia is an infection of the lung parenchyma that comprises over 11% of all nosocomial infections, making it the third most common type of nosocomial infection[35]. Nosocomial pneumonia can develop in any hospitalized patient, but is much most common in patients that are receiving mechanical ventilation[36]. The development of ventilator associated pneumonia (VAP) in mechanically ventilated patients increases the mortality rate of ventilator patients from 29% without pneumonia to 71% with pneumonia[37].

Factors that predispose patients to develop VAP derive predominantly from the course of clinical care. This is best illustrated by a 1999 landmark study by Drakulovic et. al demonstrating that ventilator patients with a semi-recumbent body position have a significantly reduced risk of VAP compared to patients in a supine body position[38]. Additionally, length of time on the ventilator strongly correlates with the development of infection, as do comorbidities such as preexisting lung disease, diabetes, or, age[37,39].

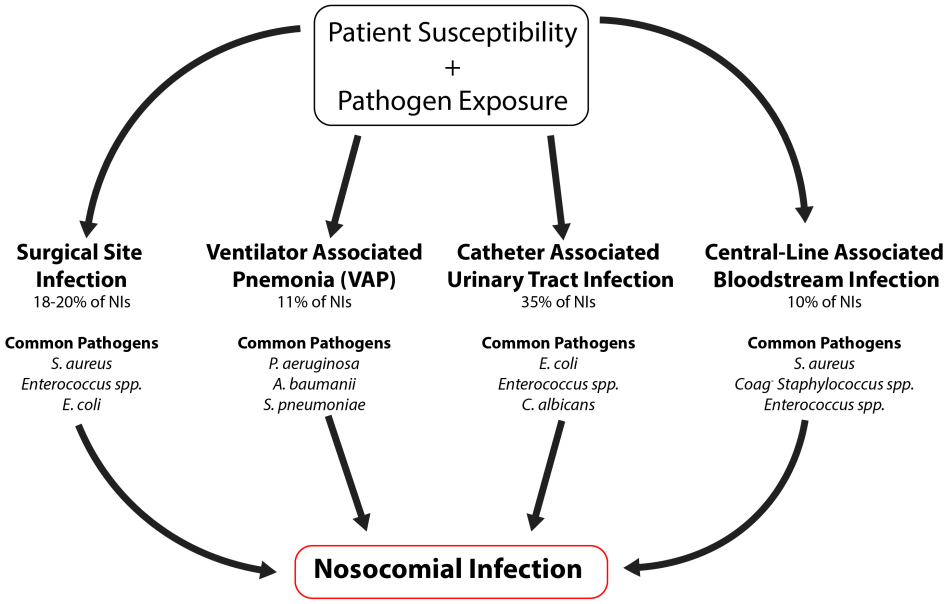


Figure I-1. Common Subtypes of Nosocomial Infection

Microbiology of Nosocomial Infection

There is significant diversity in the organisms that cause nosocomial infection. The organisms can vary due to factors such as the type of nosocomial infection or the organism prevalence in a given hospital. In addition, the most common causes of infection have changed over time. When antibiotic resistant nosocomial infection was first noticed, *S. aureus*, particularly strains with the penicillin resistant phage type 80/81, was the most common cause of nosocomial infection[40]. However, once penicillinase-resistant antibiotics were introduced in the 1950's, infections caused by Gram-negative organisms began to dramatically increase in prevalence[41,42]. By the late 1960's *Pseudomonas aeruginosa, Escherichia coli, and Klebsiella spp.* became the most common organisms of nosocomial infection[43]. Recently, methicillin resistant strains of *S. aureus* have expanded in prevalence so dramatically that *S. aureus* and other Gram-positive organisms are nearly just as common as Gram-negative isolates[14,44]. In addition, organisms that were previously not causes of nosocomial infection, such as *Acinetobacter baumanii*, *Enterococcus faecalis*, and *Clostridium difficile* have become important causes of nosocomial infection[45]. Causative organisms can also naturally vary by the type of procedure or immunosuppression that the patient endures[46]. VAP is most commonly caused by *P. aeruginosa, A. baumanii* or *S. aureus*[47]. SSIs as well as central line associated bloodstream infections are caused more commonly by *S. aureus*[32,48], but are also commonly caused by *Enterococcus spp*. and *E.coli*. Catheter-associated UTIs are most commonly caused by *E. coli*[14,49].

Treatment of Nosocomial Infection

Once a nosocomial infection develops, rapid treatment with an appropriate antibiotic is essential. A 2004 study by Kang et. al. found that when patients receive inappropriate antibiotics first, mortality rises from 27% to 38%[50]. Appropriate antibiotic treatment can be made once an organism is cultured and the antibiotic sensitivities of the causative organism are known. However, determining the causative organism is not always possible, and if it is possible, it often takes significant time and delays appropriate antibiotic selection. For this reason, many patients are initially started on expensive and toxic broad-spectrum antibiotics until the identity and sensitivities of the causative organism are known[51]. This type of broad-spectrum treatment, without knowledge of the causative organism or organism susceptibility, is known as empiric therapy. Due to the high prevalence of methicillin resistant *S. aureus* (MRSA), some of the most common empirically prescribed antibiotic are the glycopeptide antibiotic vancomycin, which has MRSA coverage, as well as aminoglycosides or cephalosporins. Even though they have broad coverage, these antibiotics can remain inadequate in killing the causative microorganism, prompting administration of additional broad-spectrum antibiotics[52]. Moreover, these antibiotics are toxic, and administration of overly aggressive antibiotics to patients that do not require such intensive treatment can actually worsen outcomes[53]. Improper empiric antibiotic therapy is a strong predictor of mortality in patients with a nosocomial infection[54]. Unfortunately, due to the rapid onset and severity of nosocomial infection, inadequate antimicrobial treatment is significantly more common in nosocomial infection compared to community-acquired infection[55].

If antibiotics are not immediately effective at treating the infection, sepsis may ensue. According to the Society of Critical Care Medicine in 2016, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[56]. Sepsis can also progress to septic shock, which includes life-threatening circulatory and metabolic abnormalities. Current management of sepsis is primarily supportive and involves maintaining patient blood pressure by administering intravenous fluids and vasopressors[57]. While supportive care significantly improves outcomes, overall mortality from sepsis remains around 29%[58].

Thus, despite the presence of antibiotics, supportive therapy, and advances in critical care, the incidence of and mortality from nosocomial infections have remained high. Moreover, it is unlikely that antibiotic treatment programs will improve in the coming years due to the dissemination of antibiotic resistance and the lack of new antibiotics. Therefore, in order to minimize the significant morbidity, mortality, and cost of nosocomial infection, it is imperative that we improve our strategies to combat the problem.

Prevention of Nosocomial Infection

Nosocomial infection arises from a combination of microbial exposure and patient susceptibility[59]. Therefore, the major strategies of infection prevention include reducing the microbial burden within the hospital and decreasing patient susceptibility. The mainstay of reducing microbial exposure is environmental disinfection[14]. Additionally, prophylactic antibiotics effectively target and eliminate pathogenic microbes before they can cause infection. While there has been significant debate over efficacy, it is generally believed that disinfection and prophylactic antibiotics have reduced the incidence of

infection over the past 100 years[60-63]. Strategies that reduce patient susceptibility are less broadly applied, and include identifying clinical situations, such as supine body position during ventilation or certain catheter insertion methods, that inappropriately precipitate infection[38,64]. However, many of the factors that increase patient susceptibility are a result of immunosuppression caused by preexisting comorbidities or routine patient care, so reducing patient susceptibility remains a major challenge. To date, there are no approved strategies that target and restore immune competency in susceptible patients, although recent trials with the administration of specific immunostimulatory cytokines such as granulocyte colony stimulating factor (G-CSF) and interferon-gamma are encouraging[65,66]. As a result, nearly all of the efforts to reduce the incidence of nosocomial infection target microbe exposure.

Decontamination Practices

In 2002 the World Health Organization released a practical guide to prevent nosocomial infection[67]. Disinfection of the hospital environment, such as disinfecting the medical staff, the patient, and the inanimate environment is the primary focus of the guide. Complete disinfection is challenging and costly, so disinfection practices are suggested to be stratified based on the susceptibility of the patient. Disinfection of the medical staff involves effective hand washing practices, which can range from "routine" for interactions with low-risk patients, or "surgical scrub" for a surgery case or with very high-risk patients[67,68]. Additionally, wearing masks, gloves, and appropriate clothing are effective in reducing the transmission of pathogenic microbes depending on the susceptibility of the patient. Environmental disinfection ranges from low-level disinfection, which uses a simple bactericidal disinfectant to clean objects that the patient

will be in contact with, to high-level disinfection, aimed at killing all microorganisms, used for objects that will be inserted into the patient or surrounding patients that are highly susceptible to infection. Patients themselves are often sources of nosocomial infection. For example, *Enterococcus* and *Staphylococcus* species have a propensity to exist on skin and are frequent causes of nosocomial infection. In 2013, Climo et. al found that washing patients in a chlorhexidine bath every day significantly reduces the risk of hospital acquired bloodstream infections[69]. In addition to disinfection, many hospitalized patients also receive prophylactic antibiotics designed to eliminate pathogenic microbes that escape disinfection and inoculate the patient.

Antibiotics and Antibiotic Resistance

After penicillin was introduced into clinical practice, it was quickly adopted as an effective treatment and prophylactic for patients in hospital settings. Antibiotics such as penicillin act to disrupt specific cellular functions that microorganism require for survival, and thereby stall the growth or kill microorganisms. Thus, while the patients remained immunologically susceptible to nosocomial infection, prophylactic antibiotics prevented the colonization and dissemination of microorganisms. There are numerous antibiotics that can be used as prophylactics, but the most common are beta-lactamase resistant cephalosporins such as cefazolin and fluorquinolones such as ciprofloxacin[70].

In the mid-1940's, almost immediately after the introduction of penicillin as a prophylactic antibiotic for wounded WWII patients, Drs. Mary Barber and Mary Rozwadowska-Dowzenko noticed a rapidly increasing incidence of hospital infection with *Staphylococci* species that were resistant to penicillin treatment[71]. At one hospital, they reported that the incidence of resistant *Staphylococcal* nosocomial infection had

risen from 14.1% to 38% of all hospital infections in one year. It is now recognized that widespread use of antibiotics perpetuates antibiotic resistance in many bacterial and fungal species[72]. Elimination of these resistant organisms requires more aggressive broad-spectrum antibiotic therapy, and, organisms eventually also become resistant to these broad-spectrum antibiotics[73]. Thus, antibiotic use breeds a positive feedback loop driving antibiotic use and resistance.

Since 1944, antibiotic resistance has blossomed, and resistance has been found in many additional microorganisms that cause nosocomial infection, including Gram-negative bacteria such as *Pseudomonas* and *Klebsiella* species as well as fungal microbes such as *Candida* species[74-76]. In 2005, methicillin resistant *S. aureus* alone caused around 95,000 infections and over 18,650 deaths[77]. Resistance to second and third line antibiotics have developed as well, and there have been reports of microorganisms that are resistant to all currently available antibiotics[78]. Microbial resistance to antibiotics is now one of the most pressing medical problems of modern society and in April 2014 the World Health Organization stated that the problem "threatens the achievements of modern medicine"[79].

Antibiotic resistance occurs due to an evolutionary response of microbial communities to antibiotic exposure. The first recognized antibiotic resistance developed to penicillin, which is a beta-lactam antibiotic that acts to disrupt DD-transpeptidase, also named penicillin-binding protein (PBP). PBP is the enzyme that forms cross-links in the bacterial peptidoglycan cell wall, which supports the cell integrity of nearly all bacteria[80]. Beta-lactams can disrupt this enzyme because they have structural similarity to the terminal amino acid on a newly forming peptidoglycan layer[81]. Once they become

incorporated into peptidoglycan they irreversibly acylate to a catalytically active serine site on the PBP, thereby permanently disrupting future cross-link formation[82]. Bacteria such as *S. aureus* rely on peptidoglycan, and thus beta-lactams are bactericidal[83].

Repeated and indiscriminate use of beta-lactams selects for organisms that can evade beta-lactam toxicity. One of the first mechanisms of acquired resistance to beta-lactams was identified as a *S. aureus* encoded beta-lactamase enzyme, which actively degrades beta-lactams before it inhibits PBPs[84]. Methicillin was then introduced in the 1950's as a beta-lactamase resistant antibiotic, and other beta-lactamase resistant antibiotics, such as cephalosporins, have since been introduced. However, in 1981 *S. aureus* evolved a new type of PBP encoded by the bacterial gene *mecA*, called PBP2a, that acylates to beta-lactams very slowly. Thus, at therapeutic concentrations, even beta-lactamase resistant antibiotics are ineffective at inhibiting peptidoglycan synthesis[85]. To date, there are no beta-lactam antibiotics that can kill bacteria if they express PBP2a.

In addition to the spontaneous development of resistance, antibiotic resistance genes can be transmitted from bacteria within the same species, or even to compatible bacteria in other species[86]. This lateral gene transfer is likely responsible for the rapid spread of antibiotic resistance. The methicillin resistant gene *mecA* is found on a transmissible element in *Staphylococcus* species, called the *Staphylococcal* cassette chromosome (*SCCmec*), this element has been spread through lateral gene transfer[87,88]. Even non-pathogenic commensal bacteria can develop resistance to antibiotics and eventually transfer this resistance to pathogenic bacteria, such as occurred in the development of methicillin resistant *Neisseria* species[89].

The development of antibiotic resistance is simply a matter of time, and the best defense against antibiotic resistance is to decrease antibiotic use, decrease hospital length-of-stay, and decrease infections by reducing pathogen exposure[90]. Reducing antibiotic use is quite challenging, as antibiotics are often inappropriately prescribed for viral infections, and are present at low doses in animal feed throughout the United States[91]. Prophylactic antibiotics are also particularly instrumental in the propagation of antibiotic resistance, as they are used regardless of whether patients have an infection. It is unlikely that prophylactic antibiotic use will decrease given the current standard of hospital care. However, there are two major strategies that can be employed to reduce prophylactic antibiotic use. First, if the infection susceptibility of the patient is known, patients can be triaged based on their risk of developing an infection. This way, aggressive prophylactic antibiotic therapy can be reserved for the most at-risk populations. Second, non-antibiotic interventions can be developed to reduce the intrinsic susceptibility of the patient. To accomplish either of these strategies, the factors that determine susceptibility to infection must be known. However, specific cellular and molecular processes that are responsible for infection susceptibility or resistance are unclear, making assessments difficult to validate and immunoaugmentation therapies difficult to develop.

Current Strategies to Assess Infection Susceptibility

Due to the cost of disinfection and the need to limit the use of antibiotics, patients must be stratified by their risk of infection. The World Health Organization recommends stratifying patients by their degree of immunocompromise as well as the procedures that they are undergoing[67]. For example, the World Health Organization considers lowest risk

patients as those that are not immunocompromised and are engaging in non-invasive procedures, while the highest risk patients are severely immunocompromised, defined by a white blood cell (WBC) count of less than 500 WBC/ml, and are undergoing high-risk invasive procedures. Within the category of invasive procedures, such as surgery, risk of infection can be stratified based on whether wounds are classified as clean, clean-contaminated, contaminated, and dirty-infected[59].

While the quantity of leukocytes in the blood is important to immune function, these rough guidelines leave appreciable room for misclassification. Immune cells can sustain functional deficits that can be missed by a simple leukocyte count. Most attempts to characterize these functional deficits have explored elicited cytokine secretion by different leukocyte populations. For example, the adaptive immune system is often characterized by the ability of T-cells to secrete pro-inflammatory cytokines[92,93]. The innate immune system, most classically, has been characterized by the ability of blood monocytes to secrete pro-inflammatory cytokines, particularly tumor necrosis factor alpha (TNF-α), or by the ability of monocytes to up regulate surface expression of major histocompatibility complex (MHC) proteins and co-stimulatory molecules[94,95]. There are no established ways to assess neutrophil function beyond a characterization of neutrophil number, even though neutrophil function can be modulated in ways that change their efficiency of fighting infections[8,96].

While assessments of cytokine secretion are simple to acquire it is unclear whether they can predict infection susceptibility. Most studies simply assume that reduced elicited cytokine secretion indicates suppressed immune function but do not actually assess this assumption[21,94,97]. There have been studies that have attempted to

correlate cytokine expression levels with clinical outcomes. For example, Pena et al (2014) demonstrated that an endotoxin tolerance signature, indicative of reduced pro-inflammatory cytokine secretion, predicts disease severity and organ dysfunction[98]. Additionally, Hall et al (2011) showed that reduced blood TNF-α secretion in hospitalized children was associated with nosocomial infection[99]. These correlations make intuitive sense, as monocyte and T-cell cytokine responses are essential for coordinating immune responses[100]. However, sepsis pathologies can arise due to excessive cytokine secretion, and reduced cytokine secretion may actually be beneficial during severe infection[101]. As such, there have been studies demonstrating that cytokine secretion does not predict clinical outcomes. For example, Drewry et al (2016) demonstrated that blood TNF-α assessments do not predict outcomes of sepsis[102]. In general, clinical immune assessments that rely on cytokine secretion are correlative. Further, there are no studies that have demonstrated a mechanistic link between reduced cytokine responses and nosocomial infection. Monocyte HLA-DR expression may have more predictive value but further studies are needed to validate it as a reliable biomarker[102]. In contrast, studies have identified that neutrophil dysregulation precedes nosocomial infection up to 5 days in advance[103], but these strategies have not been widely adopted because neutrophil phenotyping is more complex than measuring cytokine secretion[8,104].

Summary of Nosocomial Infection

Overall, nosocomial infection is a significant contributor to morbidity and mortality in the United States. Prevention is the best way to reduce mortality, however, current strategies of pathogen elimination are stalled due to antibiotic resistance. Further,

there are no targeted interventions that improve immune competency in susceptible patients. To reduce the incidence of nosocomial infection, we must 1) improve ways to determine patient infection risk and 2) develop strategies that restore patient immune competency. To determine the factors that predispose patients to infection, and correct these factors, the pathogenesis of severe infection must be understood.

The Pathophysiology of Infection

Pathogenic microorganisms can cause a range of infection severities, from clinically undetectable to sepsis with gross clinical pathology[105]. The severity of disease is determined by a complex interplay between pathogen and host[106,107]. While specific host or microbial factors can dramatically change the course of any given infection, there are general processes that determine the course of microbial infection in humans.

If the pathogen is easy to control, macrophages, or early recruited neutrophils, will orchestrate an inflammatory response by secretion of pro-inflammatory cytokines and chemokines (discussed further below). These leukocytes will also directly contain and kill the pathogen by release of neutrophil extracellular traps (NETs) and phagocytosis[108]. However, if pathogens evade early destruction they will continue to grow and spread, and microbial products on the pathogen surface will trigger endothelial cells in local capillaries to secrete more pro-inflammatory cytokines[109]. When secreted in high quantities locally, pro-inflammatory cytokines also induce physiologic blood vessel dilation to facilitate the recruitment of additional neutrophils and monocytes to the site of infection. Pro-inflammatory cytokines that leak into the bloodstream also induce mild systemic effects, including fever and sickness behavior. These physiologic, cytokine-

mediated, responses manifest as swelling, pain, warmth, and redness, typically considered the classic signs of inflammation.

As an uncontrolled infection progresses, the microorganism may eventually invade the bloodstream. If the pathogen is well suited for living in blood, such as *S. aureus*, it will drive excessive pro-inflammatory cytokine release and bloodstream inflammation, also known as "cytokine storm"[110]. Cytokine storm can dysregulate physiologic processes such as parenchymal cell metabolism, the complement system, and the coagulation cascade. As dysregulation of these processes worsens, multi-organ dysfunction, a condition in which many bodily organs are stunned, can develop[111,112]. Additionally, endothelial cells will increase expression of tissue factor, which can precipitate inappropriate intravascular coagulation[113]. If coagulation is excessive, clots can form within intact vessels and clog blood flow to many organs simultaneously, a condition called disseminated intravascular coagulation (DIC)[114]. Cytokine storm can also drive systemic blood vessel dilation to become severe, leading to profound hypotension and distributive shock[115]. Finally, as chemokines become widely distributed at high concentrations, neutrophils and monocytes will be unable to find the source of infection[116]. Death will occur if the hypotension and multi-organ dysfunction are severe enough. Uncontrolled infections can progress to sepsis within hours to days, making it particularly difficult for therapeutic interventions to improve sepsis outcomes. To date, no targeted therapeutics have been approved for the treatment of sepsis[117].

Overall, to prevent nosocomial infection and sepsis, the pathogen must be contained early. As the macrophage is the first leukocyte to detect, respond to, and

contain pathogens, macrophages are ideal targets for therapies aiming to improve early pathogen containment.

Macrophages

All bodily organs contain long-lived, resident, amoeboid-like macrophages[118]. Macrophages are mobile leukocytes that search for and repair disruptions to organ homeostasis, such as the presence of a pathogen in a sterile environment. Macrophages were first described by Dr. Elie Metchnikoff in the late 1800's when he noticed that starfish larvae contained microscopic cells that would try to eat away at foreign objects that penetrated the surface of the larvae[119]. Metchnikoff called these cells phagocytes or "eating cells", and went on to prove that these cells could eat many targets, from dead host cells to pathogenic microorganisms. Thus, from the earliest discoveries of macrophages it was appreciated that they had a significant diversity of function.

In the 1960's, van Furth and Cohen demonstrated that macrophages arising at sites of inflammation are derived from circulating blood monocytes[120]. However, it has recently become clear that most tissue resident macrophages are derived from yolk sac or fetal liver progenitors at birth[121,122]. These tissue resident macrophages are specialized in maintaining homeostasis of the organ in which they reside. Because each organ requires different basal processes to maintain homeostasis, macrophages are appreciably different depending on their organ of origin. For example, splenic macrophages are specialized in phagocytosis of dead red blood cells that can accumulate within the spleen, and brain macrophages, also called microglia, are responsible for neuron pruning[123,124].

Macrophage Activation

Beyond these diverse homeostatic processes, all macrophages can execute a set of shared inducible functions that are induced by common perturbations to homeostasis (Figure I-2). Inducible macrophage functions are commonly characterized on a spectrum between pro-inflammatory and anti-inflammatory[125]. Pro-inflammatory macrophages, also called classically activated or M1 macrophages, are induced by microbial products like lipopolysaccharide (LPS) and cytokines like interferon-gamma (IFN-γ), whereas anti-inflammatory macrophages, also called alternatively activated or M2 macrophages, are induced by IL-4[126,127]. Indeed, these polarizing stimuli drive macrophages to secrete large quantities of either pro-inflammatory or anti-inflammatory cytokines. However, most inducible macrophage functions do not fall within these strict classifications, and are exhibited by both pro- and anti-inflammatory macrophages. For example, phagocytosis is induced in pro-inflammatory macrophages to facilitate pathogen clearance, but phagocytosis is also induced in anti-inflammatory macrophages to facilitate efferocytosis[128,129]. In addition, macrophages integrate signals from the environment that can change their response to these stimuli. For example, comparable doses of LPS induce only a mild pro-inflammatory phenotype in alveolar macrophages, but a profound pro-inflammatory phenotype in peritoneal macrophages. As such, macrophages should be thought of as dynamic and diverse leukocytes that can integrate both external and internal signals and respond to homeostatic disturbances as needed.

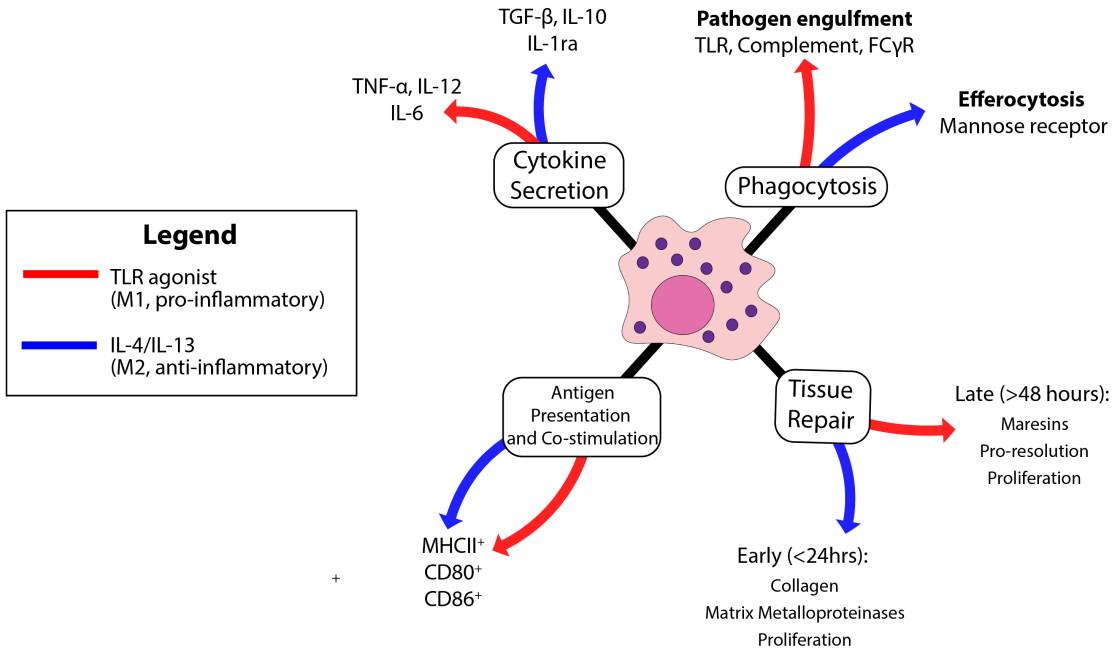


Figure I-2. Inducible Macrophage Functions

Schematic representing major categories of inducible macrophage functions. Schematic depicts the specific manifestations of these functions in pro-inflammatory macrophages (M1, red lines) and anti-inflammatory macrophages (M2, blue lines).

To detect the presence of pathogens, macrophages contain pattern recognition receptors (PRRs) that identify conserved motifs found on microbes[130]. One of the most highly expressed and sensitive classes of PRRs includes the Toll-like receptors (TLRs). TLR ligation initiates a series of events that aim to clear the detected pathogen and return the region to homeostasis. TLR ligation induces macrophage to produce pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β[131]. In addition to these cytokines, macrophages secrete chemokines to recruit neutrophils, monocytes, and lymphocytes. Secreted chemokines will diffuse and form a gradient, and leukocytes, which mobilize in the direction of progressively increasing concentrations of chemokine, will be recruited to sites of infection[132]. In general, neutrophil recruitment precedes

monocyte and lymphocyte recruitment[133]. Neutrophils express the chemokine receptors CXCR1 and CXCR2, and macrophages secrete the CXCR1/CXCR2 binding CXC-motif containing chemokine IL-8, or the mouse homologues CXCL1 (KC) and CXCL2 (MIP-2)[134,135]. To recruit monocytes, or to recruit other tissue macrophages to the infection, macrophages secrete chemokines that bind the chemokine receptors CCR1, CCR2, or CCR5. These include the CC-motif containing chemokines MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5), and MCP-2 (CCL8)[136]. T and B cells also express these chemokine receptors, so this process primes adaptive immune responses.

After recognizing environmental stimuli and secreting cytokines, macrophages do not die, but rather continue to regulate the response to infection. This is best evidenced as macrophages are also essential for bacterial clearance, antigen presentation, and tissue repair after infection[118,137]. If the adaptive immune system has previously responded to the detected pathogen, memory T- and B- lymphocytes will initiate a robust and early adaptive immune response[138]. However, if the adaptive immune system does not recognize the pathogen, it is the responsibility of the innate immune system to eradicate the pathogen until the adaptive immune system becomes activated, which occurs around 7-14 days later[139]. Macrophage processes that mediate bacterial clearance include engulfment of extracellular pathogens by phagocytosis and endocytosis, and killing of pathogens by acidification, oxidative stress, or nitrogen stress.

Macrophage Phagocytosis and Microbial Killing

Central to the ability of macrophages to clear extracellular microorganisms is the capacity to engulf them and kill them. This process occurs through a series of steps that involve recognition of a pathogen, cytoskeletal rearrangements to engulf the pathogen

inside of a vacuole termed the 'phagosome', and activation of targeted killing mechanisms[140]. Macrophages can directly recognize pathogens using innate PRRs, such as TLRs, scavenger receptors, and mannose receptors[141]. Identification of pathogens or other molecules can be facilitated by the attachment of proteins such as antibodies or complement to the pathogen, a process known as opsonization[140]. If a pathogen is coated in antibody, the Fc portion of the antibody can bind Fc receptors, such as Fc gamma receptor 1, expressed on the surface of macrophages[142]. Fc receptor ligation results in phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) on the Fc receptor cytosolic tail, which initiates profound actin-mediated cytoskeletal rearrangements[143]. Complement coated pathogens bind complement receptors, such as the transmembrane protein complement receptor 1 (CR1), and induce protein kinase C activation, which in turn induces cytoskeletal rearrangements that result in engulfment.

Once a pathogen is internalized, the phagosome will begin to mature. The phagosome first interacts with endosomes to form an early phagosome that contains a high concentration of the trafficking Rab GTPase Rab5. Rab5-positive phagosomes recruit phosphoinositide-3-kinases (PI3Ks), which are serine/threonine kinases, to the phagosome membrane. PI3K phosphorylates the lipid phosphoinositide-2-phosphate (PIP_2) embedded in the phagosome membrane to form phosphoinositide-3-phosphate (PIP_3). Once formed, PIP_3 recruits additional proteins such as the NADPH oxidase and inducible nitric oxide (iNOS), to the phagosome. NADPH oxidase pumps reactive oxygen species (ROS) such as superoxide into the phagosome and iNOS pumps nitric oxide (NO) into the phagosome[144]. TLR activation further drives early reactive oxygen and reactive nitrogen burst[145,146]. Eventually the early phagosome will shed Rab5 and

acquire Rab7, in a process mediated by the protein Mon1. Mon1 is able to intrinsically displace Rab5 from the membrane, and recruit Rab7 binding proteins such as Ccz-1[141]. Once on the membrane, Rab7 drives the recruitment of proton pumps such as the vacuolar ATPase (V-ATPase) as well as the cystic fibrosis transmembrane conductance regulator to the phagosome[147-149]. V-ATPases consume ATP to pump H^+ across the phagosome membrane and acidify the phagosome to around pH 5.5. The combination of NADPH oxidase-induced oxidative stress and V-ATPase acidification is destructive and can denature pathogens in the phagosome. Pathogens have evolved a diversity of strategies to try and avoid phagosomal killing, but strong TLR activation or macrophage activation by external cytokines such as IFN-γ can significantly increase the quantity of toxic mediators, which can overwhelm many initially evasive pathogens[100].

If pathogen clearance is effective, macrophages are tasked with cleaning and repairing the local environment. Recruited neutrophils die rapidly at sites of infection and release their toxic mediators into the local environment, causing host tissue damage[150]. Macrophages are responsible for resolving this neutrophil-mediated damage after it has occurred[151]. In addition to clearing out the neutrophilic debris, macrophages recruit fibroblasts and endothelial cells by secreting growth factors like transforming growth factor alpha and vascular endothelial growth factor[152]. Macrophages cleave previously secreted chemokines using matrix metalloproteinases, thereby creating anti-inflammatory molecules that help resolve the inflammation[153]. Additionally, macrophages produce lipid mediators, such as resolvins and maresins, that further promote anti-inflammatory wound healing and organ protection[154].

In sum, macrophages are highly specialized plastic cells that play a role in all parts of infection. They have the ability to initiate specific processes in a temporal manner, actively participate in these processes, as well as recruit specialized cells to help with the goal at hand. Macrophage function is profoundly modulated by TLRs, and TLR ligation is known to drive more efficient bacterial killing. Thus, TLR agonists may be useful in clinically modulating macrophage antimicrobial function to promote early pathogen clearance and prevent severe infection and sepsis.

Toll-like Receptors and TLR-induced Metabolic Reprogramming

In the early 1980's Toll genes were recognized by Drs. Katherine Anderson and Christiane Nüsslein-Volhard as important proteins for the generation of dorsovental polarity during Drosophila development[155,156]. In 1991 Drs. Nicholas Gay and Fionna Keith recognized that the structure of the Drosophila toll protein was remarkably similar to the mammalian IL-1 receptor, suggesting it may have a role in immune function[157]. In 1996 the mammalian homologues of Toll, TLRs, were confirmed to associate with mammalian immune function, when Drs. Ruslan Medzhitov and Charles Janeway, Jr. demonstrated that ectopic overexpression of the TLR genes caused NF-kB activation and an up-regulation of pro-inflammatory cytokine genes[158]. While many additional scientists contributed to early TLR research, TLR4 was finally identified as a PRR in 1998 when Dr. Bruce Beutler demonstrated that the spontaneously mutated C3H/HEJ mouse strain, which is unable to respond to LPS, exhibited a missense mutation in its TLR4 gene[159] This discovery was recognized by the 2011 Nobel Prize in Physiology or Medicine. This finding then gave way to the identification of a variety of mammalian TLRs that currently

includes 13 members TLR1-13, each of which can respond to a variety of microbial and non-microbial ligands[160].

TLRs are type-I integral membrane glycoproteins that have an extracellular domain with a leucine-rich repeat (LRR) and an intracellular domain with a Toll/IL-1 receptor (TIR) domain[161]. Despite structural similarity, the genes for each TLR map to different places in the genome, although they can be grouped into super-families. TLRs are localized in one of either two cellular locations: cell surface or endosomal. TLRs 1, 2, 4, 5, and 6 localize to the cell surface, whereas TLRs 3, 4, 7, 8, and 9 localize to the endosome. TLR4 can localize to both the cell surface and the endosome; monomeric TLR4 is found at the cell surface, but once dimerized will traffic to the endosome.

TLR4 uses a variety of adaptor and shuttling proteins to help bind ligands such as LPS (Figure I-3). Lipopolysaccharide binding protein (LBP) is a soluble plasma protein that forms high affinity stoichiometric complexes with the lipid A moiety of LPS[162]. Once bound to LPS, LBP can bind to CD14[163]. CD14 is a glycophosphotidylinositol-linked glycoprotein that has a hydrophobic binding pocket for LBP. The hydrophobic pocket of CD14 is quite large, allowing it to accommodate a wide variety of ligands[164]. Once CD14 binds LBP, it can transfer the LPS molecule to MD-2 a soluble protein that contains another large hydrophobic pocket for LPS[165,166]. Due to this redundant LPS binding scheme, the TLR4/MD-2 complex can exhibit CD14-dependent or independent binding, although CD14-dependent binding is significantly more effective in activating TLR4[167]. Once MD-2 binds LPS, it associates with the TLR4 receptor, effectively dimerizing or oligomerizing TLR4 on the cell surface. This induces a conformational

change that brings two cytosolic TIR domains close enough to bind intracellular signaling proteins[168].

All TLRs contain a TIR domain on their cytoplasmic tail. Once the receptor is dimerized, these TIR domains each bind one of two intracellular TIR domain containing adaptor proteins: myeloid differentiation factor 88 (MyD88) and TIR-domain containing adaptor-inducing interferon-beta (TRIF). There are additional TIR domain containing proteins, such as MAL, TRAM, and SARM, but these simply act as bridging molecules that encourage or discourage binding of MyD88 or TRIF[168]. TLRs 1, 2, 4, 5, 6, 7, 8 and 9 activate MyD88-dependent signaling whereas TLRs 3 and 4 can activate TRIF-dependent signaling. TLR4 is the only TLR that is known to adapt to both MyD88 and TRIF[169]. The activation of MyD88 or TRIF induces a specific signaling cascade that results in transcription factor activation. MyD88 signals through a IRAK/TRAF6 complex that eventually results in the phosphorylation of IKK and activation of NFκB. Additionally, the transcription factor AP-1 is activated through MyD88-dependent activation of MAP kinases such as p38, JNK, and ERK. TRIF signals through a RIP1/TRAF6-mediated process that also results in NFκB and AP-1 activation. TRIF-induced signaling cascades also activate TRAF3, which phosphorylate and activate the viral associated transcription factor IRF-3. While both MyD88 and TRIF activate NFκB, the TRIF-mediated RIP1/TRAF6-dependent activation of NFκB appears to be less potent than IRAK/TRAF6-dependent activation of NFκB[169]. Additionally, TRIF is only recruited to TLR4 after receptor internalization, whereas MyD88 signals from the cell surface, so TRIF-dependent signaling is delayed compared to MyD88-dependent signaling.

NFκB, AP-1, and IRF3 drive the transcription of immunologically relevant mRNA, including pro-inflammatory cytokines, chemokines, phagocytosis and antimicrobial proteins, and cell adhesion proteins. The collective transcription factor response can be identified by the presence of certain cell surface, secreted, and intracellular molecules, such as IKK and IRF3 phosphorylation, TNF-α and IL-6 secretion, and co-stimulatory molecule expression[170]. In addition to activating transcription of immunologic proteins, TLRs also profoundly reorganize macrophage metabolism. There are diverse molecular strategies by which TLR ligands reprogram macrophage metabolism; however, one of the most well characterized TLR-induced metabolic signaling networks is the PI3K/mTOR/HIF-1α signaling axis.

The PI3K/mTOR/HIF-1α Signaling Axis

TLR4 activates proteins that induce allosteric regulation of metabolism as well as transcription factors that transcribe metabolic genes. Most implicated in these processes is PI3K, the membrane-associated kinase that phosphorylates PIP_2 to PIP_3 (Figure I-3). It is not entirely clear how TLRs activate PI3K, but two research groups have reported that B-cell adaptor protein (BCAP) recruits PI3K to TLR4 and induces PI3K activation[171,172]. Once activated, PIP_3 recruits proteins that contain pleckstrin homology domains to the surface, most notably the kinase Akt. Once localized to the cell membrane, Akt is phosphorylated and can phosphorylate a wide variety of proteins, including tuberous sclerosis complex 1 and 2 (TSC1/TSC2). TSC1/TSC2 typically restrains the activation of the mechanistic target of rapamycin complex 1 (mTORC1) via inhibition of the GTP-binding protein Rheb. Yet, once Akt phosphorylates TSC1/TSC2, TSC1/TSC2 becomes

inhibited and Rheb can activate mTORC1[173].

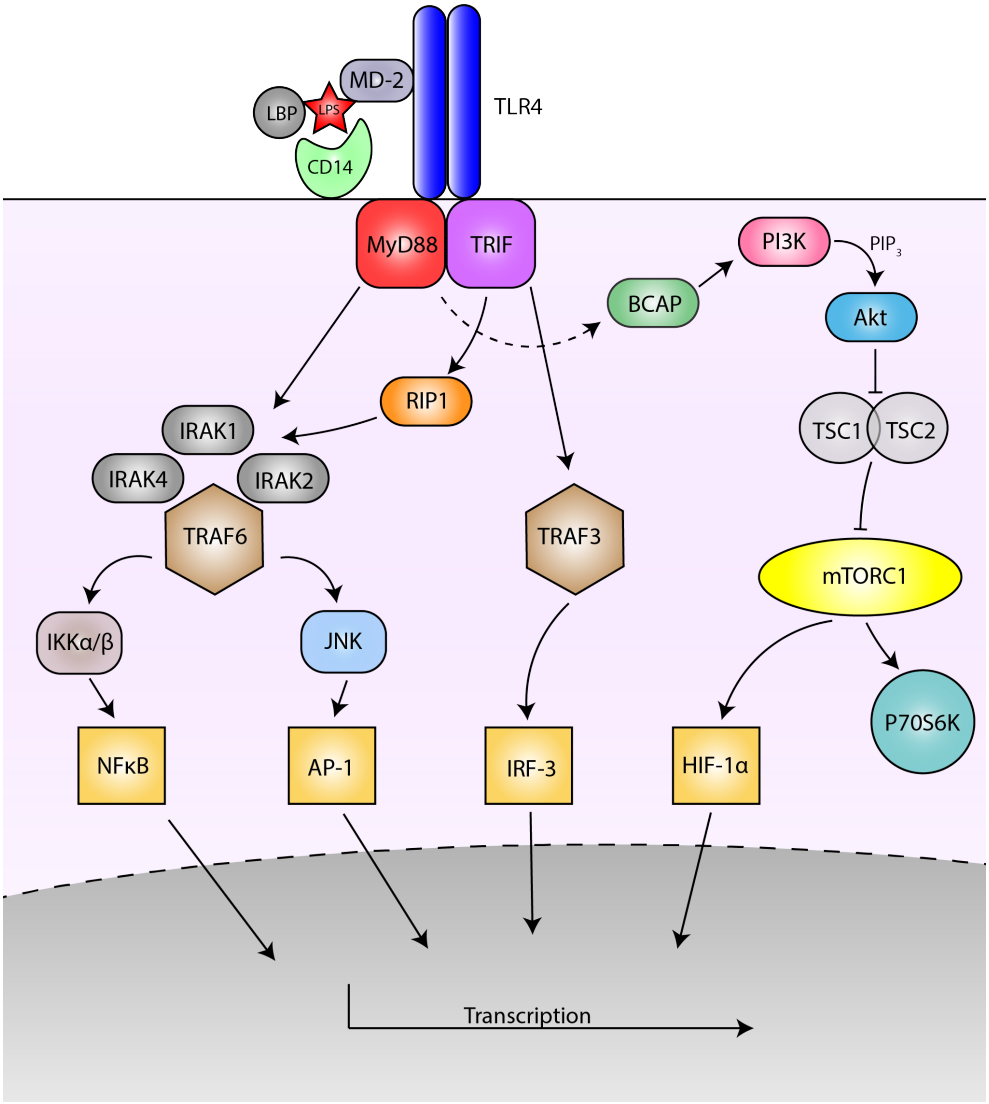


Figure I-3. Toll-like Receptor 4 Signaling

Schematic of the known signaling cascade that occurs after LPS is bound to the TLR4/MD-2 complex. Figure inspired by O'Neill, Golenbock, & Bowie. *Nat. Rev. Immunol*. (2013)[174]

The mTORC1 is comprised of five proteins: the serine/threonine kinase mTOR, which is the primary catalytic protein, Raptor, mLST8, PRAS40, and Deptor[175]. The functions of the complex proteins beyond mTOR are not well established, however they

are grossly characterized as regulating the catalytic activity of mTOR. Once mTORC1 is activated, it functions to promote a variety of anabolic processes in the cell. These include protein synthesis, lipid synthesis, and the generation of new organelles such as mitochondria. In order to promote protein synthesis, mTOR activates ribosomal proteins P70S6K and S6, which control ribosomal protein translation as well as the generation of new ribosomes[176]. mTOR also activates transcription factors such as sterol regulatory element-binding proteins (SREBPs) to initiate lipid biosynthesis, and can activate mitochondrial biogenesis through regulation of the 4E-BPs and activation of the transcriptional co-activator PGC-1 α and the transcription factor YY1[175,177,178]. Additionally, mTOR can inhibit catabolic processes in the cell such as fatty acid oxidation and organelle autophagy[179,180].

Metabolic substrates are required for the synthesis of macromolecules, and mTOR has the ability to detect cellular metabolite levels and positively regulate nutrient uptake pathways, particularly glucose consumption. For example, mTOR can sense the concentration of amino acids leucine, arginine, and glutamine, through a yet undiscovered amino acid sensing mechanism[181,182]. If sufficient metabolic substrates are detected mTOR positively regulates glycolysis, largely through activation of HIF-1α[180,183].

HIF-1α is a transcription factor that can induce expression of proteins required for glucose transport such as the glucose transporter GLUT1, and glycolysis such as hexokinase-2 (HK-2), phosphofructokinase-2 (PFK-2), and pyruvate dehydrogenase kinase-1 (PDK-1)[184-186]. Under normal conditions, HIF-1α abundance is controlled by oxygen sensing 2-oxoglutarate (alpha-ketoglutarate)-dependent dioxygenases such as

prolyl-4-hydroxylase proteins (PHDs). PHDs require oxygen for their hydroxylation action, and if HIF-1α is hydroxylated by PHDs, it will be degraded by the proteasome. Thus, under conditions of reduced environmental oxygen, PHDs will be unable to act, and HIF-1α activity will be unleashed[186]. Interestingly, HIF-1α activation can also occur during normoxia through a variety of mechanisms[187]. For example, succinate accumulation in the cytosol, which can occur in situations of altered cellular metabolic activity, directly inhibits PHDs, thereby stabilizing HIF-1α[188,189]. Additionally, reactive oxygen species inhibit PHDs, and, when produced in great amounts, will promote HIF-1α stabilization[190].

In summary, TLR activation profoundly reorganizes cellular metabolism, largely through the activation of PI3K and mTOR. The reason for this is that macrophages require alterations in metabolic composition to accomplish different tasks[191]. The collective change in cellular metabolism, from one metabolic state to another, is called 'metabolic reprogramming'[192]. The specific ways in which TLR signaling reprograms macrophage metabolism have only recently begun to be uncovered.

Macrophage Metabolic Reprogramming

Consumed glucose can be utilized by a plethora of metabolic pathways. The carbon from glucose can be used for the generation of cellular building blocks by fueling fatty acid synthesis, nucleotide synthesis, or amino acid synthesis. Additionally, glucose can be converted into pyruvate via glycolysis. Pyruvate can be transported into the mitochondrial tricarboxylic acid (TCA) cycle where it fuels the production of NADH and $FADH_2$, or it can be converted to lactate. In addition to glucose, cells can metabolize additional substrates, such as glutamine or fatty acids, to also support NADH and $FADH_2$

generation. The mitochondrial electron transport chain can utilize the electrons donated by NADH and $FADH_2$ to form a proton gradient across the inner mitochondrial membrane. The gradient then flows through ATP synthase, and generates ATP. In order to neutralize the hydrogen flow from ATP synthase, the mitochondria consume oxygen to create water. This prototypical metabolic programming can change depending on cellular requirements or metabolite restriction. For example, if oxygen is limited or rapid ATP generation is needed, cells can switch to anaerobic metabolism by running glycolysis more quickly, thereby generating ATP[193]. These stress adaptations are typically short lived, and will revert to basal conditions once oxygen is restored.

One of the first persistent atypical metabolic phenotypes was identified in 1927 when Dr. Otto Warburg noticed that cancer cells exhibit a metabolism of profoundly increased glycolysis despite adequate environmental oxygen[194]. This metabolic phenotype, called aerobic glycolysis or the 'Warburg effect', allows cancer cells to proliferate with or without the presence of oxygen, leaving them adapted to many environments. Upon further study it was determined that cancer cells mostly utilize this increase in glycolysis to generate nucleic acids, fatty acids, and amino acids, for the generation of daughter cells[193].

Compared to leukocyte precursors in the bone marrow, mature macrophages have a slow growth rate[195]. However, it has been long appreciated that pro-inflammatory macrophages are more reliant on glycolysis than oxidative metabolism during periods of activation[196]. This inflammatory metabolic phenotype was initially thought to improve cellular fitness during hypoxia, but in 1995, it was demonstrated that aerobic glycolysis could actually change the pro-inflammatory response, and that hypoxia itself could

increase the production of LPS-elicited TNF-α[197,198]. This observation was expanded when, in 2003, Cramer et al demonstrated that macrophages lacking HIF-1α have reduced LPS-elicited TNF-α secretion, even in normoxia, compared to wild-type cells[187]. In 2010, Rodriguez-Prados et. al. confirmed that TLR ligands drive increases in glycolysis, even during normoxia, to support cellular activation[199].

The carbon derived from aerobic glycolysis is essential for pro-inflammatory activation for a number of reasons. For example, glycolysis-fueled NADPH production, via the pentose phosphate pathway, is the primary substrate for macrophage respiratory burst. Additionally, glycolysis fueled fatty acid synthesis is required for phagocytic processes[200,201]. In 2014, Pearce et al. demonstrated that glycolysis-derived fatty acids also make up the endoplasmic reticulum and Golgi apparatus that cells use to package and secrete cytokines[202]. Inhibition of glycolysis or loss of the primary glucose transporter GLUT1 profoundly decreases macrophage pro-inflammatory processes such as cytokine secretion and ROS generation[203,204]. Further, decreased reliance on the TCA cycle provides some advantages to pro-inflammatory macrophages. Notably, they are indeed able to traverse hypoxic environments more successfully and can utilize TCA cycle metabolites, such as succinate or citrate, for pro-inflammatory or antimicrobial purposes[188,205].

Anti-inflammatory stimuli alter macrophage metabolism as well, although these anti-inflammatory macrophages rely more heavily on mitochondrial metabolism. For example, IL-4 activated anti-inflammatory macrophages exhibit a STAT6 and PGC-1β driven metabolic phenotype of fatty acid oxidation and mitochondrial biogenesis[206]. While there is some debate about the functional relevance of oxidative metabolism for

anti-inflammatory macrophage polarization[207,208], this metabolic phenotype appears to be required for IL-4 induced arginase activity and can control diseases that are dependent on anti-inflammatory macrophages[209]. Phagocytosis and cytokine secretion are also functional outcomes of anti-inflammatory macrophages, and, although these cells are not engaged in aerobic glycolysis, glycolysis has been demonstrated to be required for anti-inflammatory macrophage polarization[210]. In general, it appears that pro-inflammatory macrophages are more reliant on glycolysis whereas anti-inflammatory processes are associated with mitochondrial function, however there can be significant overlap between these two polarization states[211].

Through PI3K and mTOR, TLR agonists reprogram macrophage metabolism to support the functional purposes of induced immunologic proteins. Thus, disrupting or modulating metabolic processes has a broad impact on macrophage function. When administered to a whole organism, TLR agonists induce immunologic and metabolic reprogramming on an organism wide scale, and, interestingly, change host responses to immunologic stimuli for a lengthy period of time. This makes them obvious candidates as immunomodulatory agents.

Persistent Modulation of Host Immunity By TLR Ligands

Among the TLR ligands, LPS is among the most potent and has been widely studied. Knowledge of bacterial endotoxins, such as LPS, extends into the 19th century. LPS was isolated by Lüderitz and Westphal, and named lipopolysaccharide due to the presence of carbohydrates and lipids, but no protein[212]. The most noticeable effect of LPS administration in humans was fever, so it was first recognized as a pyrogen in bacterial infection[213]. While toxic effects were commonly observed, other early studies indicated it

offered a therapeutic benefit[212]. In the 1930's, Dr. William Coley recognized that LPS could induce the remission of some human malignancies, a phenomenon that was later attributed to TNF-α release[214]. Further studies in 1956 by Maurice Landy and Louis Pillemer noted that animals exposed to LPS prior to infection had improved resistance to infection[215]. Despite attempts at using endotoxins for a myriad of medical conditions, and despite Drs. Bennett and Cluff's statement that, "there is no doubt regarding the beneficial action of endotoxin", many studies ran into trouble as LPS is toxic at even modest concentration[216,217]. Thus, all studies on LPS began to focus on its toxicity, and its ability to induce fever and a sepsis-like state[218]. Moreover, many early studies on LPS were not well controlled and studies on the therapeutic benefits of LPS were largely abandoned. However, these early studies demonstrated that LPS elicits persistent immunomodulation, even at sub-toxic doses. The two most well studied phenomena are endotoxin tolerance, and antibody formation. As this dissertation largely focuses on the role of endotoxin in innate immunity, the phenomenon of endotoxin tolerance will be discussed.

Endotoxin Tolerance

Physicians first noticed progressive tolerance to endotoxins in the 1920's after attempting to use it as a therapeutic[219]. However, it wasn't until 1947 that Dr. Paul Beeson performed the first definitive study on endotoxin tolerance, and demonstrated that animals exposed to sub-lethal doses of LPS experienced a state refractory to LPS-induced toxicity[220]. Today, endotoxin tolerance is more widely understood to be a state of reduced LPS-elicited pro-inflammatory cytokine secretion, particularly in myeloid cells, that is mediated by inducible protein inhibitors of TLR signaling[221]. Once induced,

endotoxin tolerance lasts approximately 2 weeks[220]. In macrophages, endotoxin tolerance occurs due to the persistent expression of proteins such as IRAK-M and SHIP-1[222]. IRAK-M is thought to be the primary mediator of tolerance in humans and animals[223,224]. IRAK-M prevents the dissociation of other IRAK molecules from MyD88, an event essential for formation of the IRAK-TRAF6 complex[224]. The expression of IRAK-M reduces TLR signaling, and, most characteristically, reduces activation of NFκB and cytokines that depend on NFκB for transcription[225], including TNF-α, IL-12, IL-6, IL-1β, CCL2, and CXCL10[221].

Even though endotoxin tolerant macrophages are refractory to LPS-elicited cytokine secretion, they are not anergic. Rather, they persistently express proteins that modulate immune activity, particularly antimicrobial function. For example, endotoxin tolerant macrophages and human monocytes have been observed to exhibit increased phagocytic ability as well as improved antimicrobial capacity[226,227]. It is not clear how LPS primes certain macrophage functions but suppresses others, but epigenetic modifications have been implicated as instrumental in this process[228]. Despite these additional phenotypic manifestations, most studies on endotoxin tolerant macrophages compare the pro-inflammatory cytokine response of LPS-stimulated control macrophages with LPS-stimulated tolerant macrophages, and few studies have focused on the phenotype and function of tolerant macrophages outside of repeated LPS exposure.

Potential beneficial effects of endotoxin tolerance

TLR agonist-primed endotoxin tolerant animals have improved resistance to infection. Why is this? Despite knowledge of this phenomenon for decades, the mechanism of protection is still speculative. Most studies that have observed resistance to

infection after LPS priming found that animals have reduced serum pro-inflammatory cytokine concentrations and improvements in antimicrobial ability in response to infection[229-231]. The reduction in pro-inflammatory cytokine secretion is commonly thought to be due to endotoxin tolerance. As pro-inflammatory cytokines are thought to be responsible for the development of sepsis and sepsis-like pathologies, endotoxin tolerance has been speculated as the mechanism of protection. However, improved leukocyte recruitment to sites of infection leads to efficient bacterial clearance that can also drive the cytokine burden down. While it is not entirely clear which phenomenon is more important to protection, studies from the Sherwood group have found that when granulocyte-colony stimulating factor (G-CSF) is blocked prior to TLR4 agonist administration, control and TLR4 agonist-primed animals have similar concentrations of serum cytokines during infection[232]. Thus, even though G-CSF neutralized animals are endotoxin tolerant, their cytokine burden is high because they are unable to clear the pathogen. This observation indicates that the augmentation of microbial clearance is likely the primary mechanism conferring protection after LPS priming.

Potential negative effects of endotoxin tolerance on infection

Despite the decades of research on the beneficial effects of TLR4 agonists, there is a significant body of literature that suggests that endotoxin tolerance is a persistent state of immunosuppression[8,93]. This view is derived primarily from an alternative interpretation of endotoxin tolerance. Cytokines are essential to immunity, so many assume that the inability to produce cytokines, such as that observed in endotoxin tolerance, is detrimental to immunity[233]. It has also been repeatedly observed that endotoxin tolerant monocytes cannot readily up-regulate MHCII complexes[234].

Furthermore, there are clinical studies that have correlated the development of endotoxin tolerance with worsened outcomes in humans in the ICU[95,98]. While these correlative studies are interesting, a cause and effect relationship between endotoxin tolerance and increased susceptibility to infection has not been established [235-237]. There are several other factors that have been shown to contribute to immune suppression in critically ill patients such as lymphocyte apoptosis, neutrophil dysfunction, and aberrations in lymphocyte function[8]. The contribution of endotoxin tolerance to immune suppression in critically ill patients had not been widely studied, since few investigators had evaluated the response of endotoxin tolerant animals to live infections. Overall, the experimental evidence suggests that TLR agonists improve infection outcomes, despite correlations with endotoxin tolerance and poor outcomes.

Natural TLR ligands

Nearly all microorganisms have conserved molecular structures that can be detected by TLRs[169]. For example, Gram-negative bacteria express the TLR4 agonist LPS which is a cell-surface molecule critical for cell wall integrity. Bacteria also contain peptidoglycan, a sugar that provides support to the bacterial cell wall, and this molecule is recognized by TLR2. Fungal pathogens contain the structural cell wall molecule β-glucan, which is also recognized by TLR2 as well as dectin-1. Bacterial DNA, which is rich is CpG motifs, can be recognized by TLR9. Viruses have significant diversity in their surface proteins and are recognized by their pathogenic nucleic acids, such as ssRNA and dsRNA, recognized by TLR7/8 and TLR3, respectively. In addition to the mentioned ligands, each TLR can recognize a variety of microbial products as well as endogenous proteins released by cells during injury or infection such as mitochondrial DNA and HMGB1[238]. This receptor

promiscuity allows for the development of synthetic molecules, which are analogues to these natural TLR ligands that can be used to activate innate immunity. These analogues allow for more tailored activation of TLRs depending on the desired response.

Monophosphoryl lipid A

The toxicity associated with LPS administration in humans precludes its use as an immunomodulator. To address that issue, in the 1980's Dr. Edgar Ribi developed a modified form of LPS, called monophosphoryl lipid A (MPLA)[239]. MPLA is generated by selective hydrolysis of the C1 phosphate group found on lipid A from naturally occurring *Salmonella spp.* LPS (Figure I-4)[239,240]. Despite the structural changes, MPLA remains a TLR4 ligand. However, MPLA has altered activity at the TLR4 receptor. Notably, MPLA elicits a greatly reduced pro-inflammatory cytokine response compared to LPS and is estimated to be 100-1,000 times less pro-inflammatory[241]. A recent paper published by the Sherwood group shows that much of the decreased pro-inflammatory activity is due to the inability of MPLA to activate the NLRP3 inflammasome whereas LPS is a potent activator[242]. Due to reduced induction of pro-inflammatory mediators, MPLA is less toxic than LPS[240]. As such, MPLA is safe to administer to humans and is used as an adjuvant in the hepatitis B and human papilloma virus vaccines[243,244].

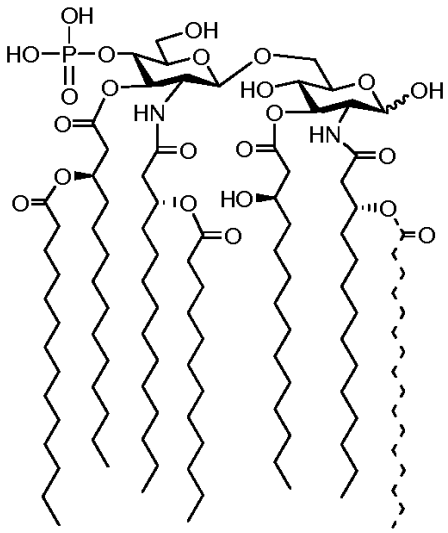


Figure I-4. The Structure of Monophosphoryl lipid A

Structure of MPLA derived from *S. minnesota*. Image derived from U.S. Patent WO2011014771 A1[245].

In addition to inadequate NLRP3 inflammasome activation, other mechanisms may also contribute to the decreased toxicity of MPLA as compared to LPS. Both Tanimura et. al. (2013) and Casella and Mitchell (2013) have found that MPLA does not efficiently dimerize the TLR4 receptor[246,247]. Mata Haro et. al. (2007) found that, while MPLA activates both MyD88 and TRIF, it has a greater propensity to activate TRIF pathways, whereas LPS predominantly activates MyD88[248]. As MyD88 and TRIF compete for TLR4 TIR domain, and MyD88 is a more potent inducer of NFkB, MPLA may less potently activate NFkB. Interestingly, MPLA and LPS compete for TLR4, and administration of MPLA may reduce the inflammatory response to LPS[249]. However, despite reduced toxicity, MPLA still induces an attenuated pro-inflammatory cytokine response after administration. Additionally, MPLA retains similar immunomodulatory activities as LPS, such as induction of endotoxin tolerance and B-cell antibody production[250].

Despite reduced toxicity, some of the earliest studies on the activity of MPLA found that it induces resistance to infection in a similar manner to LPS[251]. Those studies have been greatly expanded upon by the Sherwood group. We demonstrated that MPLA will protect against a variety of clinically relevant infection models, including a model of polymicrobial sepsis caused by cecal ligation and puncture as well as *P. aeruginosa* burn wound infection[232,252]. Similarly to LPS, MPLA prophylaxis improves pathogen clearance and reduces pro-inflammatory cytokine burden[252]. Thus, MPLA is a prime candidate for re-exploration as a TLR ligand with significant potential for use as an immunomodulator to augment host resistance against common nosocomial pathogens[253]. To date, MPLA has been used exclusively in the clinical setting as a vaccine adjuvant [254]. A greater understanding of the mechanisms by which MPLA improves innate host resistance to infection could unlock it for use as an immunomodulatory prophylactic to restore immune function in hospitalized patients.

Significance of Research and Summary of The Data

Hospital acquired infection is a major cause of morbidity and mortality in the United States. These infections are precipitated by the development of immunosuppression in hospitalized patients and compounded by the development of antibiotic resistance among common nosocomial pathogens. Identification of the processes that predispose patients to infection will allow for efficient stratification of patients by infection risk and lead to intelligent application of new prophylactic and therapeutic approaches. TLR agonists are particularly attractive as prophylactics due to their ability to augment innate antimicrobial functions, their efficacy in experimental models of infection, and their track record for safety as vaccine adjuvants in the clinical

setting. However, the molecular mechanisms by which TLR agonists augment innate resistance to bacterial infections are poorly understood.

In this dissertation, I use a variety of TLR agonists to show that the magnitude of the pro-inflammatory cytokine response to LPS challenge, a commonly used measure of innate immune function, is an inappropriate way to measure the potential host response to infection. Rather, cellular antimicrobial capacity is more predictive of infection susceptibility. Moreover, I identify the cellular and molecular mechanisms by which the TLR4 agonist MPLA improves host immunity to infection against *S. aureus* bloodstream infection. I find that persistent modulation of tissue macrophages is an essential process by which MPLA induces resistance. Interestingly, my studies identify that MPLA induces a novel and persistent mTOR-initiated metabolic phenotype in macrophages that helps them sustain heightened antimicrobial function and persistent chemokine secretion despite reduced pro-inflammatory cytokine secretion. Moreover, I demonstrate that induction of this metabolic phenotype is essential for MPLA-induced resistance to infection. Through the utilization of *in vivo* and *in vitro* models, these studies significantly advance both the mechanistic study of nosocomial infection and our understanding of macrophage biology.

CHAPTER II

THE CYTOKINE RESPONSE TO LIPOPOLYSACCHARIDE DOES NOT PREDICT THE HOST RESPONSE TO INFECTION

Scientific Goal

Determining the factors that precipitate infection susceptibility is essential for the risk stratification of hospitalized patients. One of the most frequently used assessments of innate immune function in clinical research settings is the magnitude of the cytokine response to LPS. However, the relationship between the magnitude of the cytokine response to LPS and infection susceptibility is merely correlative. In this chapter, I will experimentally test whether the magnitude of the cytokine response to LPS is an appropriate measure of infection susceptibility to severe *P. aeruginosa* infection in mice primed with different TLR ligands. These studies leverage the ability of TLR ligands to change the cytokine response to LPS and modulate immune response to live infection. Furthermore, I identify antimicrobial activity as consistent predictor of infection susceptibility. Overall, these studies establish that cytokine responses to LPS are not an appropriate measure of infection susceptibility and suggest antimicrobial capacity as an alternative.

Introduction

Endotoxin tolerance is the immunological phenomenon wherein prior exposure to TLR ligands renders innate leukocytes refractory to LPS-elicited pro-inflammatory cytokine secretion[255]. This immunoregulatory process has received widespread attention by the scientific community regarding both the molecular mechanism of induction and

clinical significance[221]. Yet, while the molecular mechanisms of induction are largely understood, the clinical significance remains debated.

There is a body of literature suggesting that endotoxin tolerance represents a state of immunoparalysis and susceptibility to infection[221,233,234]. In support of this hypothesis, an endotoxin tolerance gene signature has been associated with worse patient outcomes [98,256], and a high incidence of secondary nosocomial infections in critically ill patients [8]. Concurrent with this suggestion, an alternative body of literature reports that treatment with TLR4 ligands such as LPS and monophosphoryl lipid A (MPLA), the prototypical ligands used to induce endotoxin tolerance, augments host resistance to infection while facilitating suppressed cytokine production in a variety of infection models[229,230,252]. While seemingly in contradiction, these hypotheses both assume that the magnitude of the LPS-elicited cytokine response dictates the host response to infection. As such, the LPS-elicited pro-inflammatory cytokine response is commonly used to assess innate immune function in research settings [93,99]. Despite this, it has not been sufficiently demonstrated that the LPS-elicited cytokine response provides an accurate predictive assessment of the host response to live infection. One novel hypothesis to resolve this contradiction is that the magnitude of the LPS-elicited cytokine response is not relevant to, and thus does not predict, the host response to infection.

Endotoxin tolerance can be induced by TLR ligands other than LPS, and some TLR ligands have been reported to induce endotoxin sensitization, or augmented LPS-elicited cytokine production [257]. This diversity among TLR ligands offers a novel strategy, which I utilize in this study, to dissect whether an altered LPS-elicited cytokine response impacts host responses to infection. I hypothesize that the LPS-elicited pro-

inflammatory cytokine response does not predict the host response to infection following exposure to TLR ligands. To address this hypothesis, I examined the ability of various TLR ligands to alter the LPS-elicited pro-inflammatory cytokine response and to alter antimicrobial immunity against *P. aeruginosa*, one of the most common nosocomial Gram-negative pathogens [45]. I demonstrate that the magnitude of the LPS-elicited cytokine response does not correlate with host resistance to infection, and provide evidence that TLR immunoprophylaxis can occur with or without endotoxin tolerance.

TLR ligands differentially alter the LPS-elicited pro-inflammatory cytokine response.

To determine how different TLR ligands alter the pro-inflammatory cytokine response to LPS, mice were injected with a non-toxic dose of either the TLR4 ligands LPS or MPLA, the TLR9 ligand CpG-ODN, the TLR3 ligand poly(I:C), or lactated Ringer's solution (LR, vehicle) via the intraperitoneal route (Figure II-1A). GpC-ODN was used as an additional control. Three days following the injection, mice were challenged with a toxic dose of LPS. Six hours following LPS challenge, mice were sacrificed and plasma pro-inflammatory cytokine concentrations were measured.

In response to LPS challenge, mice primed with LPS or MPLA demonstrated significantly reduced plasma IL-6, IFN-γ, IL-1β, and KC concentrations, and mice primed with poly(I:C) demonstrated reduced IFN-γ and with a trend towards reduced IL-6 and KC (Figure II-1B), as compared to vehicle control. In contrast, CpG-ODN-primed mice demonstrated significantly higher plasma concentrations of IL-6, IFN-γ, and IL-1β compared to vehicle control. Priming mice with GpC-ODN did not significantly alter LPS-induced cytokine production. To use a more clinically feasible assessment of LPS

responses, as well as to detect TNF-α which clears from the plasma within 2 hours after *in vivo* LPS, heparinized whole blood was harvested from TLR-ligand primed mice and exposed to LPS for 6 hours *ex vivo* followed by measurement of TNF-α concentrations in the plasma supernatant fraction. The supernatant fraction from MPLA, LPS, and poly(I:C)-primed mice had reduced TNF-α concentrations compared to control, whereas CpG-ODN primed blood was found to have elevated TNF-α (Figure II-1C). Thus, various TLR ligands differentially alter the response to LPS *in vivo* and *ex vivo*. The TLR4 ligands LPS and MPLA potently induce sustained endotoxin tolerance whereas poly(I:C) weakly elicits endotoxin tolerance. In contrast, CpG-ODN potently augments LPS-induced cytokine production.

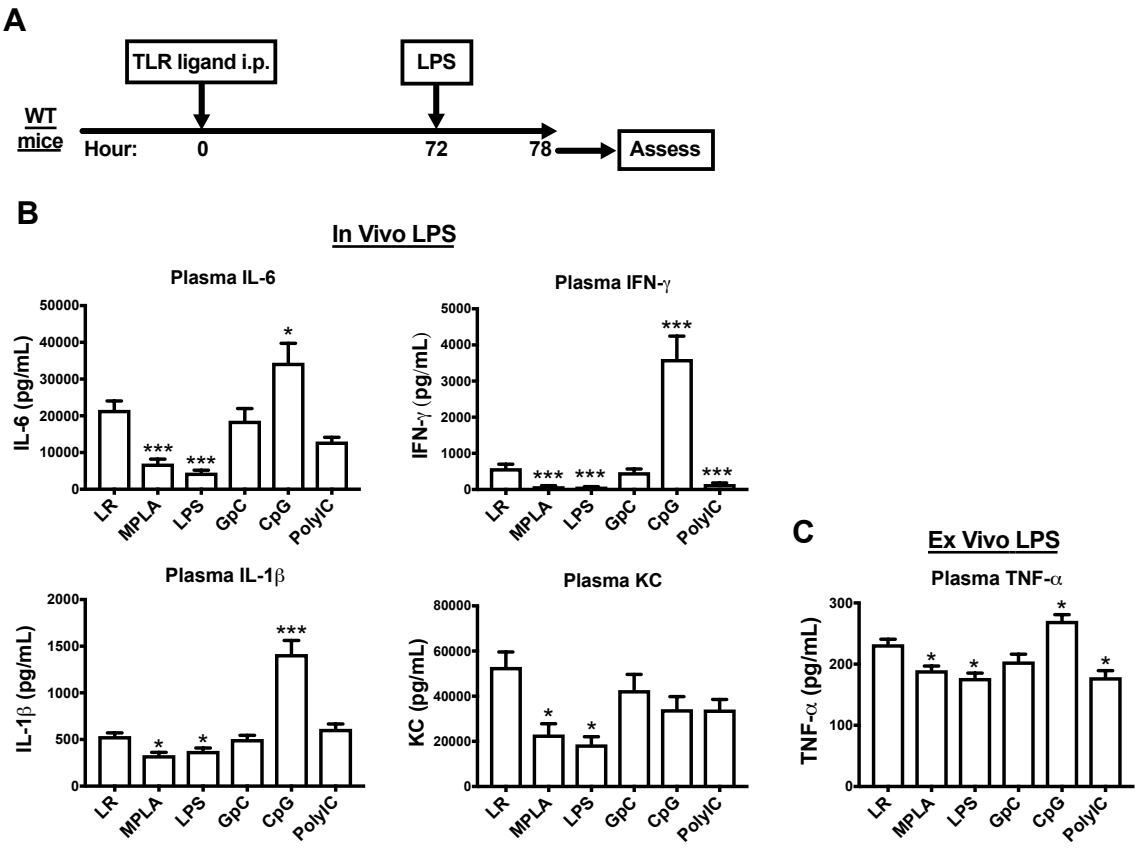


Figure II-1. TLR ligands differentially alter the pro-inflammatory cytokine response to LPS.

A) Male wild-type BALB/c mice received intraperitoneal injection with 2μg LPS, 20μg MPLA, 20μg GpC-ODN, 20μg CpG-ODN, 20μg poly(I:C) or vehicle followed 3 days later by intraperitoneal challenge with 100μg LPS. Blood was harvested for cytokine measurements at 6 hours after LPS challenge. For experiments assessing *ex vivo* cytokine production, blood was harvested at 3 days after TLR ligand priming. B) Plasma IL-6, IFN-γ, IL-1β, and KC six hours after *in vivo* LPS. (n=15 mice per group). C) TNF-α six hours after blood exposure to 100ng/mL LPS ex vivo (n=5 mice per group). Error bars indicate SEM. ***, $p< 0.001$, *, $p<.05$ as compared to LR determined by one-way ANOVA with Dunnett's multiple comparison's test.

The magnitude of the LPS-elicited pro-inflammatory cytokine response does not indicate infection susceptibility.

I next determined the ability of TLR ligands to change the host susceptibility to live *P. aeruginosa* infection. Mice were treated with either LPS, MPLA, GpC-ODN, CpG-ODN, poly(I:C), or LR (vehicle) and three days later were challenged with *P. aeruginosa* (Figure II-2A). Core temperature, bacterial counts, leukocyte numbers at the site of infection and plasma cytokines were measured six hours after *P. aeruginosa* challenge. Core body temperature provides an accurate surrogate for physiologic integrity during infection since mice reliably develop hypothermia as the severity of infection progresses [258]. Mice primed with LPS, MPLA, or CpG-ODN were robustly resistant to infection, as indicated by maintenance of normal core body temperature (Figure II-2B), whereas mice primed with LR or GpC-ODN showed dramatic reductions in core body temperature. Core body temperature in mice primed with poly(I:C) was statistically higher than in vehicle-primed mice but significantly lower than in non-infected mice or mice primed with LPS, MPLA or CpG-ODN. Mice primed with LPS, MPLA, or CpG-ODN demonstrated a significantly greater capacity to clear bacteria from the site of infection as indicated by a reduction in intraperitoneal *P. aeruginosa* (Figure II-2C), whereas mice primed with GpC-ODN, poly(I:C), or vehicle did not. Overall, the magnitude of the LPS-elicited pro-inflammatory cytokine response found in Figure II-1 was not predictive of the host response to infection. TLR ligands that increase (CpG-ODN) and decrease (MPLA, LPS) LPS-elicited cytokine responses both improved the host response to *P. aeruginosa* infection.

Protective TLR ligands improve the recruitment of phagocytes.

Next, the cellular mechanisms by which TLR ligands generate protection from infection were investigated. Given that mice resistant to infection had reduced bacterial burden, the recruitment of neutrophils and macrophages to the site of infection was characterized. Six hours after intraperitoneal *P. aeruginosa* infection, the peritoneal cavity of infected mice was lavaged with PBS and leukocytes in the peritoneal lavage fluid were characterized using flow cytometry. Both the total number and percentage of neutrophils were significantly elevated in LPS, MPLA, and CpG-ODN primed mice as compared to LR-primed controls (Figure II-2D-E). Poly(I:C) primed mice demonstrated an increase in percentage, but not total number, of neutrophils recruited to the peritoneal cavity. Additionally, a significant increase in intraperitoneal macrophage numbers and percentage was observed after priming with MPLA and CpG-ODN compared to LR-primed controls. Priming with LPS, GpC-ODN, or poly(I:C) did not elicit a significant increase in the total number or percentage of macrophages compared with LR-primed controls. These data indicate that persistent improvements in the recruitment of phagocytes to the site of infection are a likely mechanism by which many TLR ligands exert their protective effect, a finding that is supported by our previous studies with MPLA[252].

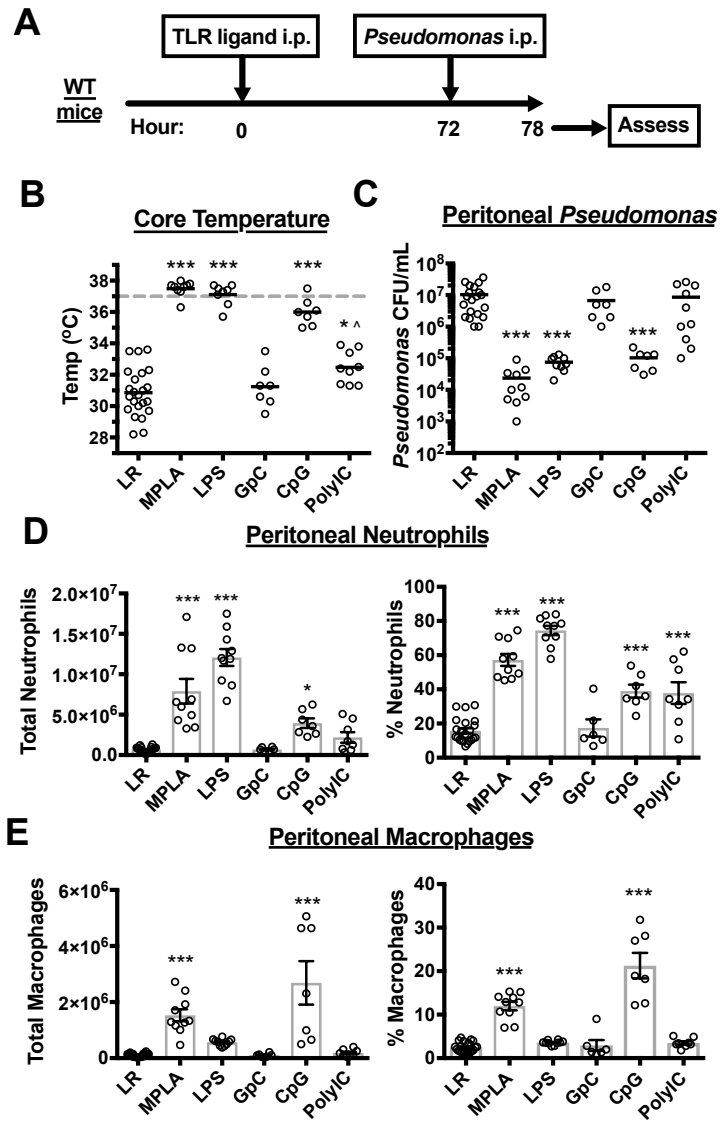


Figure II-2. TLR ligands differentially affect host resistance to infection.

A) Mice received intraperitoneal injection with 2μg LPS, 20μg MPLA, 20μg GpC-ODN, 20μg CpG-ODN, 20μg poly(I:C) (n=8-10 mice per group) or LR (vehicle) (n=23 mice) followed by intraperitoneal challenge with $1x10^8$ CFU *P. aeruginosa* 3 days later. Temperature, bacterial burden, leukocyte recruitment and cytokine concentrations were measured at 6 hours after bacterial challenge. B) Core body temperature as measured by a rectal probe 6 hours after infection; murine baseline core body temperature of uninfected mice is 37˚C, as indicated by dashed line. C) *P. aeruginosa* CFU/mL of peritoneal lavage fluid isolated from the peritoneal cavity after a PBS wash. D) Total number and percentage of neutrophils ($Ly6G^+F4/80^-$) in the peritoneal cavity. E) Total number and percentage of macrophages ($Ly6G^-F4/80^+$) in the peritoneal cavity. SEM .***, $p< 0.001$, *, $p<0.05$, as compared to LR, ^, $p<0.001$ as compared to LPS, MPLA and CpG-ODN, all determined by one-way ANOVA with Dunnett's multiple comparison's test.

Systemic pro-inflammatory cytokine concentrations correlate with bacterial burden. Plasma pro-inflammatory cytokine concentrations at six hours following *P. aeruginosa* infection were then quantified (Figure II-3). Plasma concentrations of IL-6, IFN-γ, IL-1β, and CXCL1 (KC) (Figure II-3A-D) were significantly reduced in mice primed with LPS or MPLA compared to LR-primed animals. Even though CpG-ODN increased plasma pro-inflammatory cytokines after LPS challenge (Figure II-1), CpG-ODN-primed mice had a significant reduction in plasma IL-6, IL-1β, and KC, but not IFN-γ, after *P. aeruginosa* infection. Plasma cytokine concentrations in mice primed with GpC-ODN were not significantly different from vehicle-primed controls whereas poly I:C treatment resulted in decrease IFNγ and IL-1β but not IL-6 or KC. Thus, these data indicate that TLR ligands that augment bacterial clearance will facilitate reduced systemic pro-inflammatory cytokines during infection, even if they sensitize the response to LPS.

TLR4 ligand-induced immunoprophylaxis persists for up to 15 days but is independent of the adaptive immune system

Despite many studies on the prophylactic benefit of TLR4 ligands, it remains unclear how long TLR4-induced resistance to infection lasts. To determine how long TLR immunoprophylaxis lasts, mice were challenged with *P. aeruginosa* 1, 10, and 15 days following MPLA priming. Mice infected 10 days following MPLA treatment had significantly higher core body temperature than vehicle controls and demonstrated significant reductions in bacterial burden (Figure II-4A-B). Mice infected 15 days following MPLA treatement had improvements in core temperature over controls, but did not show statistically significant improvements in bacterial clearance. Thus, the effects of MPLA-induced prophylaxis last up to 15 days.

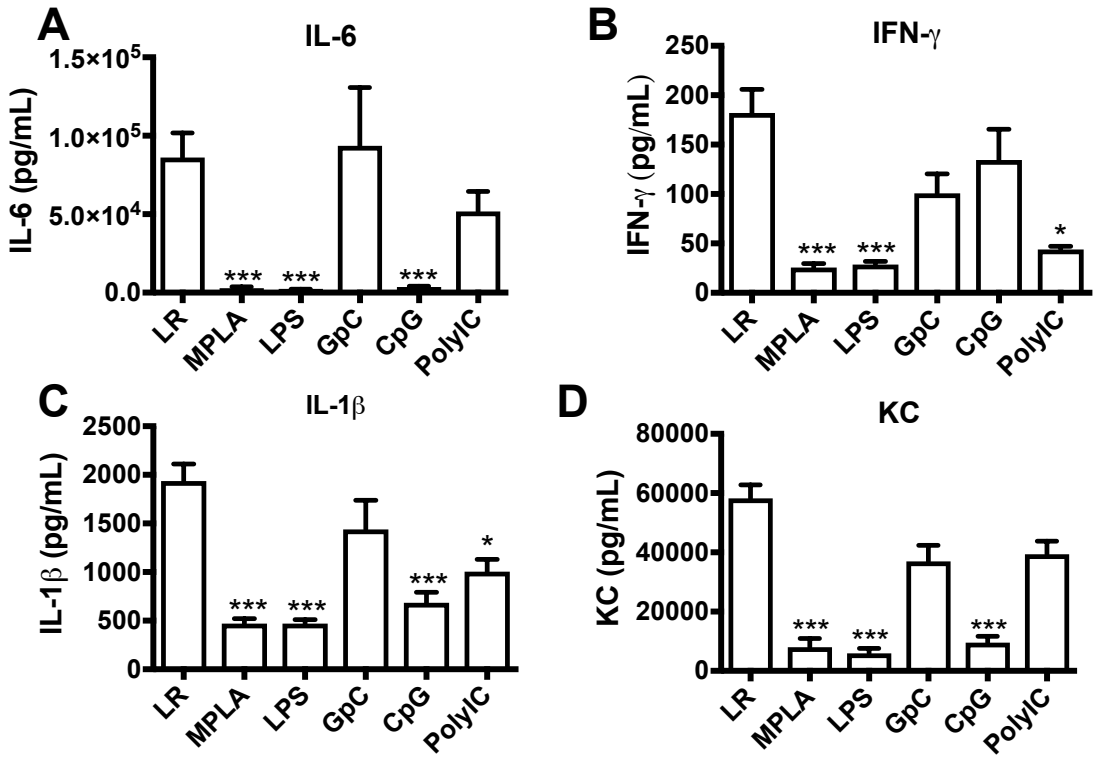


Figure II-3. Systemic pro-inflammatory cytokine concentrations correlate with resistance to infection.

Mice were primed with TLR ligands and infected with *P. aeruginosa* 3 day later. Blood was harvested by carotid laceration and plasma was assessed for cytokines concentrations. A) Plasma IL-6, B) IFN-γ, C) IL-1β, and D) KC were measured 6 hours after *P. aeruginosa* infection by BioPlex Assay. SEM .***, $p< 0.001$, *, $p<0.05$, as compared to LR. All determined by one-way ANOVA with Dunnett's multiple comparison's test..

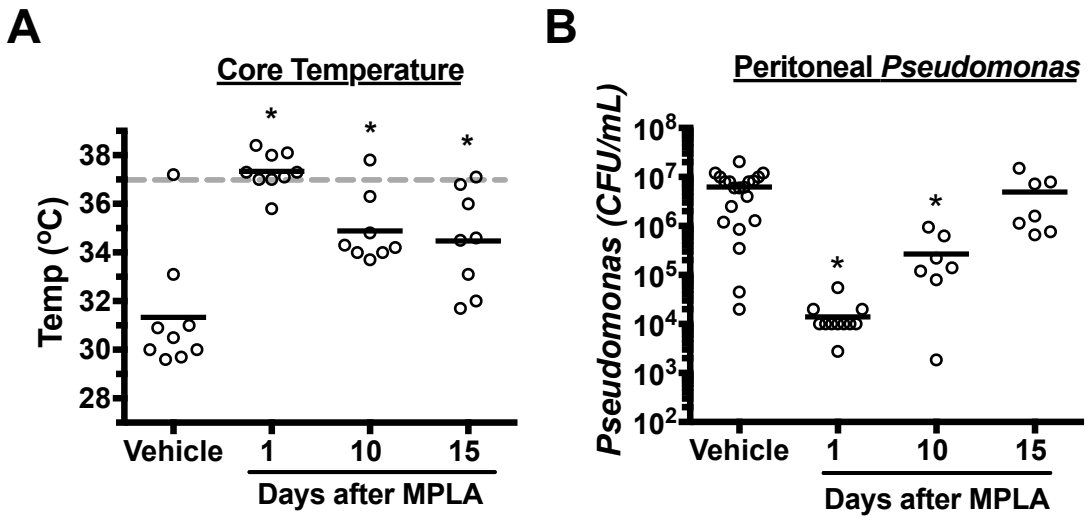


Figure II-4. TLR4 ligand-induced immunoprophylaxis persists for up to 15 days.

Mice were primed with two intraperitoneal injections of MPLA 24 hours apart, then challenged with 10^8 CFU *P. aeruginosa* 1, 10, or 15 days following priming and assessed 6 hours after the challenge (n=8-20 mice per group). A) Core body temperature as measured by rectal probe 6 hours after *P. aeruginosa*. B) *P. aeruginosa* CFU/mL of peritoneal lavage fluid isolated from the peritoneal cavity after a PBS wash. *, $p<.05$ compared to LR via one-way ANOVA with Dunnett's multiple comparison's test.

There are no established mechanisms for the innate immune system to sustain a phenotype two weeks after a stimulus. While the Sherwood lab has previously published on the requirement of the innate immune system for TLR ligands to protect animals [252], the importance of the adaptive immune system for protection against *P. aeruginosa* is unknown. Therefore, I investigated the hypothesis that the adaptive immune system mediates the persistent protection and improved phagocyte recruitment seen after TLR ligand priming. Wild-type and RAG2 knockout ($RAG2^{-/-}$) mice, which are deficient in functional T- and B-cells, were primed with MPLA or LR (vehicle) and challenged 3 days later with *P. aeruginosa* (Figure II-5A). MPLA- primed $RAG2^{-/-}$ mice maintained body temperature and demonstrated reduced bacterial burden in response to infection, as compared to LR-primed $RAG2^{-/-}$ mice (Figure II-5B-C). Additionally, MPLA- primed $RAG2^{-/-}$ mice demonstrated a significant elevation in both total and percent neutrophils in the peritoneal cavity as well as total and percent macrophages, compared with LR-primed $RAG2^{-/-}$ mice (Figure II-5D-E). Similar to wild-type animals, pro-inflammatory cytokine levels in MPLA-primed $RAG2^{-/-}$ mice were significantly lower than in controls (Figure II-5F). Thus, the adaptive immune system is not required to maintain TLR4-mediated augmentation of host resistance to infection.

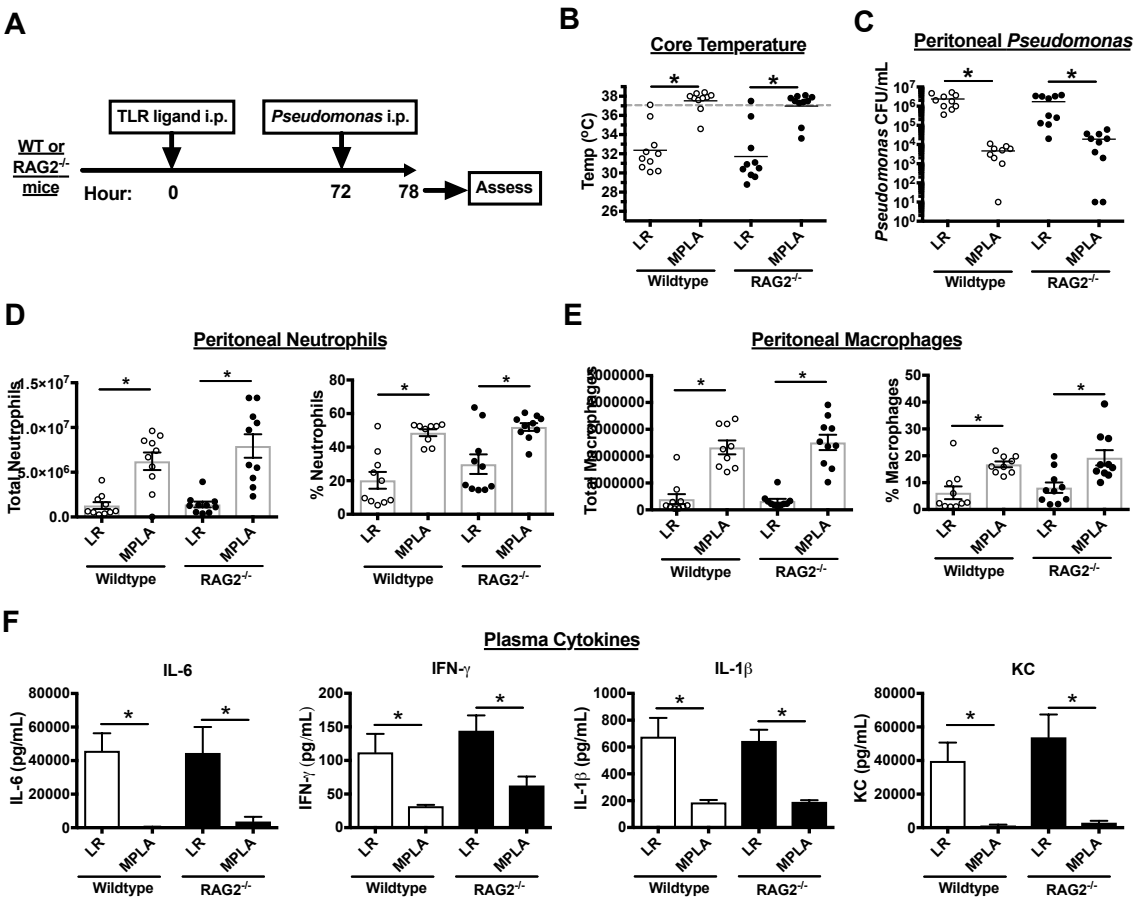


Figure II-5. The adaptive immune system is not required to maintain resistance to *P. aeruginosa* infection.

A) Wild-type and RAG2$^{-/-}$ mice were received intraperitoneal injection with 20μg MPLA or LR (n=10 mice per group) followed by intraperitoneal challenge with 1x10^{8} CFU *P. aerugionsa* 3 days later. B) Core body temperature was measured 6 hours after infection by rectal probe. C) *P. aeruginosa* CFU/mL of peritoneal lavage fluid isolated from the peritoneal cavity after a PBS wash. D) Total number and percentage of neutrophils (Ly6G^{+}F4/80^{-}) in the peritoneal cavity. E) Total number and percentage macrophages (Ly6G^{-}F4/80^{+}) in the peritoneal cavity. F) Plasma IL-6, IFN-γ, IL-1β, and KC were measured 6 hours after *P. aeruginosa* infection. Error bars indicate SEM. *, p<.05 via one-way ANOVA with Dunnett's multiple comparison's test.

Discussion

Taken together, these data support the argument that the LPS-elicited cytokine response is not indicative of infection susceptibility, especially after prior exposure to TLR ligands. I discovered that many TLR ligands persistently alter the magnitude of the LPS-elicited pro-inflammatory cytokine response, but that both decreased (LPS, MPLA) and increased (CpG-ODN) LPS-elicited cytokine responses occurred concurrent with resistance to infection. To my knowledge, this is the first study to identify an incongruity between the magnitude of the cytokine response to LPS and the response to live infection. This finding, while simple, may have significant implications. It has previously been assumed that the cytokine response to LPS is predictive of the response to infection. Investigators continue to use LPS-induced cytokine shock as a surrogate model for bacterial sepsis [259,260], and the LPS cytokine response assay to determine immune-competency in human patients [93,261]. Moreover, the conclusion that a reduced LPS-elicited cytokine response is immunosuppressive continues to receive wide attention [233,262]. This study suggests that the magnitude of the LPS-elicited cytokine response should not be used as the sole measure of immune function, and its value should be questioned.

The finding that LPS and MPLA protect against infection reiterates previous conclusions that endotoxin tolerance is not a state of immunoparalysis. While the expression of an endotoxin tolerance gene signature has been associated with worse outcomes in critically ill patients [256], the increased expression of these genes may simply correlate with the severity of the primary inflammatory insult [224,228]. Severe inflammation can lead to a myriad of pathologies, including widespread organ and endothelial cell

dysfunction, T cell apoptosis, metabolic instability and hypothalamic dysregulation, each of which is also associated with worse patient outcomes [117,263-265]. Thus, although an LPS tolerant phenotype may be present in patients with critical illnesses, a cause and effect relationship has not been established. Other causes of impaired antimicrobial immunity, such as T cell loss and dysfunction, may be present. Loss and dysfunction of T cells has been widely reported in critically ill humans and in animal models of critical illness [266-268]. Animal models of critical illness demonstrate that T cell dysfunction contributes to impaired antimicrobial immunity [269,270]. Thus, it remains possible that the relationship between endotoxin tolerance and poor ICU outcomes is simply correlative.

The finding that CpG-ODN elevates *in vivo* LPS-elicited cytokine responses yet still protects mice from *P. aeruginosa* infection disputes the hypothesis that endotoxin tolerance is the mechanism underlying TLR ligand immunoprophylaxis. Further supporting this conclusion, poly(I:C) was able to induce mild endotoxin tolerance yet was unable to induce infection prophylaxis. Mice protected by a reduced cytokine burden alone do not typically display concurrent increases in bacterial clearance and phagocyte recruitment [271], yet MPLA- and LPS- primed mice exhibited improved bacterial clearance and phagocyte recruitment. Previous work from the Sherwood lab has found that MPLA induces robust neutrophil and monocyte proliferation and mobilization [170], and that G-CSF, and the accompanying antimicrobial response, is essential for MPLA to reduce cytokine levels in response to *P. aeruginosa* [232]. Thus, my data continue to support the hypothesis that TLR ligands protect animals from infection by improving host antimicrobial efficiency, rather than by altering LPS-elicited cytokine responses.

The duration of TLR4 ligand-induced immunoprophylaxis had not been previously determined. The finding that MPLA protects RAG2$^{-/-}$ mice and that MPLA protects mice for at least 10 days and up to15 days after a single injection suggests that the innate immune system has mechanisms to sustain improved antimicrobial responses. These results are surprising considering the lack of mechanisms for the innate immune system to sustain such a phenotype. Mechanisms of persistence may lie in sustained epigenetic alterations, as have recently been observed following LPS administration, and in non-TLR-ligand immunoprophylaxis[272,273]. Further work on TLR ligand-induced persistent epigenetic modifications will likely discover novel ways for the innate immune system to sustain phenotypes long after a stimulus has been cleared.

Finally, many therapeutic trials in sepsis have focused on altering pro-inflammatory cytokine levels during sepsis, such as with anti-TNF-α, IL-1 receptor antagonist, and a TLR4 antagonist, all of which have been met with failure[274-276]. The findings presented here suggest that augmenting the host antimicrobial response may be a more effective way to reduce systemic inflammation and resolve sepsis. Hence, these data support clinical trials with immunostimulatory compounds that can improve antimicrobial function in immune cells. Ongoing studies of this nature include trials with interleukin-7, which aim to reverse T-cell anergy observed during post-sepsis immunosuppression [266], as well as trials with GM-CSF [277]. Our findings support the initiation of clinical trials with MPLA or CpG-ODN to improve antimicrobial efficiency in anticipation of sepsis in immunocompromised patients. More accurate assessments of immune function, combined with therapies that boost antimicrobial processes, may effectively mitigate the risk of infection in hospitalized patients.

CHAPTER III

MONOPHOSPHORYL LIPID A PROTECTS AGAINST SYSTEMIC INFECTION VIA MODULATION OF TISSUE MACROPHAGES AND NEUTROPHILS

Scientific Goal

Nosocomial bloodstream infections, such as central line associated bloodstream infections, drive significant mortality and cost in the hospital. MPLA prophylaxis has previously been demonstrated to protect against local *P. aeruginosa* and polymicrobial Gram-negative peritoneal sepsis, such as would be encountered in a SSI or burn wound infection, but has not been demonstrated against *S. aureus*, the most common causative agents of nosocomial bloodstream infections. In this chapter, I will determine the efficacy of MPLA in inducing resistance to *S. aureus*, and determine the cellular mechanism by which this resistance occurs. These studies will establish MPLA as a protective agent against one of the most dangerous Gram-positive nosocomial pathogens and further elucidate the mechanisms by which MPLA induces broad-spectrum resistance to infection.

Introduction

Infection with antibiotic resistant pathogens, particularly in the hospital setting, is one of the greatest modern threats to public health in Western nations[278]. Critically ill and high-risk surgical patients are highly vulnerable to infection, as are patients who are immunosuppressed due to sepsis, organ transplantation and treatment of autoimmune diseases [6,8,94]. Further, central line associated bloodstream infections are one of the most common types of nosocomial infection, and the risk of developing infection is

precipitated by iatrogenic central line placement. Despite wide appreciation of this risk upon line placement, prophylactic antibiotics are the only medical strategy available to reduce infection incidence[279]. Thus, there is a need for alternative medical interventions that can reduce the risk of infection without propagating antibiotic resistance. The Sherwood lab previously explored MPLA as a safe and effective agent to induce resistance to Gram-negative infection[252]. My studies in Chapter II, along with previous studies by the Sherwood lab, suggest that TLR ligands induce persistent resistance to Gram-negative infection by augmenting cellular antimicrobial responses and bacterial clearance, rather than by inducing endotoxin tolerance[252]. This prompts the question of whether MPLA can also protect against non-endotoxin-containing organisms, such as *S. aureus*. *S. aureus* is one of the most common causes of nosocomial bloodstream infection in the United States and is an organism identified by the CDC as a significant threat for antibiotic resistant infections[280]. It has previously been demonstrated that LPS pretreatment can protect against *S. aureus* challenge; however, the mechanism of protection against *S. aureus* was not clear and assumed to be some form of pro-inflammatory cytokine tolerance[229].

My studies in chapter II, as well as studies conducted by Romero et al, have identified that TLR agonist induced resistance to peritoneal *P. aeruginosa* infection is independent of the adaptive immune system and dependent on neutrophil activity[252]. However, the specific mechanisms by which TLR agonists change resistance to infection may vary on the type of infection and the causative organism. Neutrophils have been demonstrated to play a key role in the clearance of *S. aureus*, however, macrophages have been reported to be the primary mediators of bacterial clearance from the

bloodstream[230]. Moreover, macrophages express some of the highest levels of TLR4, and their function can be significantly altered by TLR4 stimulation. Thus it remains unclear whether macrophages play a role in TLR agonist-induced resistance to potential pathogens.

In this chapter, I hypothesize that MPLA priming improves the host antimicrobial response to *S. aureus*. In order to determine the specific phenomena that mediate resistance, I also explore the cellular mechanisms by which MPLA changes the host response to *S. aureus* infection. I employed mice deficient in various immune populations to demonstrate that MPLA robustly protects mice from severe systemic and local *S. aureus* infection in a mechanism dependent on augmented tissue macrophage antimicrobial functions with help from the neutrophil pool, but that resistance is not dependent on adaptive immunity or pro-inflammatory monocytes.

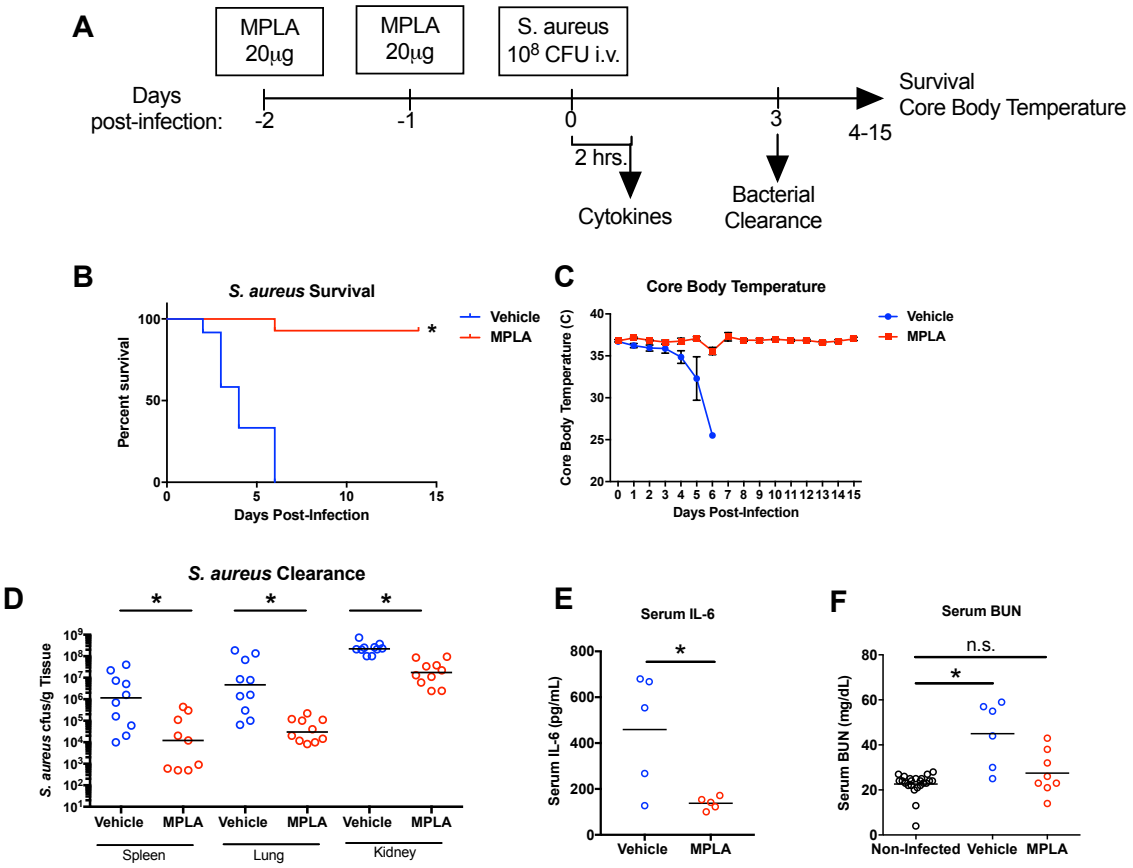


Figure III-1 MPLA induces resistance to intravenous *S. aureus* infection

A) WT BALB/c mice were injected with intravenous MPLA (20μg/mouse) or vehicle one and two days prior to intravenous inoculation with 10^8 cfu *S. aureus*. Following inoculation, mice were sacrificed at different times points to assess various endpoints. For survival, mice were followed 14 days after inoculation. B) Kaplan Meier survival curve of MPLA primed and vehicle mice. (n=12 mice/group) C) Core (rectal) body temperature assessed daily after inoculation. D) *S. aureus* cfu per gram of tissue recoverable from whole spleen, lung, and kidney in vehicle and MPLA-primed mice at 3 days after S. aureus inoculation. E) Concentration of serum IL-6 2 hours after infection. F) Concentration of serum blood urea nitrogen (BUN) 3 days after inoculation. Data shown as mean +/- SEM. *, p< .05 as determined by t-test or ANOVA with Tukey's *post-hoc* multiple comparison analysis.

MPLA primed mice are broadly resistant to systemic infection

To investigate the impact of MPLA on *S. aureus* infection, I primed mice with intravenous MPLA or vehicle and then challenged them with 10^8 cfu intravenous *S. aureus* (Figure III-1A). While all vehicle mice succumbed to the *S. aureus* infection, greater than 90% of MPLA-primed mice survived the infection (Figure III-1B). This was further reflected in core body temperature, as MPLA-primed mice maintained body temperature significantly better than naïve mice (Figure III-1C). Upon assessment of the burden of *S. aureus* in tissues, MPLA-primed mice had significantly lower *S. aureus* cfu in the spleen, lung, and kidney compared to vehicle mice (Figure III-1D). Two hours following S. aureus infection, MPLA-primed mice had significantly lower IL-6 than vehicle mice (Figure III-1E). *S. aureus* infection also induced kidney injury, and MPLA primed mice had reduced serum BUN when compared to vehicle mice (Figure III-1. Additional studies conducted in collaboration with Dr. Julia Bohannon demonstrated that MPLA-primed mice challenged with systemic *Candida albicans* infection have improved survival and preserved core body temperature (data not shown). Thus, MPLA induces resistance to a broad spectrum of pathogens, including *P. aeruginosa*, *S. aureus*, and *C. albicans*.

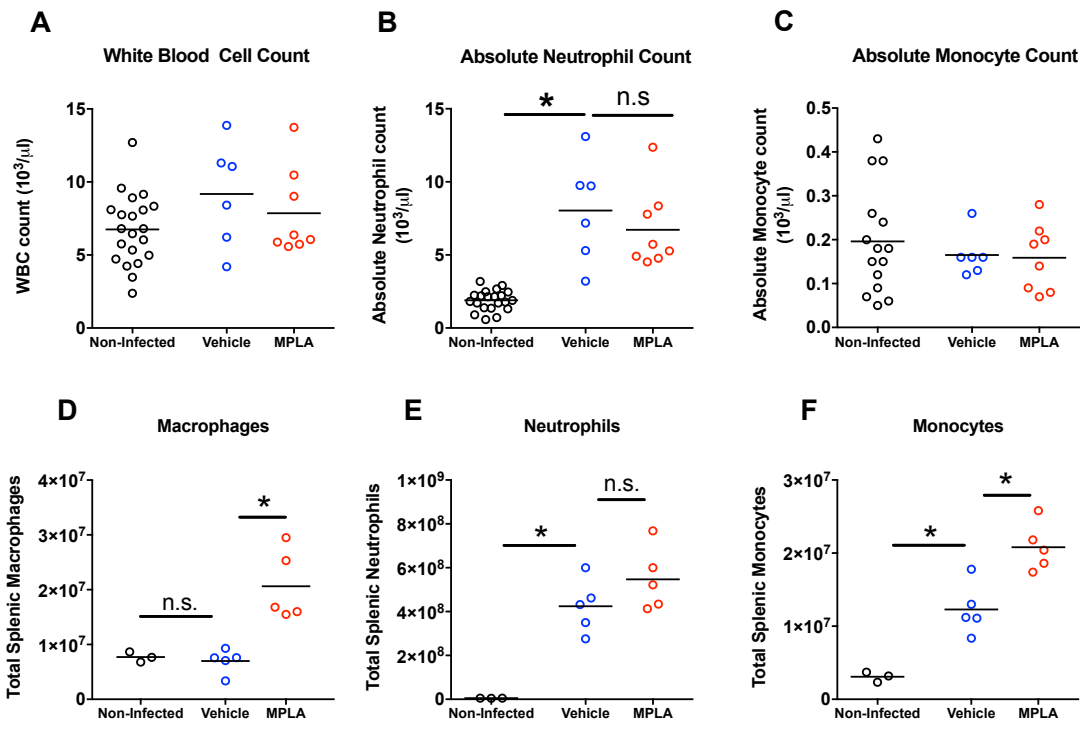


Figure III-2 MPLA priming induces changes to tissue, but not blood, leukocyte populations after *S. aureus* infection.

WT BALB/c mice were injected with intravenous MPLA (20µg/mouse) or vehicle one and two days prior to intravenous inoculation with 10^8 cfu *S. aureus*. Following 3 days, blood and spleen were harvested. A) Total white blood cell, B) Total neutrophil and C) total monocytes in the blood. D) Total macrophage E) neutrophil and F) monocytes in the spleen. Data shown as mean +/- SEM. *, p< .05 as determined by t-test or ANOVA with Tukeys post-hoc multiple comparison analysis

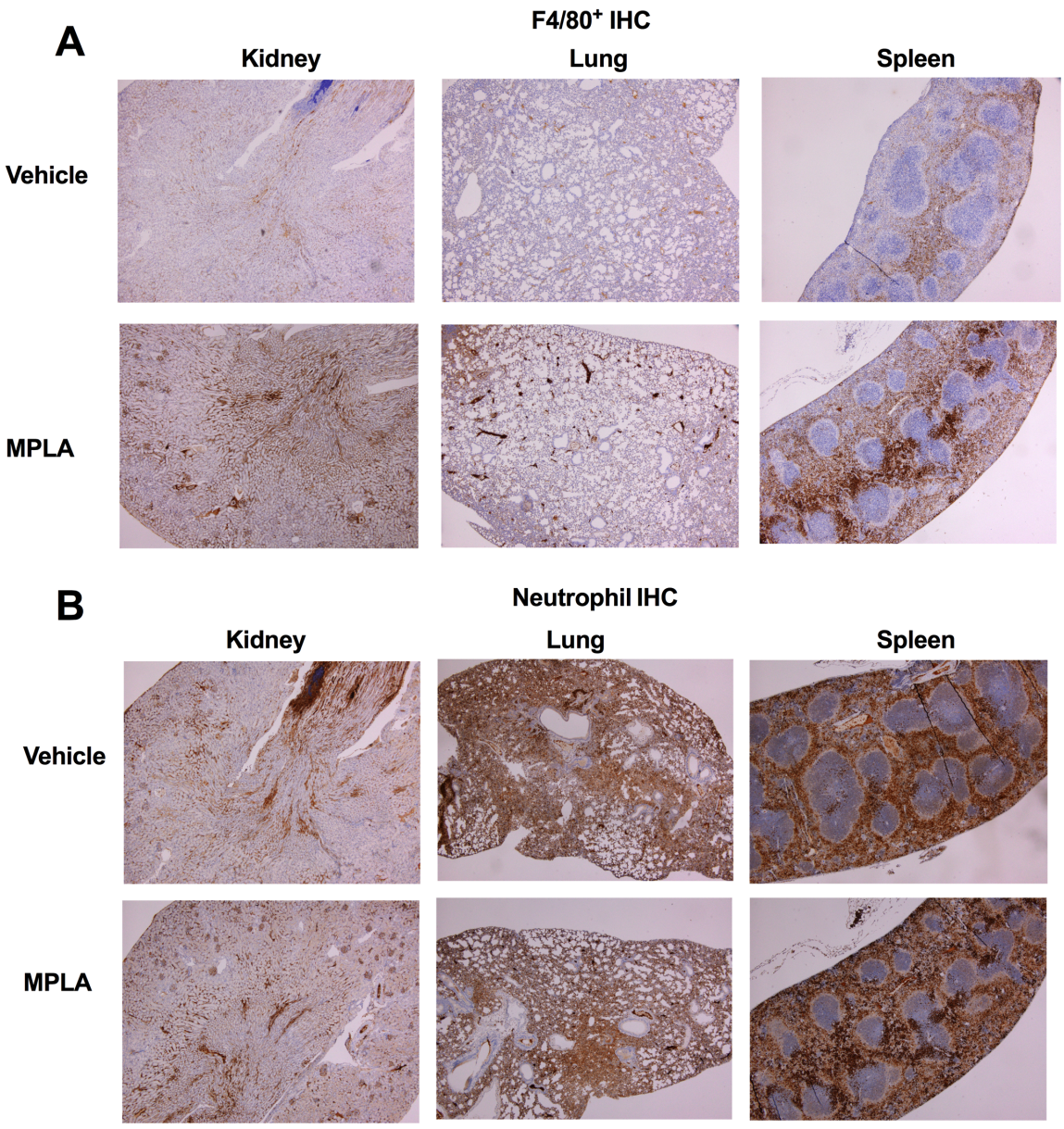


Figure III-3. MPLA-primed macrophages have significant alterations to their tissue macrophage pool.

A) F4/80^{+} immunohistochemical staining in kidney, lung and spleen sections taken 3 days after *S. aureus* inoculation from vehicle and MPLA primed mice. B) Neutrophil immunohistochemical staining in kidney, lung and spleen sections taken 3 days after S. *aureus* inoculation from vehicle and MPLA primed mice. Images shown are representative of 5 mice per group.

Assessments of innate leukocyte populations were then undertaken in collaboration with Dr. Naeem Patil. *S. aureus* infection increased absolute blood neutrophil counts, although did not change absolute blood monocyte counts. There was no significant difference in absolute total blood leukocyte, neutrophil, or monocyte numbers between vehicle- and MPLA-primed mice (Figure III-2A-C). *S. aureus* infection also elevated tissue leukocytes counts and, compared to vehicle-primed mice, MPLA-primed mice displayed significant increases in splenic macrophages and monocytes, but not neutrophils (Figure III-2D-F).

In order to clarify the specific populations of leukocytes through tissues, F4/80 (macrophage) and neutrophil immunohistochemical analysis of the kidney, lung, and spleens of mice infected with *S. aureus* was undertaken. Images of tissues from mice infected with *S. aureus* demonstrated that MPLA-primed mice had a stark increase in the number of $F4/80^+$ macrophages within the kidney, lung, and spleen (Figure III-3A). Of note, macrophages from MPLA-primed mice demonstrated darker staining, and formed spots of condensed $F4/80^+$ staining throughout organs, particularly the kidney and lung. Neutrophil staining in kidney, lung, and spleen was comparable between MPLA and vehicle mice (Figure III-3B). Despite no differences in overall staining between groups, MPLA-primed animals exhibited spots of condensed neutrophil staining that corresponded to spots of dark $F4/80^+$ staining, demonstrating that macrophage and neutrophils co-localize to foci. Thus, MPLA priming significantly changes the tissue macrophage and monocyte pool after *S. aureus* infection, but does not appreciably alter neutrophil populations.

Tissue macrophages and neutrophils and required for MPLA-mediated resistance to infection

I next explored the cellular mechanism by which MPLA improves resistance to *S. aureus*. Increased neutrophil recruitment to sites of infection was previously identified as essential for protection against local Gram-negative infections [252], however the cellular mechanism by which MPLA protects against systemic *S. aureus* infection is unknown. In order to determine which leukocytes are responsible for protection by MPLA, I selectively disrupted different leukocyte populations and assessed the efficacy of MPLA protection. First, HeJ mice, which do not have a functional TLR4 receptor, were primed with MPLA to establish that protection by MPLA requires its canonical receptor TLR4. As expected, MPLA-primed HeJ mice did not receive any benefit from MPLA prophylaxis, and MPLA-primed HeJ mice experienced survival similar to control HeOuJ mice (Figure III-4A). We then assessed the requirement of the adaptive immune system for MPLA-mediated protection. Protection by MPLA is surprisingly persistent, and adaptive immune cells are known to influence neutrophil recruitment, particularly through the Th17 T-cell compartment [281]. However, in Chapter II, I determined that resistance to peritoneal *P.aeruginosa* infection does not require the adaptive immune system. In order to assess the requirement of adaptive immunity against *S. aureus*, I primed RAG2$^{-/-}$ mice with MPLA and assessed their resistance to *S. aureus*. MPLA was able to induce robust protection against *S. aureus* in RAG2$^{-/-}$ mice, demonstrated as MPLA-primed WT and RAG2$^{-/-}$ had similar survival after *S. aureus* (Figure III-4B). Taken together, these results indicate that the antimicrobial effects of MPLA on *S. aureus* are dependent on TLR4 and independent of the adaptive immune system.

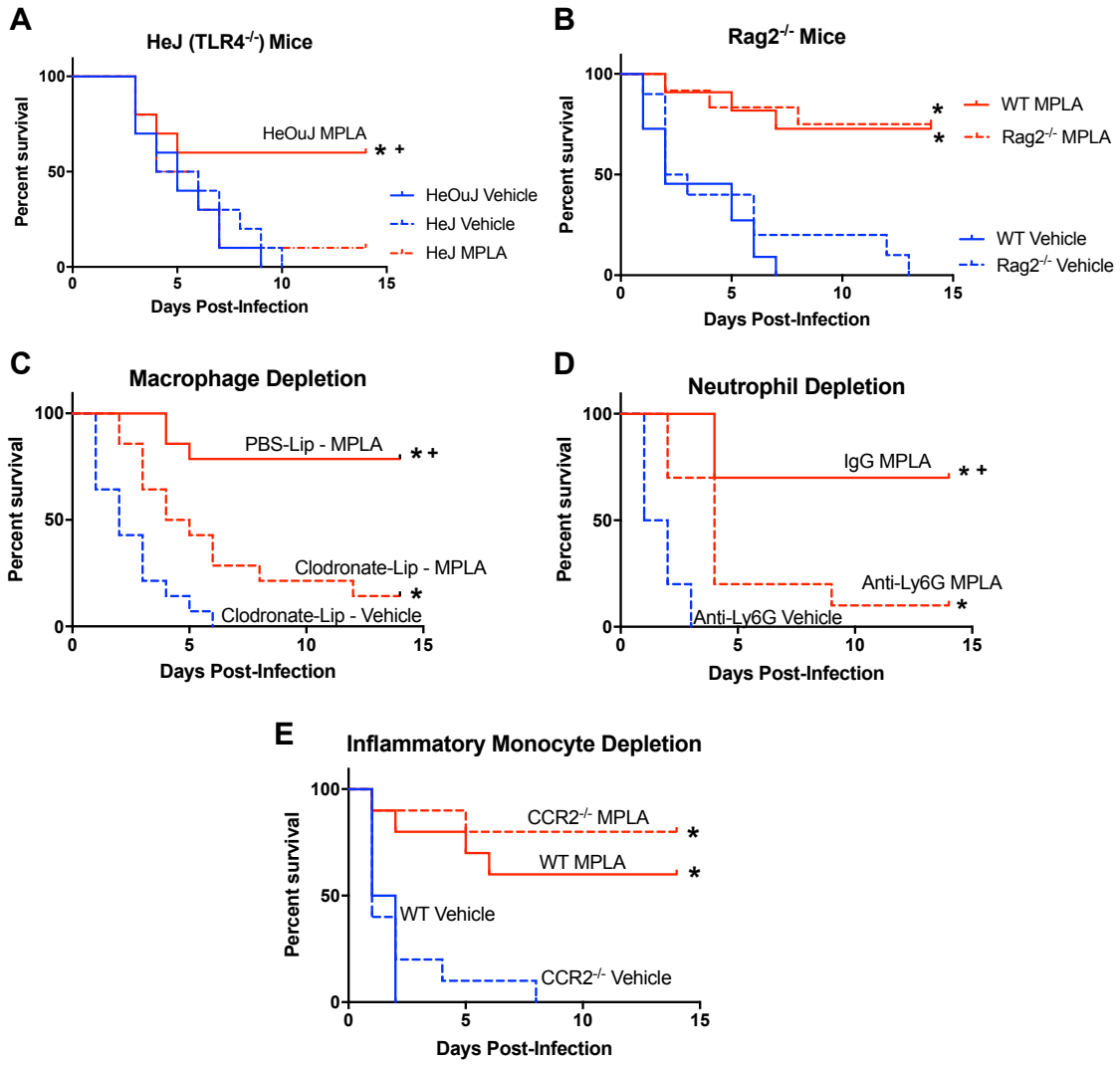


Figure III-4. MPLA mediates resistance to *S. aureus* via tissue macrophages and neutrophils

A) HEJ and HeOuJ mice were primed with MPLA and then challenged with 10^8 cfu intravenous *S. aureus*. Kaplan-Meier survival plot of mice after infection (n=10 mice/group). B) RAG2$^{-/-}$ and WT mice were primed with MPLA and then challenged with 10^8 cfu intravenous *S. aureus*. Kaplan-Meier survival plot of mice after infection.(n=10 mice/group). C) Mice were administered intravenous clodronate-liposomes 24 hours prior to the first MPLA injection. Mice were then infected with 10^8 cfu *S. aureus*. Kaplan-Meier survival plot of mice after infection (n=15 mice/group). D) Mice were administered intravenous anti-Ly6G antibody 24 hours prior to the first MPLA injection. Mice were then infected with 10^8 cfu *S. aureus*. Kaplan-Meier survival plot after infection (n=10 mice/group) E) Kaplan-Meier survival plot of WT and CCR$^{-/-}$ mice primed with MPLA or vehicle and infected with intravenous *S. aureus*. n=10 mice/group . Data shown as mean +/- SEM. For Kaplan-Meier plots *, p<.05 compared to WT vehicle, $^+$, p<.05 compared to depleted-MPLA via log-rank Mantel-Cox test.

To determine the contributions of innate leukocyte subsets to MPLA-induced resistance to *S. aureus*, different leukocyte populations were selectively ablated prior to vehicle or MPLA treatment. Tissue macrophages were depleted with clodronate-liposomes 24 hours prior to the first MPLA injection. Intravenous clodronate-liposomes initially deplete monocyte as well as tissue macrophage populations. However, blood monocytes quickly regenerate following a single administration of clodronate-liposomes, whereas tissue macrophages do not [282]. Macrophage depletion greatly reduced the resistance of MPLA-primed mice to infection (Figure III-4C). MPLA still induced slight but significant protection in macrophage-depleted mice when compared to macrophage-depleted naïve mice indicating there may be additional leukocytes involved. To investigate this, we assessed the importance of neutrophils for MPLA-mediated protection against *S. aureus.* Similarly to macrophages, depletion of neutrophils by treatment with anti-Ly6G markedly worsened survival in MPLA-primed mice challenged with *S. aureus* (Figure III-4D). I then assessed whether the protective macrophages were derived from inflammatory monocytes by employing $CCR2^{-/-}$ mice. CCR2 is required for inflammatory monocyte emigration from the bone marrow, and $CCR2^{-/-}$ mice have a diminished circulating $Ly6C^{+}$ inflammatory monocyte pool[283]. Wild type and $CCR2^{-/-}$ mice had similar survival rates during *S. aureus* infection and MPLA successfully protected both $CCR2^{-/-}$ and wild type mice (Figure III-4E).

Monocytes can fuel increasing macrophage numbers, so it was interesting that $CCR2^{-/-}$ mice were protected by MPLA. In order to assess this further, histological studies of $CCR2^{-/-}$ mice were performed. Upon immunohistochemical analysis, MPLA-primed $CCR2^{-/-}$ mice demonstrated improvements in bacterial clearance and $F4/80^{+}$

staining in kidney and lung that was comparable to wild type (Figure III-5A-D). Thus, MPLA protects against *S. aureus* through a mechanism involving the augmented antimicrobial capacity and recruitment of tissue macrophages to microbial foci, with help from the neutrophil pool.

MPLA induces persistent augmentation of tissue macrophage and neutrophil anti-microbial function

Macrophages persist after inflammatory stimuli such as MPLA, suggesting that they sustain an augmented antimicrobial phenotype that facilitates improved *S. aureus* clearance. In order to determine if MPLA induces persistent alterations to leukocyte antimicrobial capacity *in vivo*, I employed a model of intraperitoneal *S. aureus* infection. In collaboration with Dr. Julia Bohannon, I first assessed whether MPLA could induce resistance to peritoneal *S. aureus* infection. WT mice were injected with intraperitoneal MPLA and then challenged with 10^8 cfu intraperitoneal *S. aureus* 24 hours later. Similar to intravenous *S. aureus*, MPLA-primed mice had significantly reduced bacterial burden (Figure III-6A). Further, experiments in collaboration with Dr. Julia Bohannon demonstrated that MPLA increased the number of neutrophils, macrophages, and monocytes in the peritoneal cavity (data not shown).

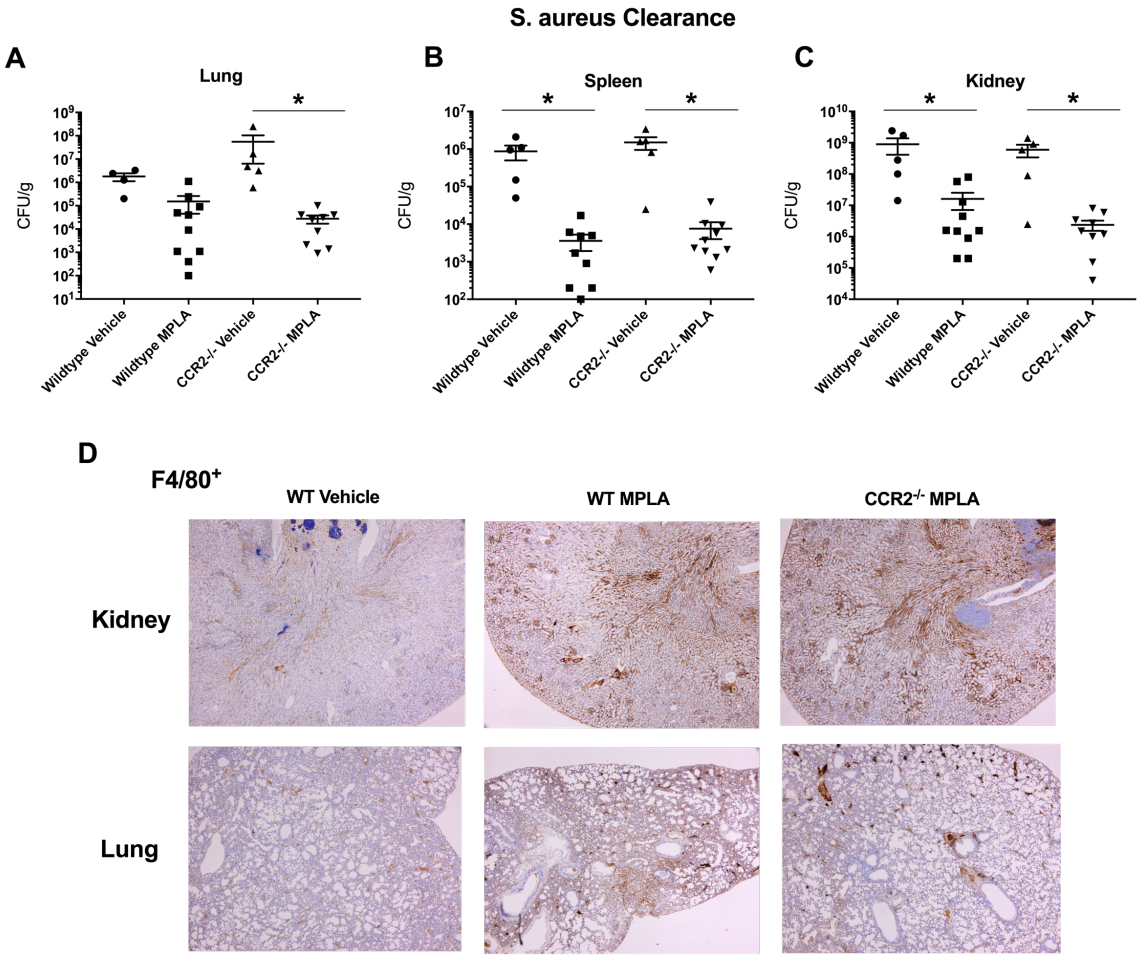


Figure III-5 Loss of inflammatory monocytes does not diminish MPLA-mediated alterations to bacterial clearance or the macrophage pool.

WT and CCR$^{-/-}$ mice were primed with MPLA and then challenged with 10^8 S. aureus. A) Kaplan-Meier survival plot of mice after *S. aureus* infection. (n=5 mice per group). Mice were sacrificed 3 days after inoculation. *S. aureus* cfu per gram of tissue recoverable from whole A) lung, B) spleen and C) kidney. D) F4/80$^+$ immunohistochemical staining in kidney and lung sections. Data shown as mean +/- SEM. *, $p<.05$ as determined via one-way ANOVA with Tukey's *post hoc* test.

In order to assess leukocyte phagocytosis, pHrodo-tagged *S. aureus* was injected into the periotoneal cavity of MPLA-primed mice 24 hours after the last MPLA injection. pHrodo only fluoresces upon a significantly decreased pH, such as that found in the phago-lysosome. Six hours after pHrodo-tagged *S. aureus* injection, peritoneal fluid was harvested and different myeloid populations were assessed for pHrodo MFI by flow cytometry (Figure III-6B). On a per cell basis, there was no difference between the level of neutrophil pHrodo MFI in vehicle and MPLA primed animals. Similarly, monocytes showed no differences in pHrodo MFI. However, macrophages in MPLA-primed animals had significantly increased phagocytosis of *S. aureus* bioparticles. Next, the ability of these leukocytes to engage in respiratory burst, another key antimicrobial function, was assessed. Peritoneal leukocytes were exposed to dihydrorhodamine (DHR) 123, a non-fluorescent molecule that becomes fluorescent when exposed to reactive oxygen species, and then challenged the cells with a PMA stimulus (Figure III-6C). Neutrophils from MPLA primed animals also demonstrated significantly more DHR than neutrophils from vehicle mice whereas monocytes from vehicle and MPLA-primed mice had similar levels of DHR. Macrophages from MPLA-primed mice exhibited significantly elevated PMA-elicited DHR staining. Thus, MPLA priming elevates the capacity of neutrophil and macrophage antimicrobial processes in response to *S. aureus*.

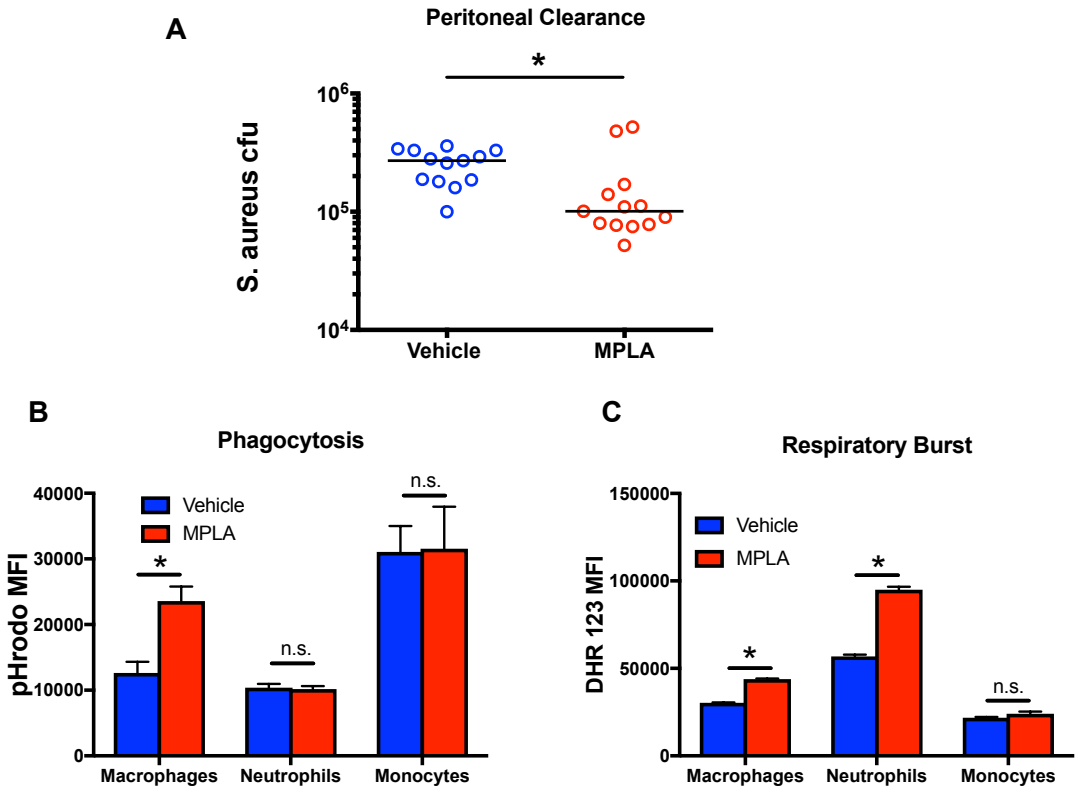


Figure III-6. MPLA augments the antimicrobial capacity of peritoneal macrophages and neutrophils.

WT BALB/c mice were primed with intraperitoneal MPLA for two consecutive days and then challenged with intraperitoneal 10^8 S. aureus 24 hours after the last dose of MPLA. A) Bacterial burden in S. aureus infected mice 6 hours after inoculation. B) Mice were primed with MPLA or vehicle via intraperitoneal injection and 24 hours later pHrodo tagged *S. aureus* bioparticles were injected into the peritoneal cavity. Peritoneal leukocytes were harvested 6 hours later and assessed for pHrodo MFI via flow cytometry. C) Mice were primed with MPLA or vehicle via intraperitoneal injection and 24 hours later leukocytes were harvested from the peritoneal cavity. Respiratory burst was elicited by 45 minutes of PMA stimulation and DHR 123 MFI of macrophages, neutrophils, and monocytes by.flow cytometry. Data shown as mean +/- SEM. *, $p < .05$ as determined by t-test or ANOVA with Tukeys post-hoc multiple comparison analysis

Discussion

Overall, these results demonstrate that MPLA prophylaxis induces resistance to *S. aureus* bloodstream infection by modulation of macrophage and neutrophil antimicrobial capacity. I found that when mice were depleted of either macrophages or neutrophils, resistance was significantly attenuated. I demonstrated striking histological evidence that the tissue macrophage pool is increased after infection, further implicating the role of macrophages in resistance. Additionally, tissue macrophages and neutrophils, but not monocytes demonstrated augmented *in vivo* functional capacities. These findings underscore the ability of MPLA to induce persistent beneficial alterations to innate immunity in a non-specific manner.

While the Sherwood lab has previously established a role for neutrophils and G-CSF in MPLA-mediated resistance to local peritoneal *P. aeruginosa* infection[232], here I find that both tissue macrophages and neutrophils are required for MPLA-mediated protection against systemic *S. aureus*. It has been suggested that tissue resident macrophages are responsible for clearing the majority of bacteria from the bloodstream[230,284]. These mature tissue macrophages are generated prior to birth, persist throughout inflammation, and can undergo a proliferative burst after inflammatory stimuli are cleared[285-287]. My data suggest that tissue resident macrophages present at the time of MPLA exposure, rather than monocyte recruited macrophages, are mediating MPLA-induced resistance to infection. While these data show that MPLA increases monocyte tissue infiltration, MPLA successfully protected $CCR2^{-/-}$ mice, suggesting that recruitment of CCR2-dependent $Ly6C^{high}$ inflammatory monocytes is dispensable for MPLA-mediated resistance to *S. aureus*. Despite deficiencies in monocyte emigration

from the bone marrow, CCR2$^{-/-}$ mice retain normal macrophage populations[288], and these data show that MPLA induces similar increases in macrophage numbers in WT and CCR2$^{-/-}$ mice. Thus, my data show that macrophages increase their presence around infectious foci though improved migration, improved survival during infection, and a proliferative burst, rather than contribution from the inflammatory monocyte pool. CCR2$^{-/-}$ mice do retain CCR2 independent Ly6C^{-} monocytes, but it is unlikely that these monocytes are contributing to the increasing F4/80^{+} macrophage pool as they are anti-inflammatory[289]. It is possible that monocyte recruitment is important for other causes of systemic bloodstream infection, but this remains to be explored.

The Sherwood lab has previously found that macrophages are dispensable for early resistance to intraperitoneal *P. aeruginosa* model, yet in the systemic *S. aureus*, macrophages are essential. Why the discrepancy? First, the requirement for neutrophils in early resistance to intraperitoneal *P. aeruginosa* is consistent with their roles as early responders to bacterial infection and as mediators of tissue pathogen clearance. MPLA can elicit G-CSF upon administration, and this G-CSF can support the generation and survival of neutrophils[232]. Additionally, the experiments in Romero et. al. (2011) that established the requirement for neutrophils against *P. aeruginosa* only assessed the first 6 hours of infection[252]. The experiments with systemic *S. aureus* presented here were conducted over 14 days[252]. It is possible that as peritoneal *P. aeruginosa* disseminates throughout the body, macrophages will become more important in host resistance to infection. The Sherwood lab has also shown that neutrophils are required for resistance to slowly progressive models of *P. aeruginosa* infection, such as a burn wound infection, but the requirement of macrophages in these models remains to be determined [232].

Histological analysis of organs from *S. aureus* infected mice demonstrated areas of co-localization between macrophages and neutrophils. There are a variety of explanations for this observed phenomenon. First, macrophages can direct neutrophils to infectious foci by the release of chemokines[290,291]. If macrophages persist at the site of chemokine release, they will co-localize with neutrophils, which can increase the antimicrobial capacity of macrophages[292]. An alternative explanation is that macrophages are neutrophils are both recruited by the same stimulus through receptors that initiate chemotaxis to bacterial products. For example, both macrophages and neutrophils contain G-protein coupled n-formyl peptide receptors, which, similarly to chemokine receptors, potently mobilize neutrophils and macrophages to areas of increasing n-formyl peptides such as n-formyl methionine produced by bacteria[293]. Regardless of the mechanism of co-localization, macrophages and neutrophils can complement each other's antimicrobial capabilities[292]. Both cell types can phagocytose and kill pathogens, and macrophages have the ability to clear out dead neutrophils, making room for newly recruited neutrophils[294]. Thus, the areas of cellular co-localization likely indicate successfully mitigated infectious foci, as they are only present in MPLA primed mice.

Broad changes to leukocytes are a hallmark of immunosuppression in the critically ill [8,21]. For example, patients with recent sepsis have significant apoptosis of both innate and adaptive cells [8]. Burn patients, some of the most at risk of nosocomial infection, undergo an immunological shift towards T-lymphocytes expressing Th2 markers, and increase the development of T_{regs} [295]. In human adults, a loss of effective adaptive processes places a greater burden on the innate immune system to fight infections. When innate processes are not strong enough, or are dysfunctional themselves,

severe infections can develop. Patients that succumb to sepsis have been found to have an elevated bacterial burden near death, indicating that an inability to mount efficient antimicrobial processes and clear bacteria plays an important role in facilitating mortality [296]. Modulating neutrophil and macrophage antimicrobial function is a promising strategy to restore immune competency in these immune-compromised hospitalized patients.

Overall, *S.aureus* is the leading cause of catheter associated bloodstream infections and, these data demonstrate that MPLA is an effective prophylaxis against *S. aureus* bloodstream infection. There are additional studies that could be undertaken to demonstrate greater clinical relevance of this resistance. First, future studies should explore whether this resistance applies to some of the most difficult to treat multi-drug resistant *S. aureus* isolates. Moreover, future studies should explore the ability of MPLA to protect against *S. aureus* infection in models of immunodeficiency, such as a burn injury or surgical model. Clinical assessments of the ability of MPLA to induce resistance to infection should be undertaken. Finally, while these data clearly implicate macrophages in MPLA-induced resistance to *S. aureus,* the molecular mechanisms by which MPLA alters macrophage biology to achieve this end are unknown. Chapter IV and Chapter V begin to dissect the molecular mechanisms underlying this resistance.

CHAPTER IV

TLR4 AGONISTS DRIVE PERSISTENT AND DYNAMIC METABOLIC REPROGRAMMING OF MACROPHAGES

Scientific Goal

TLR agonists induce persistent resistance to infection through modulation of the innate immune system. Specifically, this resistance is due to alterations to tissue macrophage and neutrophil populations. Confusingly, evidence that TLR primed macrophages are endotoxin tolerant has led to the repeated assertion that they are non-functional and permit infections to occur. Largely because of this assertion, the molecular mechanisms by which TLR agonists augment resistance to infection are unknown. In this chapter, I begin to unravel the molecular mechanisms of resistance by exploring how TLR agonists, particularly the TLR4 agonist MPLA, alters macrophage metabolism. These studies demonstrate that endotoxin tolerance does not entail metabolic quiescence in macrophages. Further, these studies pave the way for future studies to identify functional outcomes of persistent macrophage metabolic reprogramming, such as I will explore in Chapter V.

Introduction

Macrophages respond to microbial products by undergoing metabolic reprogramming that supports their functional goals. Gross analysis of this metabolic reprogramming has been studied for over 50 years[297], but the ability to dissect the specific purposes for macrophage metabolic reprogramming has been limited by technology until recently. The identification of metabolic pathways used by macrophages

to achieve functional endpoints opens up the possibility that new therapeutics can specifically target macrophage metabolism.

Numerous studies on macrophage metabolism have demonstrated that macrophages engage in a metabolic phenotype of aerobic glycolysis in response to TLR agonists[298]. Aerobic glycolysis is characterized by increased glucose consumption with unchanged or decreased oxygen consumption and is utilized by macrophages to drive cytokine production and phagocytosis[187,202]. Aerobic glycolysis is coordinated by activation of the PI3K/mTOR/HIF-1α signaling cascade, which results in expression of the positive regulators of glycolysis phosphofructokinase-2 (PFK-2) and hexokinase-2 (HK-2), shuttling of GLUT1 transporters to the plasma membrane, and up-regulation of lactate dehydrogenase[202,203,299]. This process increases the rate of glycolysis rapidly. Rapid glycolysis generates ATP as well as fatty acids for expansion of the endoplasmic reticulum and Golgi apparatus required to package and secrete cytokines[202].

Interestingly, macrophage mitochondria oxidize a small fraction of the consumed glucose. The reason for this is that TLR4 activation also causes two significant breaks in the macrophage tricarboxylic acid (TCA) cycle: one in the conversion of citrate to isocitrate, and one in the conversion of succinate to fumarate[300]. Rather than flux through the TCA cycle, glucose-derived citrate is exported to the cytosol for fatty acid synthesis or converted into the antimicrobial metabolite itaconate[301-303]. Succinate dehydrogenase (SDH) is inhibited by metabolites like itaconate, and the resulting accumulation of succinate can facilitate secretion of the pro-inflammatory cytokine IL-1β[188,304]. Further, inflammatory macrophages generate significant quantities of nitric oxide, which can inhibit complex I of the electron transport chain[305,306]. Overall, these deviations to TCA

cycle and electron transport chain (ETC) function reduce the ability of macrophages to utilize the increased glucose consumption for NADH generation and ATP production.

Despite major advances in understanding how early TLR activation reprograms macrophage metabolism, little is known about how macrophage metabolic programming evolves once a stimulus has been cleared. From a functional standpoint, it is well established that macrophages exposed to TLR4 agonists do not return to a pre-activated state following agonist clearance, rather, they endure persistent endotoxin tolerance. Thus, macrophage metabolism may be persistently altered after TLR agonist clearance.

Here, I hypothesize that early metabolic reprogramming persists after TLR agonist clearance. Prior to metabolic assessments, I first identify an improved way by which mature primary bone marrow derived macrophages can be generated for *in vitro* study. Then, I identify that early TLR-induced glycolysis is sustained following TLR agonist clearance, but that suppression of mitochondrial oxygen metabolism rapidly recovers. Further, I demonstrate that a major part of this recovery is due to a previously unrecognized mechanism of malate shuttling in macrophages that drives cytosolic NADH into the mitochondria. Overall, I demonstrate that MPLA induces a sustained but surprisingly dynamic macrophage metabolic program characterized by early aerobic glycolysis that eventually progresses to heightened mitochondrial metabolism.

Initial bone marrow cell density impacts the growth of bone marrow derived macrophages differentiated in M-CSF

Due to their relative infrequency compared to other cell types in tissues, macrophages are difficult to isolate from the host. Macrophages that can be isolated

rapidly change their phenotype once removed from the host[307]. Thus, in order to probe macrophage function, better *in vitro* models are required.

The most heavily used *in vitro* macrophage today is the murine bone marrow derived macrophage (BMDM)[308,309]. These macrophages can be produced in large quantities from primary mouse bone marrow. However, the conditions with which these cells are grown, particularly the growth density and type of growth factor supplementation, can vary significantly between research groups[308]. The two major differentiation methods include growth in L929 fibroblast conditioned media or M-CSF alone[310]. L929 fibroblasts produce M-CSF and growth factors such as GM-CSF, and IL-3, that can encourage the growth of macrophages[307]. These additional growth factors can support the health of BMDMs, but can also polarize them in unexpected ways. For this reason, M-CSF alone is considered the gold standard for macrophage differentiation[311].

A recent study by Lee et. al. (2013) demonstrated that bone marrow cell density during BMDM differentiation in L929 growth media has a significant impact on the maturity of bone marrow derived macrophages[312]. This study found that more mature, pure macrophage populations arise when cells are grown at a low density of starting bone marrow, compared to a high density. However, there are no studies evaluating differences between cell densities at differentiation for BMDMs differentiated with M-CSF.

In order to assess this, I differentiated mouse bone marrow in M-CSF alone with an initial bone marrow cell density of $1x10^7$, $2x10^6$, or $4x10^5$ cells per $10cm^2$ petri dish. Following 7 days, I assessed the resulting cells. Cells grown with a starting bone marrow density of $1x10^7$ resulted in the greatest number of BMDMs per plate (Figure IV-1A).

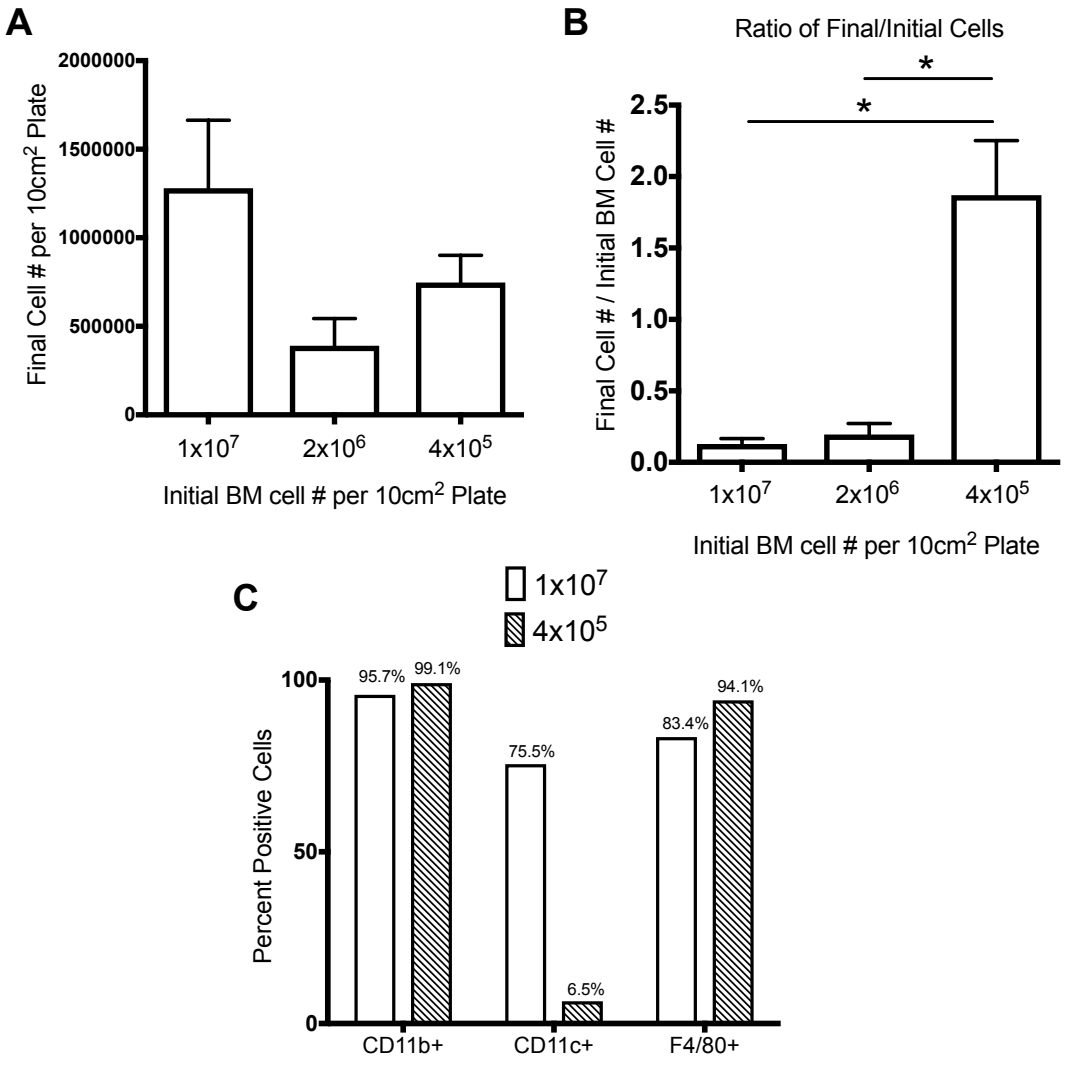


Figure IV-1. Bone marrow cell density during M-CSF macrophage differentiation alters the growth and phenotype of macrophages.

Bone marrow was collected from mouse femurs and re-suspended and plated in 10cm2 petri dishes at either $1x10^7$, $2x10^6$, or $4x10^5$bone marrow cells per dish. A) Total cells attached to the petri dish following a 7 day incubation. B) Relative yield of the final cell numbers obtained compared with the starting bone marrow density. C) Percent positive CD11b, CD11c, and F4/80 cells isolated from petri dishes following differentiation.

However, cells differentiated at $4x10^5$ bone marrow cells per plate produced a significantly greater yield than cells at $1x10^7$ or $2x10^6$ (Figure IV-1B). Additionally, I compared cells grown from $1x10^7$ and $4x10^5$ by surface marker expression. Cells grown with a starting density of $4x10^5$ had greater $CD11b^+$ staining, lower $CD11c^+$ staining, and greater $F4/80^+$ staining than cells grown with a starting density of $1x10^7$ (Figure IV-1C). Thus, bone marrow density during differentiation in M-CSF has a significant impact on the yield and phenotype of the resulting BMDMs, and a starting concentration of $4x10^5$ bone marrow cell per $10cm^2$ plate produces more mature macrophages at a better yield. All experiments using bone marrow derived macrophages in this dissertation utilize BMDMs grown in M-CSF with an initial bone marrow concentration of $4x10^5$ cells per $10cm^2$.

Endotoxin tolerant macrophages exhibit persistent alterations to glycolytic and oxidative metabolism, despite endotoxin tolerance

My *in vivo* data suggest that the innate immune system can sustain an immunoprotective phenotype despite endotoxin tolerance. To explore this phenomenon on a cellular level, I examined the metabolism and function of bone marrow derived macrophages (BMDMs) following exposure to multiple TLR ligands (Figure IV-2A). MPLA-, LPS-, CpG-ODN-, and poly(I:C)- primed BMDMs exhibited reduced IL-6 secretion in response to LPS, reflective of endotoxin tolerance (Figure IV-2B). A similar tolerance profile was noted after exposure to heat-killed *P. aeruginosa* (Figure IV-2C). To assess broader macrophage activity, macrophage glycolytic metabolism was examined by the Seahorse glycolysis stress test. MPLA-, LPS-, CpG-ODN- and poly(I:C)- primed BMDMs displayed an significantly elevated glycolytic rate compared to control (Figure IV-2D-E).

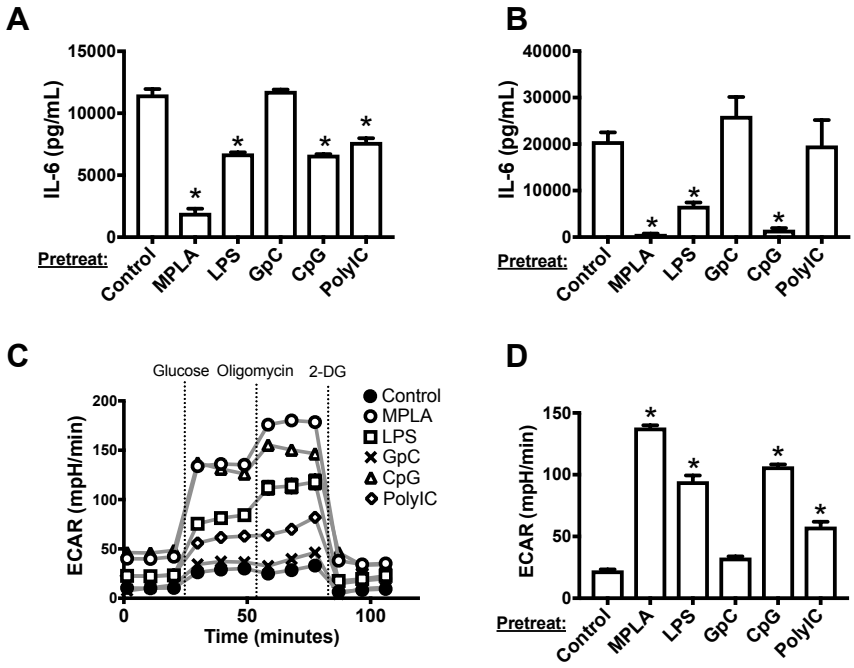


Figure IV-2 TLR-ligand primed macrophages exhibit persistent alterations to glycolytic metabolism.

A) Bone marrow derived macrophages (BMDMs) were exposed to 100ng/mL LPS, 1μg/mL MPLA, 10μg/mL CpG-ODN, 10μg/mL GpC-ODN, or 10μg/mL poly(I:C) for 24 hours, washed, rested for 3 days in media without TLR ligands and then assessed. For all assays, all BMDMs were plated at a density of $5x10^5$ cells/mL. **B)** BMDMs were stimulated with 100ng/mL LPS for 24 hours and IL-6 was measured by ELISA. **C)** BMDMs were stimulated with $1x10^6$ CFU/mL of heat-killed *P. aeruginosa* for 24 hours and IL-6 was measured by ELISA. **D)** Glycolysis stress test of primed BMDMs on the Seahorse XF^e96. BMDMs were sequentially exposed to 10mM glucose, 1μM oligomycin, and 50mM 2-deoxyglucose (2-DG) **E)** Glycolytic capacity as determined by the Seahorse XF^e96. Glycolytic capacity is determined following the addition of oligomycin. Data shown as mean +/- SEM. *, $p< .05$ as determined by ANOVA with Tukeys post-hoc multiple comparison analysis. n=3-5 replicates for each experiment. Figures shown are representative of at least two repeated experiments.

MPLA-primed macrophages switch from aerobic glycolysis to energy metabolism

Cellular metabolism governs immune activity, and elevated glycolytic metabolism during endotoxin tolerance may be a reflection of a broadly modified metabolic state. Because TLR agonists induce aerobic glycolysis, I first wondered whether this metabolic reprogramming reflected a persistence of early aerobic glycolysis or an adapted metabolic program. In order to investigate this, I exposed bone marrow derived macrophages to MPLA for 24 hours, washed and rested the cells for 3 days, and then performed metabolic assessments. These macrophages are referred to as 3 days post MPLA for the rest of the dissertation (3dp macrophages). I compared 3dp macrophages to pro-inflammatory macrophages exposed to MPLA for 24 hours prior to assessment (24hr macrophages), as well as control macrophages (Figure IV-3A). First, I determined the extracellular acidification rate (ECAR) of the macrophages by the Seahorse glycolysis stress test. I found that the glycolytic rate and the maximal glycolytic rate was significantly higher in 24hr and 3dp macrophages compared to control (Figure IV-3B-C). The Seahorse data were confirmed by glucose consumption and lactate production measurements, demonstrating that 3dp MPLA macrophages sustain an elevated glycolytic rate (Figure IV-3D-E).

Mitochondrial function recovers following MPLA clearance

The aerobic glycolysis phenotype is associated with an unchanged or reduced oxygen consumption rate, so I next performed a Seahorse mitochondrial stress test to determine the basal and maximal oxidative rate. 3dp macrophages exhibited a significantly elevated basal and maximal oxidative rate, whereas the basal oxidative rate of 24hr macrophages was unchanged and the maximal oxidative rate was suppressed

compared to controls (Figure IV-4A-B). The combination of augmented glycolysis and oxidative metabolism in 3dp macrophages suggests these macrophages are producing abundant ATP. Indeed, I found that 3dp macrophages were producing more ATP than control macrophages or 24hr macrophages, and that the relative increase in ATP production could be blocked by the addition of oligomycin, but not 2-DG (Figure IV-4C). This metabolic state was not unique to MPLA, as LPS and CpG-ODN induced a similar metabolic phenotype (data not shown). Thus, early glycolysis is sustained following MPLA clearance, but suppressed oxidative metabolism recovers.

I next investigated possible mechanisms for the increased oxygen consumption. The density of mitochondria can be determined by Mito Tracker Green staining, the absolute mitochondria DNA to genomic DNA ratio, and by comparing the expression of mitochondrial enzymes, such as succinate dehydrogenase and citrate synthase. MitoTracker Green MFI was elevated in 3dp macrophages, and, to a more limited extent, in 24hr macrophages (Figure IV-4D). Relative mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA) levels were also elevated in 24hr and 3dp macrophages (Figure IV-4E). Finally, cellular succinate dehydrogenase and citrate synthase content was increased in 3dp macrophages compared to control and 24 hour macrophages (Figure IV-4F). More mitochondria do not necessarily imply functional mitochondria. TLR4 agonists induces the expression of iNOS, which disrupts oxygen consumption via nitric oxide (NO)-mediated complex 1 inhibition[313]. While 24hr macrophages had significantly increased NO production, this dropped to control levels in 3dp macrophages (Figure IV-4G). Thus, early glycolysis is sustained following MPLA priming, but early disruptions to oxygen consumption recover along with mitochondrial biogenesis, driving ATP production.

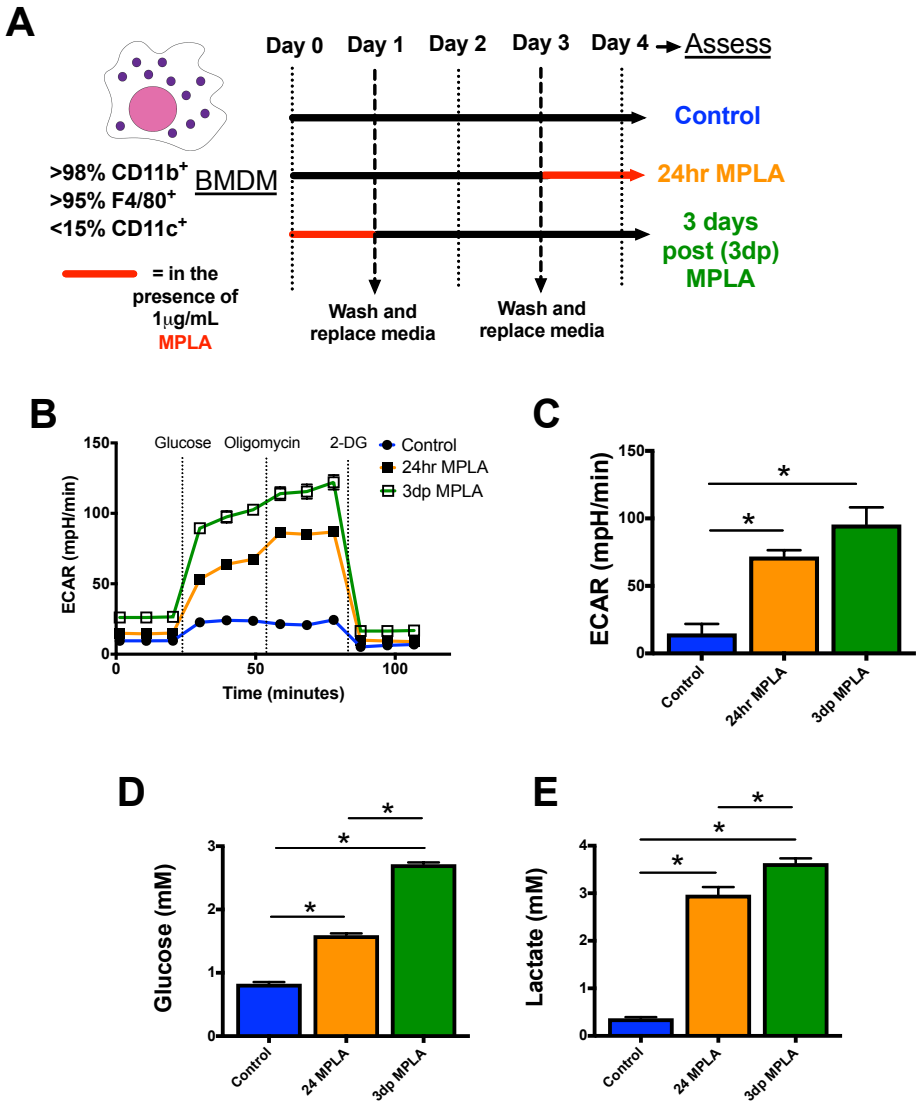


Figure IV-3 TLR-mediated glycolytic alterations are initiated during priming and are persistent

A) Bone marrow derived macrophages (BMDMs) were primed with MPLA at 1μg/mL for 24 hours, washed twice with PBS and rested for 3 days (3dp macrophages). These macrophages were compared to BMDMs stimulated with MPLA for 24 hours prior to assessment (24hr macrophages) and control BMDMs. For experiments assessing BMDMs over 24 hours, the 24hr macrophages were stimulated from day 4-5 rather than 3-4. B) Glycolysis stress of macrophages as determined by the Seahorse Xf^{e}96. C) Maximal glycolytic rate derived after the addition of oligomycin in the glycolysis stress test D) Glucose consumed from BMDMs over 24 hours. E) Lactate produced by BMDMs over 24 hours. All experiments representative of at least two replicates. Data shown as mean +/- SEM. *, p< .05 as determined by ANOVA with Tukey's *post-hoc* multiple comparison analysis. n=3-7 replicates for each experiment. Figures shown are representative of at least three repeated experiments.

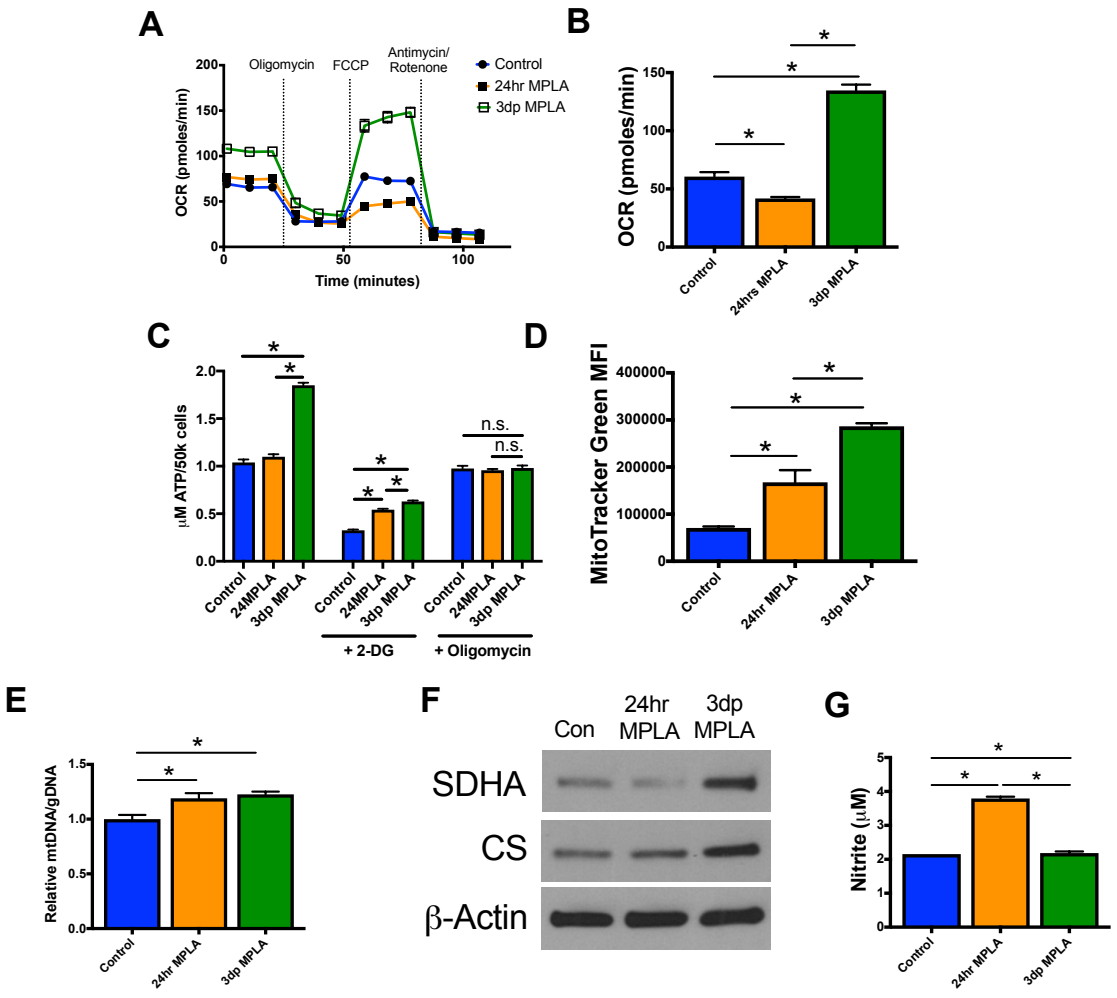


Figure IV-4. Mitochondrial function recovers following MPLA clearance

A) Mitochondrial stress test of BMDMs as determined by the Seahorse Xf^{e}96. B) Maximal oxidative rate derived after the addition of FCCP in the mitochondrial stress test. C) Intracellular ATP from BMDMs as determined by luminescence assay. Some BMDMs were exposed to 10mM 2-DG or 1µM oligomycin for 3 hours prior to the assay. D) MitoTracker Green staining as determined by flow cytometry. E) Mitochondrial DNA (mtDNA) / nuclear DNA (gDNA) as determined by qPCR. F) Succinate dehydrogenase (SDHA), citrate synthase (CS), and beta-actin as determined by western blot. Blot representative of 3 replicate experiments. G) Nitrate concentration in the media produced by BMDMs over 24 hours. All experiments representative of at least two replicates. Data shown as mean +/- SEM. *, p< .05 as determined by ANOVA with Tukey's *post-hoc* multiple comparison analysis. n=3-5 replicates for each experiment. Figures shown are representative of at least two repeated experiments.

Malate shuttling sustains elevated mitochondrial activity in MPLA-primed macrophages

I observed that, compared to 24hr macrophages, 3dp macrophages convert a lesser proportion of their consumed glucose to lactate (Figure IV-3D-E). In order to determine how macrophages are utilizing glucose, I performed ^{13}C-glucose metabolic flux analysis and, with the help of collaborators Dr. Jamey Young and Allison McAtee, modeled the flux rates of major metabolic enzymes in the TCA cycle. This model is determined by dissecting the enrichment patterns of ^{13}C into different metabolites and, combined with glucose consumption measurements and oxygen consumption measurements derived from the Seahorse XF^{e}96, the absolute flux rates in pmoles/cell/day of different metabolic enzymes can be calculated. Compared to control macrophages, 24hr macrophages expectedly had increased glycolytic flux and decreased fatty acid oxidation. Additionally, 24hr macrophages exhibited significantly increased citrate export to the cytosol, and increased compensatory glutamine anaplerosis (Figure IV-5A).

Cytosolic citrate is converted to cytosolic oxaloacetate and acetyl-coA by ATP-citrate lyase, which fuels fatty acid synthesis. Cytosolic oxaloacetate can then by converted to malate by malate dehydrogenase 1 through the incorporation of NADH[314]. If this malate is then converted to pyruvate via malic enzyme 1, the electrons from this NADH are regenerated as cytosolic NADPH. Instead, if this malate is transported into the mitochondria, the malate will be converted back to oxaloacetate by malate dehydrogenase 2, generating mitochondrial NADH. NADH is impermeable to the mitochondrial membrane and metabolite shuttling mechanisms such as this are the only way for

cytosolic NADH equivalents to enter the mitochondria. Notably, 24hr macrophages recycled all of this cytosolic oxaloacetate back to pyruvate via malic enzyme 1.

Compared to 24hr macrophages, 3dp macrophages elevated glycolysis even further; driving slightly increased citrate export, but significant up-regulation of isocitrate dehydrogenase, and decreased compensatory glutamine anaplerosis (Figure IV-5A). Most surprisingly, instead of recycling cytosolic oxaloacetate to pyruvate, 3dp macrophage shuttled a significant amount of the malate intermediate into the mitochondria, and down-regulated malic enzyme 1 flux. Correlating with increased malate shuttling, 3dp macrophages exhibited dramatically increased NADH and FADH2 flux into H_2O, indicating greater ATP synthesis via oxidative phosphorylation (Figure IV-5B).

Malate shuttling was further examined by determining the relative flux rates of each reaction that feeds citrate synthase (Figure IV-5C). These rates are calculated with the rate of each reaction set relative to the rate of citrate synthase. In 3dp macrophages, cytosolic-derived malate, compared to fumarate hydratase-derived malate, constituted a greater proportion of the malate used by malate dehydrogenase 2. Malate dehydrogenase 2-derived oxaloacetate, compared to pyruvate carboxylase-derived oxaloacetate, constituted a significantly greater proportion of the oxaloacetate used by citrate synthase. The relative contribution of fatty acid oxidation to the acetyl-coA pool was significantly decreased in 24hr macrophages, but began to return to control in 3dp macrophages. Part of the enrichment data used to generate the flux model along with the total absolute and relative flux rates are shown in Figure IV-6 as well as Table IV-1 and Table IV-2. Overall, these data demonstrate that 3dp macrophages adapt to early MPLA-induced disruptions by utilizing a malate shuttle to drive NADH into the mitochondria.

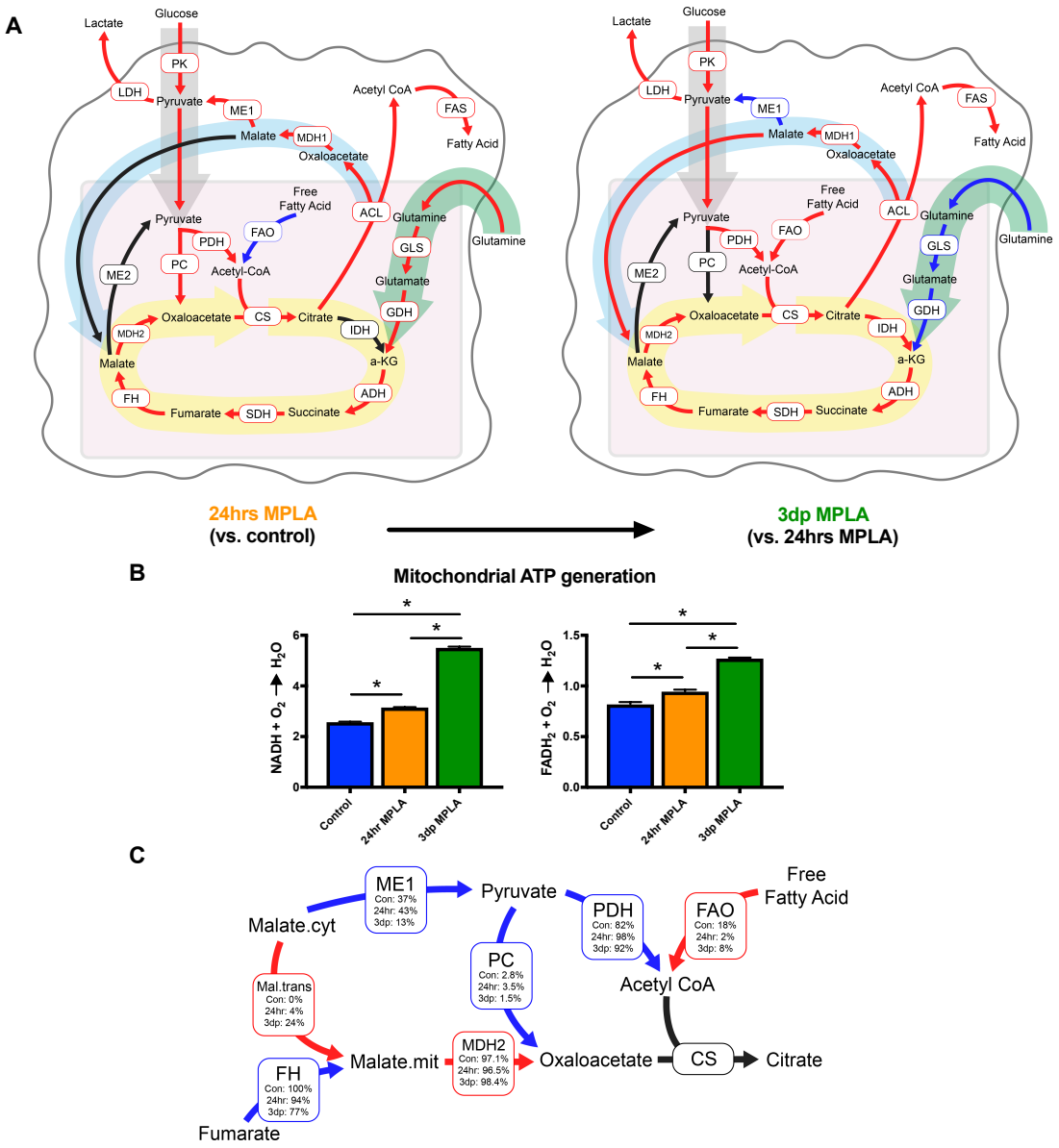


Figure IV-5. Malate shuttling sustains elevated mitochondrial activity in MPLA primed macrophages.

BMDMs were incubated in complete media with U-^{13}C-glucose for 24 hours. Metabolites were assessed by GC-MS and mapped using INCA. A) Absolute flux rate map of 24hr MPLA macrophages. Red and blue arrows indicate statistically significant increases or decreases, respectively, compared to control cells. B) Absolute flux rate map of 3dp MPLA macrophages. Red and blue arrows indicate statistically significant increases or decreases, respectively, compared to 24hr macrophages. C) Absolute NADH and $FADH_2$ into H_2O flux in pmole/cell/day as derived from INCA. D) Relative enzyme flux map of 3dp macrophages. All enzyme reactions set as relative to CS rate at 100%, then compared across groups. Red and blue arrows indicate significant increases or decreases, respectively, in 3dp macrophages compared to 24hr macrophages. Significance between groups identified by $p < .05$ by ANOVA with Tukey's *post-hoc* test.

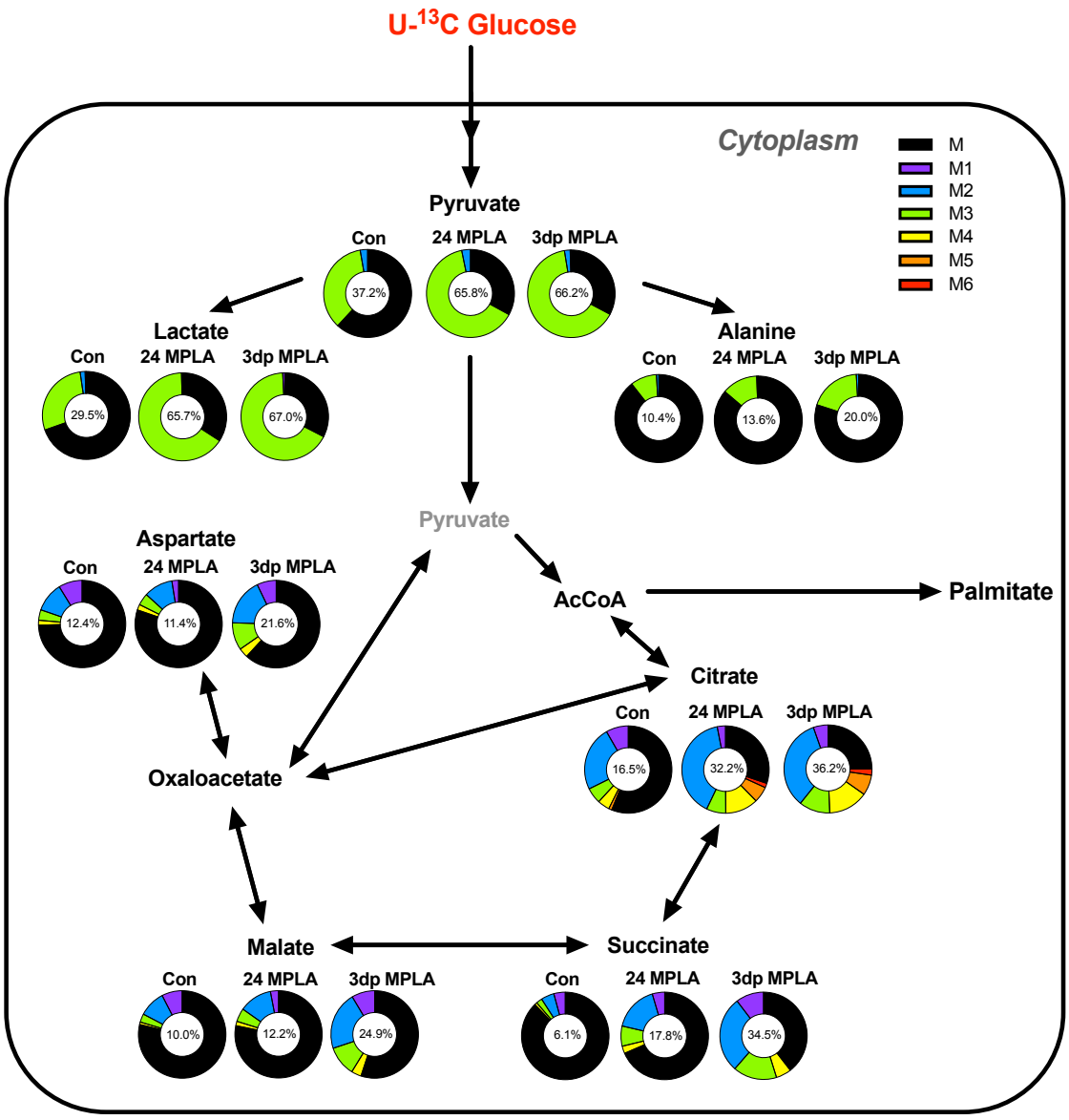


Figure IV-6 MPLA-primed macrophages have greater ^{13}C-glucose enrichment into TCA cycle metabolites

BMDMs were exposed to media where the only glucose source was U-^{13}C-glucose. After 24 hours, BMDMs were harvested and metabolites were assessed by GC-MS. Each donut represents the fraction of metabolite that contains M+X number of ^{13}C isotopes for control 24hr macrophages and 3dp macrophages. Values in the center of the donut indicate total ^{13}C enrichment of that metabolite.

	Control		24hrs MPLA		3dp MPLA	
	Mean	±SEM	Mean	±SEM	Mean	±SEM
PK	4.73	0.15	9.11	0.16	15.50	0.16
LDH net	4.09	0.17	8.22	0.21	13.11	0.20
Pyruvate-trans	1.12	0.05	1.93	0.01	3.06	0.09
PDH	1.13	0.05	1.93	0.04	3.06	0.08
PC	0.04	0.00	0.07	0.00	0.05	0.01
CS	1.38	0.03	1.96	0.01	3.40	0.10
IDH net	0.89	0.02	0.83	0.01	1.53	0.03
ADH	1.38	0.01	1.85	0.02	2.21	0.05
SDH	1.38	0.01	1.85	0.02	2.21	0.05
FH net	1.38	0.01	1.85	0.02	2.21	0.05
MDH2 net	1.34	0.03	1.89	0.01	3.34	0.11
ME2	0.04	0.01	0.06	0.03	0.05	0.02
GLS net	0.49	0.03	1.03	0.03	0.68	0.03
GDH net	0.49	0.03	1.03	0.03	0.68	0.03
FAO	0.25	0.04	0.03	0.03	0.33	0.04
ACL	0.48	0.05	1.13	0.03	1.86	0.13
MDH1 net	0.48	0.05	1.13	0.03	1.86	0.13
ME1	0.48	0.05	1.04	0.05	0.68	0.05
Malate-trans	0.00	0.00	0.09	0.04	1.19	0.05
FAS	0.48	0.05	1.13	0.03	1.86	0.13
NADH -> H_2O	2.57	0.02	3.15	0.02	5.50	0.05
$FADH_2$ -> H_2O	0.82	0.02	0.94	0.02	1.27	0.01

Table IV-1. Absolute flux rates of metabolic enzymes after MPLA as plotted in Figure IV-5A

Absolute metabolic flux rates were determined by oxygen consumption derived from the Seahorse Xf^{e}96 basal oxygen consumption measurement and glucose consumed during the ^{13}C-glucose labeling experiment. All values are in the units of pmoles/cell/day.

	Control		24hrs MPLA		3dp MPLA	
	Mean	±SEM	Mean	±SEM	Mean	±SEM
PK	77.7	40.8	146.2	65.4	70.1	7.2
LDH net	33.5	39.6	90.8	73.4	-7.2	7.2
Pyrtrans	81.6	3.2	98.9	0.3	90.7	1.9
PDH	81.9	3.0	98.3	1.7	92.2	1.1
PC	2.9	0.2	3.5	0.2	1.6	0.2
CS	100.0	0.0	100.0	0.0	100.0	0.0
IDH net	62.7	2.7	51.9	8.2	62.3	1.7
ADH	100.2	1.0	94.8	1.1	77.2	3.8
SDH	100.2	1.0	94.8	1.1	77.2	3.8
FH net	100.2	1.0	94.8	1.1	77.2	3.8
MDH2 net	97.1	0.2	96.5	0.2	98.4	0.2
ME2	3.1	1.1	3.0	1.6	3.1	1.1
GLS net	37.6	2.2	42.9	9.3	14.9	5.0
GDH net	37.6	2.2	42.9	9.3	14.9	5.0
AST1 net	0.0	0.0	0.0	0.0	0.0	0.0
FAO	18.1	3.0	1.7	1.7	7.8	1.1
ACL	37.3	2.7	48.1	8.2	37.7	1.7
MDH1 net	37.3	2.7	48.1	8.2	37.7	1.7
ME1	37.3	2.7	43.4	10.5	13.4	5.1
MALtrans	0.0	0.0	4.6	2.5	24.3	3.6
FAS	37.3	2.7	48.1	8.2	37.7	1.7

Table IV-2. Relative flux rates of metabolic enzymes after MPLA as plotted in Figure IV-5B

Relative enzyme flux rate map of MPLA macrophages. All enzyme reaction rates were set within group to an arbitrary designation of CS at 100%.

Augmented energy metabolism can be orchestrated by MyD88 or TRIF

I next aimed to dissect the molecular processes underlying the observed metabolic phenotype. TLR4 signaling is propagated by two TIR containing adaptor proteins MyD88 and TRIF. Canonically, MyD88 activates the transcription factors NFκB and AP-1 while TRIF activates interferon regulatory factors such as IRF3, although there is significant cross talk between these two pathways. I sought to determine the relative contribution of the TLR4 signaling adaptors in inducing the metabolic phenotype observed in 3dp macrophages. MPLA is a selective TLR4 agonist and, as expected, MPLA was unable to induce sustained glycolysis or oxygen consumption in $TLR4^{-/-}$ macrophages (Figure IV-7A). $MyD88^{-/-}$ macrophages had a significantly reduced ECAR at 24 hours after MPLA treatment as compared to controls or $TRIF^{-/-}$ macrophages (data not shown), but, surprisingly, both $MyD88^{-/-}$ and $TRIF^{-/-}$ 3dp macrophages showed elevations in ECAR and OCR that were comparable to wild type (WT) macrophages (Figure IV-7B). I investigated redundancy of MyD88- and TRIF-dependent signaling for induction of the MPLA-induced metabolic phenotype using MyD88/TRIF double knockout macrophages. MPLA-treated $MyD88^{-/-}TRIF^{-/-}$ 3dp macrophages exhibited an ECAR and OCR that was markedly decreased compared to wild type and similar to control $MyD88^{-/-}TRIF^{-/-}$ double knockout macrophages (Figure IV-7C). Thus, while MyD88 is required for early TLR metabolic reprogramming, either MyD88 or TRIF can induce the metabolic phenotype observed in 3dp macrophages, but at least one adaptor is required.

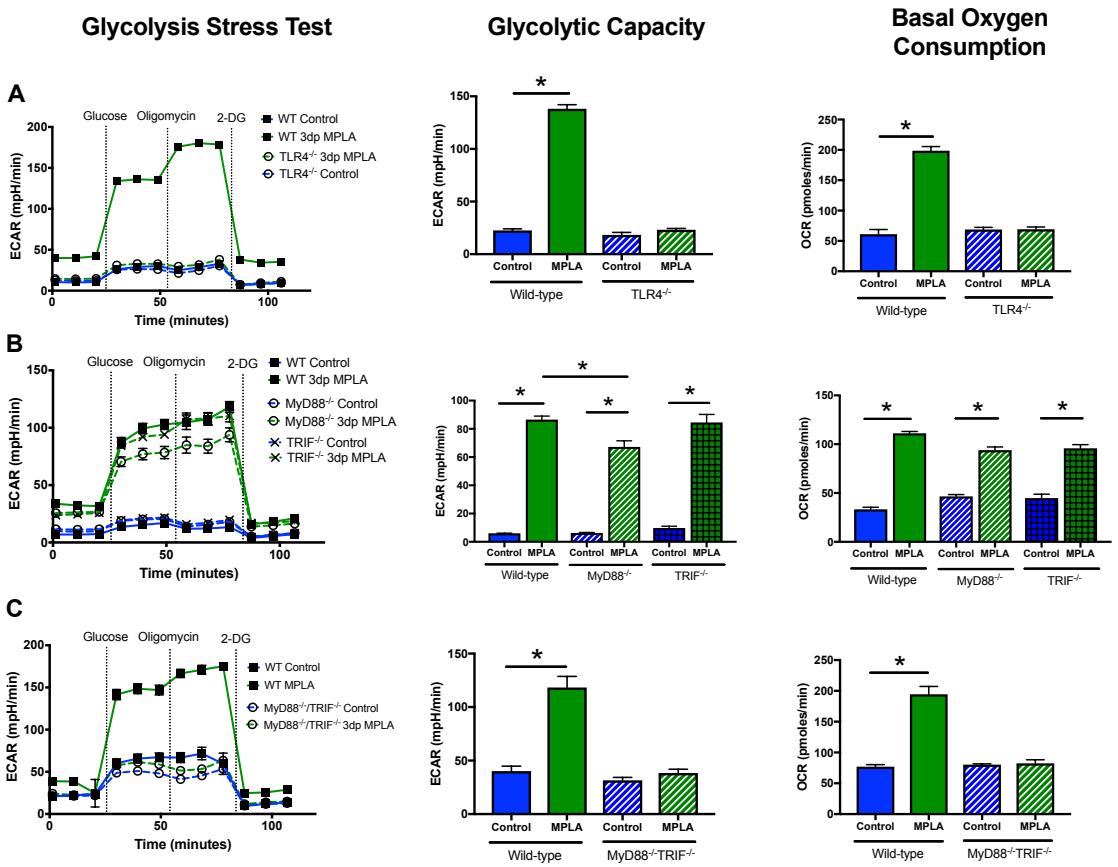


Figure IV-7. Augmented energy metabolism is orchestrated by MyD88- or TRIF-dependent signaling.

BMDMs were derived from WT or knockout mice and assessed via the Seahorse Xf^{e}96 glycolysis stress test. Glycolytic capacity was determined after the addition of oligomycin in the glycolysis stress test. Basal OCR was determined after the addition of glucose in the glycolysis stress test. A) Glycolysis stress test, maximal glycolytic rate, and basal OCR of WT or TLR4$^{-/-}$ BMDMs. B) Glycolysis stress test, maximal glycolytic rate, and basal OCR of WT, MyD88$^{-/-}$ or TRIF$^{-/-}$ BMDMs. C) Glycolysis stress test, maximal glycolytic rate, and basal OCR of WT or MyD88$^{-/-}$/TRIF$^{-/-}$ double knockout BMDMs. Data shown as mean +/- SEM. *, p< .05 as determined by ANOVA with Tukey's *post-hoc* multiple comparison analysis. n=3-5 replicates for each experiment. Figures shown are representative of at least two repeated experiments.

Discussion

In Chapter II, I demonstrated that MPLA induces resistance for up to 15 days following administration, but it was unclear how the innate immune system sustained a phenotype for this time period. These data suggest that MPLA drives persistent glycolysis and oxidative metabolism that persists long after MPLA. Supporting this hypothesis, I found that the TLR-induced metabolic reprogramming can persist up to 3 days follow ligand clearance. Interestingly, the TLR ligands most effective at inducing this persistent metabolic phenotype were the same TLR ligands that induced resistance to infection in Chapter II. Thus these data prompt the hypothesis that persistent metabolic reprogramming underlies TLR-induced resistance to infection.

Acute TLR activation in macrophages results in two significant breaks in TCA cycle glucose utilization, and these deviations require macrophages to generate lactate, rely on glutamine anaplerosis, and reduce the ability of macrophages to utilize cytosolic NADH for mitochondrial ATP production[300,315]. However, these deviations also drive macrophages to generate antimicrobial molecules, such as nitric oxide or itaconate. My gross metabolic data indicate that macrophages sustain significantly increased glucose consumption after TLR agonist removal, yet they are not actively producing nitric oxide. Rather, these macrophages are consuming oxygen and producing ATP. My glucose flux analysis identified that macrophages accomplish this by sustaining much of the metabolic reprogramming induced during TLR stimulation, and by significantly up-regulating a mitochondrial malate shuttle that drives cytosolic NADH into an expanded mitochondrial pool. Malate shuttling is known to occur under cellular conditions when fatty acid synthesis is persistently elevated, and can fuel significant NADH transport[316,317]. Even

though 24hr macrophages also have high ATP citrate lyase activity, the resulting cytosolic malate in these pro-inflammatory macrophages is largely converted to cytosolic pyruvate by malic enzyme 1. This reaction generates cytosolic NADPH for use in fatty acid synthesis or respiratory burst, and this finding is in concert with the literature[302,318]. Thus, by sustaining glycolysis, up-regulating malate shuttling, and partially restoring fatty acid oxidation, macrophages can generate significant mitochondrial ATP while maintaining the possibility of utilizing consumed glucose for antimicrobial purposes if needed[301,319,320]. This metabolic phenotype is in concert with ample functional evidence from the literature, and from my results in Chapter III, that endotoxin tolerant macrophages retain potent antimicrobial capacity[222,227].

Macrophages have been reported to engage in mitochondrial biogenesis in the early response to anti-inflammatory stimuli, such as IL-4[206]. TLR4 agonists have been reported to induce mitochondrial biogenesis in parenchymal cells such as hepatocytes, cardiomyocytes, and neurons[321-323]. Moreover, mTOR, which is strongly activated by TLR stimulation, can promote mitochondrial biogenesis[175]. Nevertheless, it was previously unclear if TLR agonists induce mitochondrial biogenesis in macrophages. Here I establish that TLR agonists indeed induce mitochondrial biogenesis in macrophages, although this occurs very late after TLR activation as the cell enters endotoxin tolerance. Moreover, these mitochondria persist in the cell and produce significant ATP. This mitochondrial biogenesis appears to be an outcome of resolving mild MPLA-induced inflammation. Mitochondrial biogenesis may not have been observed early in TLR stimulation because early-inducible glycolytic factors such as HIF-1α can inhibit biogenesis[324]. Nevertheless, mitochondrial biogenesis is a healthy

cellular response to mitochondrial damage[325,326], and it has been noticed that patients who survive sepsis undergo mitochondrial biogenesis and ATP recovery, whereas patients who succumb do not[327]. While this metabolic phenotype appears to have a beneficial impact on infection, macrophages from rheumatoid arthritis patients also exhibit an ATP^{high} ROS^{high} phenotype with elevated TCA cycle activity[328], so it is possible that the metabolic adaptations uncovered here contribute to chronic inflammatory conditions.

Increased glycolysis and oxidative metabolism does not fit into previously defined metabolic classifications of M1 or M2 macrophages. Rather, these metabolic findings demonstrate that MPLA-primed endotoxin tolerant macrophages have a metabolic phenotype reflecting features of both M1 (increased glycolytic rate) and M2 (increased oxidative rate) macrophages. This finding is in concert with data from transcriptional studies of endotoxin tolerant macrophages that demonstrate that these cells express a unique transcriptional profile with features of both M1 and M2 macrophages, although predominantly express M2 features[329,330]. As such, the identification of this phenotype raises many questions regarding the functional purpose of metabolic reprogramming in TLR-primed macrophages, and this will be explored in Chapter V.

In recognition of the many reports of increased human myeloid cell antimicrobial activity during endotoxin tolerance [226,227,331], these data support the proposal that endotoxin tolerance be thought of as a state of 'cellular reprogramming' rather than immunoparalysis or anergy[332]. Even though endotoxin tolerant macrophages secrete fewer pro-inflammatory cytokines, they remain surprisingly active.

CHAPTER V

TLR4-INDUCED METABOLIC REPROGRAMMING FACILITATES ANTI-MICROBIAL AND PRO-RECRUITMENT ACTIVITY IN MACROPHAGES

Scientific Goal

Macrophages can engage in a variety of inducible functions that mediate resistance to infection including cytokine secretion, phagocytosis, and microbial killing[118]. In Chapter IV, I identified that macrophages engage in persistent and dynamic metabolic reprogramming consisting of sustained glycolysis with reactive mitochondrial activity. Cellular metabolism governs cellular function, yet it remains unclear what macrophage functional processes are impacted by this altered metabolic state. In Chapter V, I aim to determine the functional processes that this persistent metabolic reprograming supports, particularly those that impact the host response to infection. The goal of this chapter is to explore whether macrophage metabolic reprogramming is a critical mediator of MPLA-induced resistance to infection.

Introduction

Unlike their phagocyte cousin the neutrophil, macrophages do not die during the course of an infection. Rather, they engage in a variety of processes that facilitate key steps of inflammation and infection resolution. Inducible macrophage functions generally occur across a timeline of the host response to a pathogen. For example, changes in cytokine secretion, size, granularity, phagocytic capacity, respiratory burst, microbial killing, pro-inflammatory cytokine secretion, chemokine secretion, and wound healing all occur at different stages of infection[333]. Canonically, pro-inflammatory cytokine secretion

occurs first, followed by a change in size, phagocytosis, and antimicrobial processes, followed later by up-regulation of wound healing processes. Yet, even though macrophages play dynamic roles in an infection, it remains unclear how metabolic reprogramming supports these temporal goals.

As uncovered in Chapter III, macrophages play a critical role in the ability of immunomodulatory therapies like MPLA to induce resistance to infection. TLR ligands are efficiently cleared from the bloodstream after administration, suggesting that events that happen after MPLA clearance are critical to effective prophylaxis[334,335]. Interestingly, macrophages exposed to TLR4 agonists do not return to a pre-activated state following agonist clearance. Rather, TLR4 agonist-primed macrophages exhibit endotoxin tolerance and augmented antimicrobial capacity[226,329]. While the mechanisms that result in endotoxin tolerance are well understood, the molecular mechanisms by which TLR4 agonists induce sustained antimicrobial augmentation are unknown.

Recently, it was uncovered that the dectin-1 ligand β-glucan induces sustained glycolytic reprogramming of monocytes that results in resistance to infection similar to TLR4 agonists[272]. β-glucan-induced metabolic reprogramming sensitizes monocytes to LPS, a phenomenon called trained immunity, and this LPS sensitization is the hypothesized mechanism of protection[336]. In congruence with this finding, I demonstrated in Chapter IV that TLR4 agonists also induce persistent metabolic reprogramming in macrophages[337]. Yet, in stark contrast with β-glucan, TLR4 agonist-primed macrophages are endotoxin tolerant[222,250,337]. Moreover, I demonstrated in Chapter II that sensitization or tolerance to LPS does not predict the host response to infection after TLR agonist exposure[232,337]. Thus, persistent metabolic reprogramming in macrophages likely has

diverse and unexplored functional implications. Here, I postulated that TLR4 agonists such as MPLA induce broad resistance to infection by driving sustained metabolic reprogramming of macrophages that fuels improved antimicrobial responses. I demonstrate that MPLA induces resistance to infection by inducing dynamic mTOR-initiated metabolic reprogramming that drives macrophage antimicrobial activity and chemokine secretion and is required for MPLA-mediated resistance to infection.

Protective TLR ligands persistently reprogram the antimicrobial functions of macrophages

I first primed macrophages with different TLR agonists and assessed the macrophages on a variety of antimicrobial endpoints (Figure V-1A). Macrophage morphology, such as cell size and granularity, provide one of the most basic assessments of cell phenotype, and often accompanies changes to antimicrobial activity[338]. Upon assessment, MPLA-, LPS-, CpG-ODN-, and poly(I:C)- primed macrophages were significantly larger and more granular than unprimed macrophages (Figure V-1B-C). I then assessed BMDMs on bacterial phagocytosis and respiratory burst, two key antimicrobial processes. MPLA-, LPS-, and CpG-ODN-, but not Poly (:C)- primed macrophages showed increased phagocytosis of pHrodo-labeled *E. coli* bioparticles (Figure V-1D), as well as increased PMA-elicited respiratory burst (Figure V-1E). These results correlate with my findings in Chapter II that MPLA, LPS, and CpG-ODN improve resistance to infection whereas Poly(I:C) does not.

In order to probe the persistent phenotype of 3d-macrophages further, I performed RNA-sequencing on macrophages 4 or 24 hours into priming, as well as 3 days after priming. RNA changes typically precede functional outcomes, which is why the 4hr

group was included. Upon RNA-sequencing, I found significant differential expression of mRNA during the first 4 hrs of MPLA priming and also at 24hrs of MPLA priming (Figure V-2A). However, by 3 days later, most mRNA expressed during priming had fallen significantly towards control, although some, such as phagocytosis-related genes, remained close to the expression level of 24hr macrophages (Figure V-2A-B). These persistently augmented phagocytosis genes corresponded not only with a greater phagocytic capacity, but also a more rapid induction of phagocytosis when the rate of phagocytosis in 3dp macrophages was measured (Figure V-2C).

Increased glycolytic metabolism facilitates MPLA-mediated improvements in phagocytosis and is dependent, in part, on HIF-1α

In Chapter IV, I observed that TLR agonist primed macrophages engage in a metabolic phenotype of persistent glycolysis with augmented oxygen consumption. In order to determine whether the observed alterations in metabolism influence anitmicrobial function, I assessed the requirement of glycolysis for macrophage phagocytosis. MPLA-primed BMDMs were incubated with 2-deoxyglucose (2-DG) during phagocytosis of pHrodo-tagged *E. coli* bioparticles. 2-DG rapidly inhibits glycolysis through inhibition of hexokinase. 2-DG induced a significant reduction in phagocytosis in control and MPLA-primed BMDMs (Figure V-3A), although phagocytosis in MPLA primed BMDMs remained higher than unprimed BMDMs.

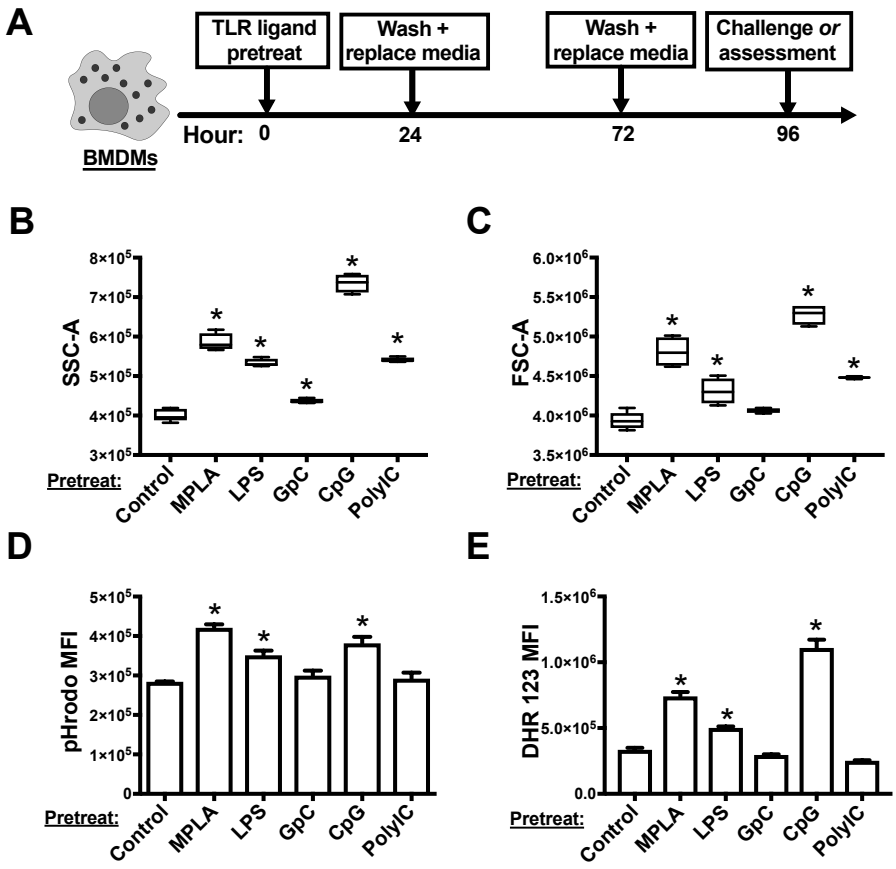


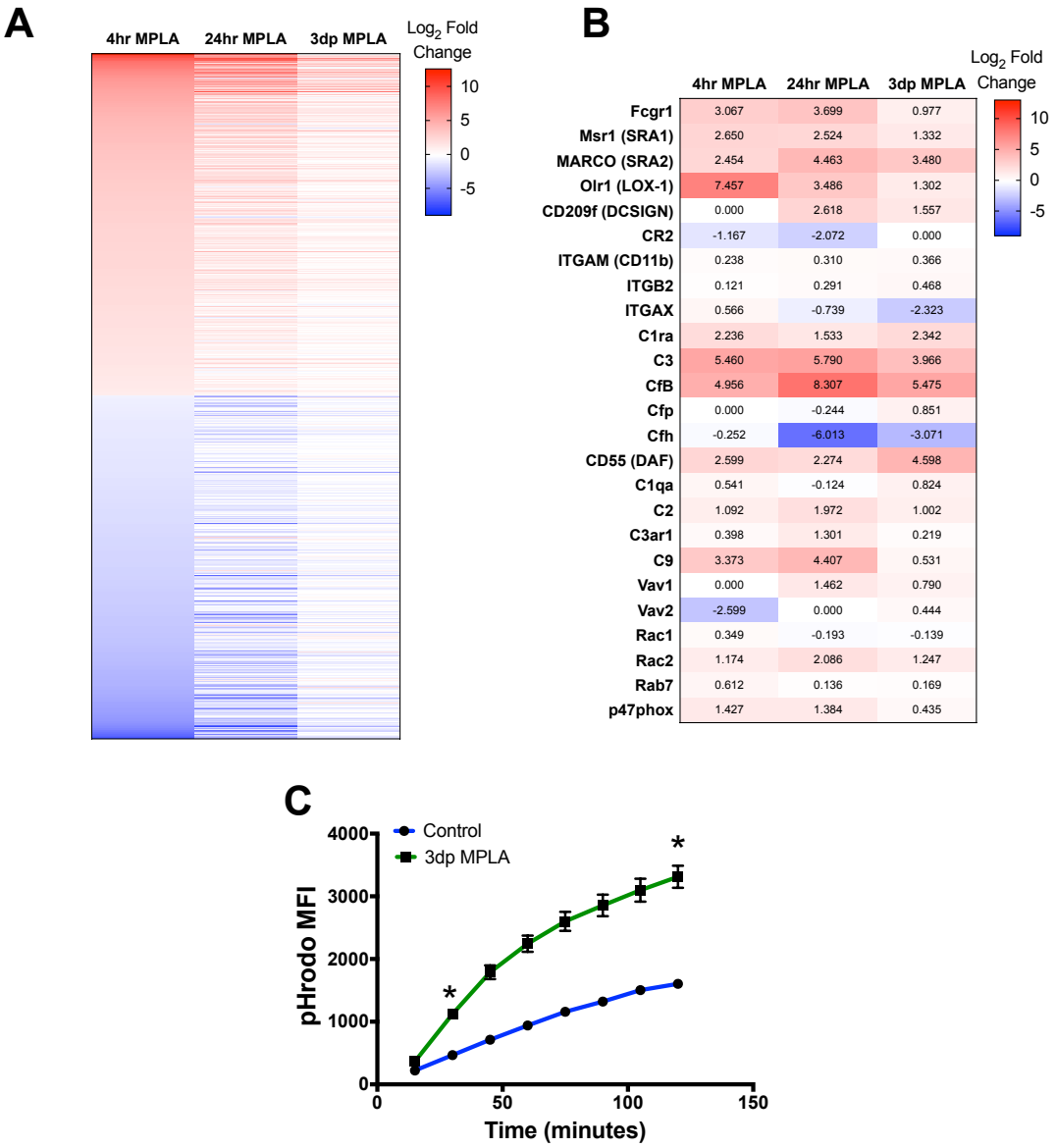
Figure V-1. TLR-primed macrophages have augmented antimicrobial function

BMDMs were primed with 100ng/mL LPS, 1μg/mL MPLA, 10μg/mL CpG-ODN, 10μg/mL GpC-ODN, or 10μg/mL poly(I:C) for 24 hours, rested for 3 days and assessed. A) Side-scatter (SSC-A) and B) Forward-scatter (FSC-A) of BMDMs as determined by flow cytometry. C) BMDM phagocytosis of pHrodo-tagged *E. coli* bioparticles. Mean fluorescence intensity (MFI) was determined by flow cytometry. D) PMA-elicited respiratory burst capacity as determined by dihydrorhodamine 123 fluorescence. Mean fluorescence intensity (MFI) was determined by flow cytometry. Error bars indicate SEM. *, $p<.05$ compared to control via one-way ANOVA with Dunnett's multiple comparison's test. n=3-5 replicates for each experiment. Figures shown are representative of at least three repeated experiments.

I next sought to determine the molcular regulators of the glycolytic phenotype and identify whether these regulators are essential for improvements in phagocytic capacity. HIF-1α is a transcription factor thought to be a critical mediator of TLR-induced glycolysis [302]. To assess the requirement of HIF-1α for TLR-induced increases in glycolysis and phagocytosis, HIF-1α knockout BMDMs were derived from $LysM^{cre}$-$HIF\text{-}1\alpha^{fl/fl}$ mice and primed with MPLA. Indeed, $HIF\text{-}1\alpha^{-/-}$ BMDMs had signficantly reduced glycolyic rate following MPLA priming compared to wild-type controls (Figure V-3B-C). Despite this decrease, $HIF\text{-}1\alpha^{-/-}$ BMDMs maintained a high rate of basal oxygen consumption (Figure V-3D). Further, MPLA-primed $HIF\text{-}1\alpha^{-/-}$ macrophages had significantly reduced phagocytosis when compared to MPLA-primed wild-type BMDMs, although phagocytosis remained significantly higher than unprimed BMDMs (Figure V-3E). Thus, persistent glycolytic metabolism supports an augmented phagocytic capacity.

Reprogrammed mitochondrial metabolism supports a persistent chemokine secretion profile

In addition to phagocytosis, one of the most critical functions of macrophages is chemokine secretion and recruitment of additional leukocytes to assist with infectious foci. Moreover, the Sherwood lab has previously shown that improved leukocyte recruitment to sites of infection is critical for MPLA prophylaxis[232,252]. Glycolytic metabolism is important for the secretion of cytokines, and mitochondrial ATP production can support protein translation[339]. Thus, I hypothesized that macrophages are engaging in the persistent secretion of cytokines or chemokines.



	4hr MPLA	24hr MPLA	3dp MPLA
Fcgr1	3.067	3.699	0.977
Msr1 (SRA1)	2.650	2.524	1.332
MARCO (SRA2)	2.454	4.463	3.480
Olr1 (LOX-1)	7.457	3.486	1.302
CD209f (DCSIGN)	0.000	2.618	1.557
CR2	-1.167	-2.072	0.000
ITGAM (CD11b)	0.238	0.310	0.366
ITGB2	0.121	0.291	0.468
ITGAX	0.566	-0.739	-2.323
C1ra	2.236	1.533	2.342
C3	5.460	5.790	3.966
CfB	4.956	8.307	5.475
Cfp	0.000	-0.244	0.851
Cfh	-0.252	-6.013	-3.071
CD55 (DAF)	2.599	2.274	4.598
C1qa	0.541	-0.124	0.824
C2	1.092	1.972	1.002
C3ar1	0.398	1.301	0.219
C9	3.373	4.407	0.531
Vav1	0.000	1.462	0.790
Vav2	-2.599	0.000	0.444
Rac1	0.349	-0.193	-0.139
Rac2	1.174	2.086	1.247
Rab7	0.612	0.136	0.169
p47phox	1.427	1.384	0.435

Figure V-2. MPLA induces persistent mRNA expression changes of phagocytosis genes.

BMDMs were harvested for mRNA and analyzed via RNA-sequencing. A) Log_2 fold change heat map of the top 1,000 most up-regulated and down-regulated genes after 4hrs of MPLA priming and the same corresponding genes after 24hrs of priming and 3 days following the end of priming. Genes without significant changes are designated at 0. B) Heat map of phagocytosis-associated genes in BMDMs. Cell values indicate Log_2 fold change value. C) Phagocytosis of pHrodo-tagged *S. aureus* bioparticles. MFI determined by the BioTek Synergy MX plate reader. Data shown as mean +/- SEM. *, $p < .05$ as determined by Student's t-test.

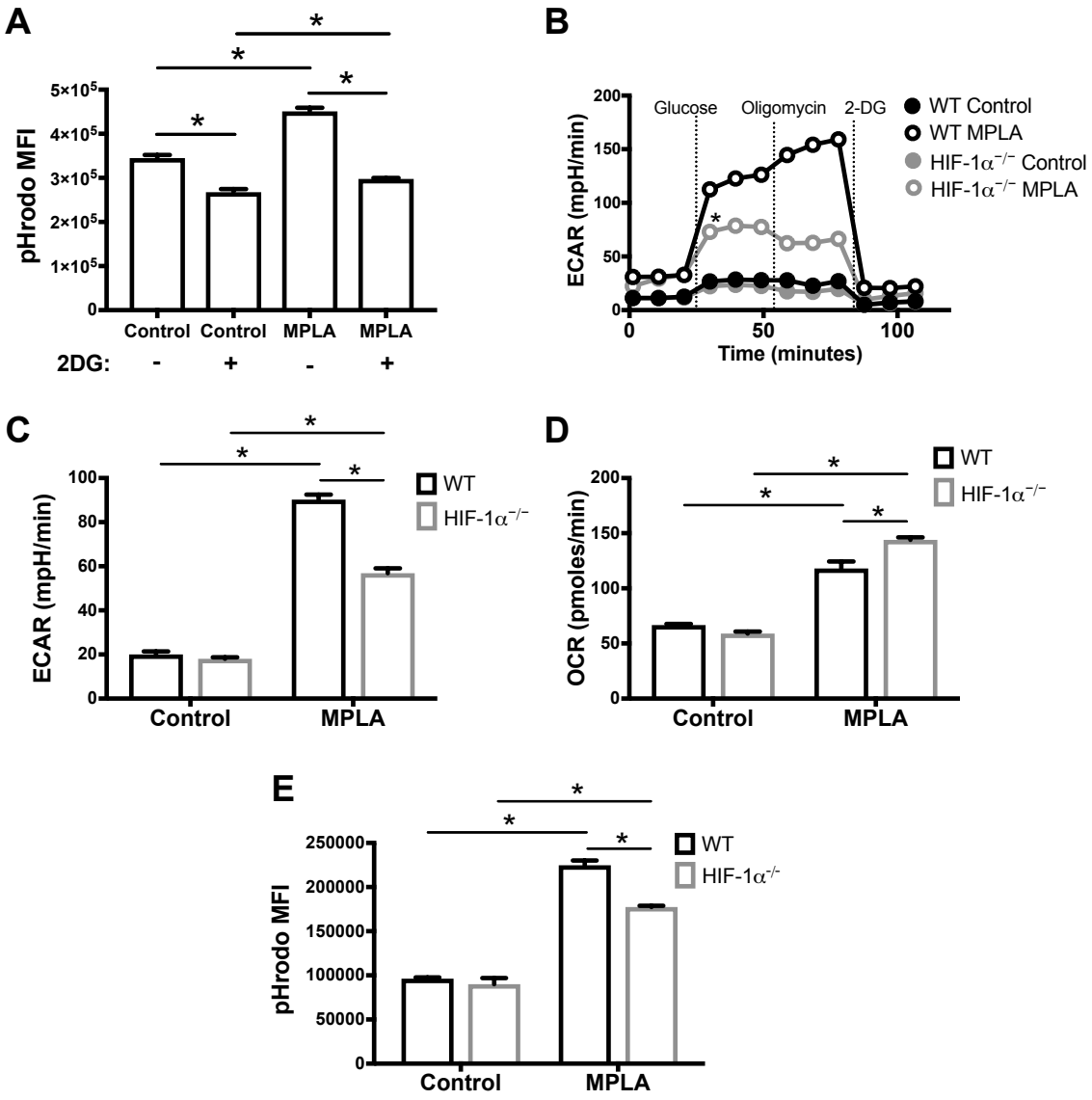


Figure V-3. Increased glycolytic metabolism facilitates MPLA-mediated improvements in phagocytosis and is dependent, in part, on HIF-1α.

A) 3dp MPLA BMDMs were exposed to 50mM 2-DG during a 1.5-hour phagocytosis of pHrodo-tagged *E. coli* bioparticles. Mean fluorescence intensity (MFI) was determined by flow cytometry. B) MPLA-primed HIF-1$\alpha^{-/-}$ BMDMs were exposed to 10mM glucose, 1μM oligomycin, and 50mM 2-deoxyglucose (2-DG) and assessed on the on the Seahorse XF^{e}96. C) Basal glycolysis as determined after the addition of glucose in the glycolysis stress test. D) Basal oxygen consumption as determined after the addition of glucose in the glycolysis stress test. E) MPLA-primed HIF-1$\alpha^{-/-}$ BMDM phagocytosis of pHrodo-tagged *E. coli* bioparticles. Error bars indicate SEM. *, $p<.05$ via one-way ANOVA with Dunnett's multiple comparison's test. n= 3-5 replicates for each experiment. Figures shown are representative of at least two repeated experiments.

In order to assess this hypothesis, I first utilized my RNA-sequencing data and ordered a rank list of immunologic cytokines, from greatest fold change increase to greatest fold change decrease individually for the 4hr MPLA, 24hr MPLA, and 3dp MPLA groups (Figure V-4A). Pro-inflammatory cytokine genes were significantly up-regulated in the 4hr MPLA group and many of these genes remained expressed at 24hrs. Interestingly, the 24hr MPLA group tended to have higher expression of chemokine genes compared to 4hr MPLA, but fewer pro-inflammatory cytokine than 4hrs MPLA. By 3 days following MPLA removal, most cytokine genes returned significantly closer to control compared to the 24hr or 4hr group. However, there were some exceptions. Expression of chemokine mRNAs associated with leukocyte recruitment, such as CCL5 and CCL2, remained elevated in 3dp macrophages. I hypothesized that the metabolic activity in 3dp macrophages supports persistent secretion of these chemokines. I assessed basal cytokine secretion over a 6 hour time period while concurrently inhibiting glycolysis, pyruvate transport, and mitochondrial ATP synthesis with the inhibitors 2-DG, UK5099, or oligomycin, respectively. The 3dp macrophages were found to constitutively secrete significant quantities of CCL5, CCL2, and CCL3 when compared to basal secretion of control macrophages (Figure V-4B-D). Further, the addition of 2-DG, UK5099, or oligomycin significantly reduced basal secretion of CCL5, CCL2, and CCL3 secretion. In contrast to these chemokines, the 3dp macrophages did not exhibit basal secretion of TNF-α, IL-6, or MIP-2 (CXCL2), and exhibited reduced TNF-α, IL-6, and MIP-2 production in response to LPS (Figure V-4E-G). Thus, persistent glycolytic and mitochondrial activity helps 3dp macrophages drive a constitutively expressed monocyte/macrophage/lymphocyte chemokine profile, even during endotoxin tolerance.

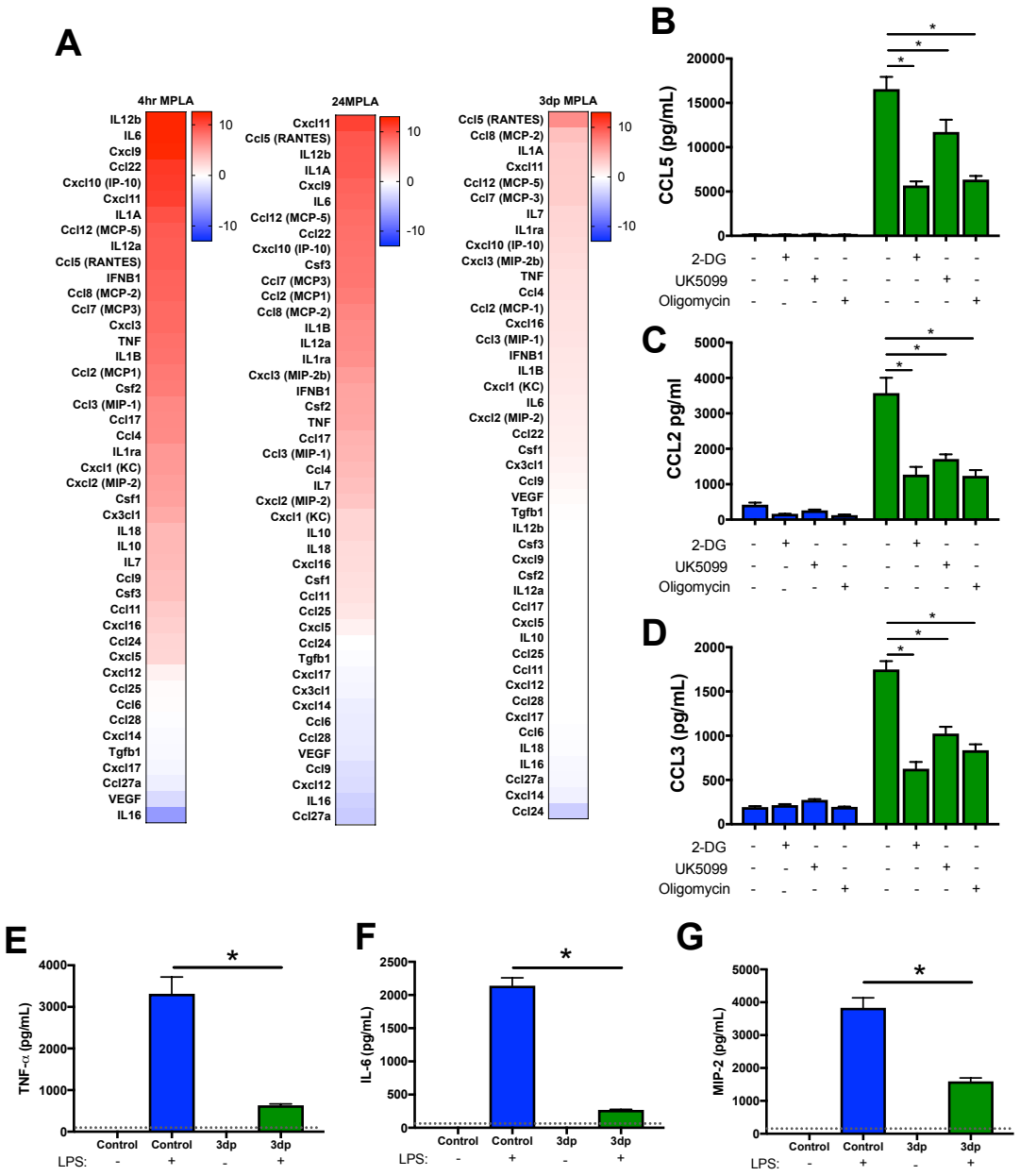


Figure V-4. Reprogrammed mitochondrial metabolism supports a persistent chemokine secretion profile.

A) List of cytokines expressed by macrophages as ordered by $\log_2$ fold change for each independent group. B-D) Control (blue) or 3dpMPLA BMDMs (green) were incubated in the presence of 10mM 2-DG, 80μM UK5099, or 1μM oligomycin. Media was harvested 6 hours after incubation. Concentration of B) CCL5 (RANTES) C) CCL2 (MCP-2) and D) CCL3 (MIP-1α) were determined by ELISA. E-G) Control or 3dp macrophages were incubated with or without 100ng/mL LPS for 6 hours. E) TNF-α and F) IL-6 and G) MIP-2 were determined by ELISA. Data shown as mean +/- SEM. *, $p < .05$ as determined by ANOVA with Tukey's *post-hoc* multiple comparison analysis. n= 3-5 replicates for each experiment. Figures shown are representative of at least two repeated experiments

mTOR-initiated metabolic programming drives MPLA-mediated resistance to infection

While HIF-1α$^{-/-}$ knockdown impaired the glycolytic rate and phagocytosis, it did not decrease oxygen consumption. Thus, I aimed to identify an upstream molecular regulator that could mediate both the glycolytic and mitochondrial metabolic programs. In Chapter IV, I identified that TLR4 can coordinate persistent metabolic reprogramming through either MyD88 or TRIF. The mammalian target of rapamycin (mTOR), a critical regulator of cellular metabolism, can be activated through both MyD88- or TRIF-dependent mechanisms and up-regulate HIF-1α activity [340-343]. Thus, I explored whether mTOR signaling is required for the development of the 3dp macrophage phenotype. I found that, while mTOR is potently activated by MPLA during priming, Akt phosphorylation and p70S6K phosphorylation, both indicators of mTOR activation are not sustained 3 days later (Figure V-5A). However, blockade of mTOR activation with rapamycin during priming significantly attenuated rates of glycolysis (Figure V-5B-C) and oxygen consumption (Figure V-5D) in 3dp macrophages. Additionally, rapamycin exposure during priming significantly reduced 3dp macrophage phagocytosis (Figure V-5E) and significantly reduced basal CCL5 secretion (Figure V-5F). I then assessed the requirement of mTOR activity for MPLA-induced resistance to infection. Rapamycin administered to mice 3 hours before MPLA or vehicle treatment completely abolished the survival benefit conferred by MPLA treatment during *S. aureus* infection (Figure V-5G). Thus, mTOR signaling during MPLA priming, while not sustained after priming, is required for MPLA to induce metabolic reprogramming, the antimicrobial phenotype, and resistance to infection.

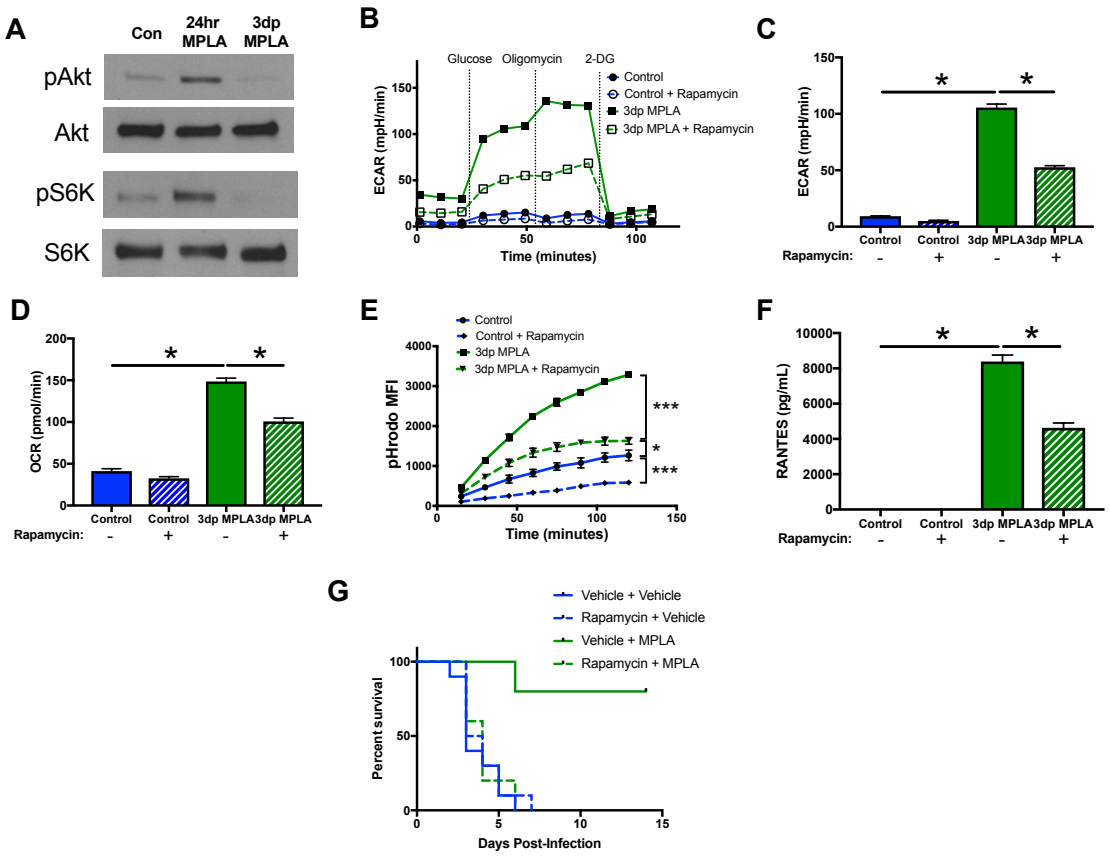


Figure V-5 mTOR-initiated metabolic reprogramming is required for MPLA-induced resistance to infection.

A) Western blot of phosphorylated Akt (pAkt), total Akt (tAkt), phosphorylated P70s6K (pP70s6K) and total P70s6K (tP70s6K). Blots representative of 3 repeated experiments. B) BMDMs were exposed to 100nM rapamycin 1 hour prior to MPLA priming and throughout the 24 hour priming period. Rapamycin was washed out after priming along with MPLA. Glycolysis stress test of 3dp macrophages with and without rapamycin during priming. C) Maximal glycolytic rate of 3dp macrophages with and without rapamycin during priming. D) Basal OCR of 3dp macrophages with and without rapamycin during priming. E) Phagocytosis over time of pHrodo-tagged S. aureus bioparticles by 3dp macrophages with and without rapamycin during priming. F) CCL5 (RANTES) secretion of 3dp macrophages with and without rapamycin during priming after 6 hours of incubation in media. G) Mice were administered 3mg/kg intraperitoneal rapamycin or vehicle 3 hours prior to the first administration of intravenous MPLA. Kaplan Meier survival plot over 14 days following *S. aureus* infection (n=10 mice/group). Data shown as mean +/- SEM. *, p< .05 as determined by ANOVA with Tukey's post-hoc multiple comparison analysis.

Discussion

Taken together, these data illustrate that MPLA induces broad resistance to infection by persistently altering macrophage metabolism and antimicrobial function. I found that TLR agonists induce persistent functional reprogramming, consisting of increased size, granularity, phagocytosis, and respiratory burst capacity. Moreover, I used 2-DG and HIF-1$\alpha^{-/-}$ macrophages to determine that sustained TLR-induced glycolytic reprogramming is essential for TLR agonists to augment antimicrobial capacity. MPLA exposure also results in reactive mitochondrial biogenesis and ATP production. I found that this ATP production helps support the persistent expression of the chemokines CCL2, CCL3, and CCL5, all which are able to recruit CCR1/CCR2/CCR5 containing leukocytes, notably monocytes, macrophages, and lymphocytes. In contrast, MPLA-primed macrophages did not secrete pro-inflammatory cytokines or neutrophil chemokines. mTOR activity during MPLA priming is critical in initiating this metabolic and functional phenotype, and rapamycin ablated the metabolic and functional alterations in macrophages, as well as MPLA-induced resistance to infection in mice.

Products of glycolysis have been associated with improved macrophage phagocytosis and respiratory burst functions [344,345]. I found that TLR ligand-primed macrophages have increased size and granularity, as well as improvements in phagocytosis and respiratory burst. Hence, these data show that endotoxin tolerance does not entail complete functional anergy, but rather that endotoxin tolerant macrophages undergo sustained adaptations to improve their antimicrobial capacity while suppressing pro-inflammatory cytokine responses. Interestingly, while poly(I:C)-primed macrophages showed increases in glycolysis, size, and granularity, they did not show improvements in

phagocytosis or respiratory burst. MPLA, LPS, and CpG-ODN each activate the TLR signaling adaptor MyD88, whereas poly(I:C) only activates the TLR signaling adaptor TRIF [346], thus, activation of MyD88-dependent signaling, while not required for metabolic reprogramming, may be required to completely induce an improved antimicrobial phenotype.

The finding that sustained glycolysis supports increased phagocytosis demonstrates that the unique metabolic programming induced by TLR agonist treatment directly supports improvements in antimicrobial function. Indeed, HIF-1$\alpha^{-/-}$ macrophages, which were partially deficient in TLR-induced glycolysis, were unable to increase phagocytosis as much as wild-type macrophages. There is only limited knowledge regarding the purpose of TLR-mediated metabolic reprogramming in macrophages for functions other than cytokine secretion. Glycolysis directly supports the production of fatty acids that are essential for monocytes to differentiate into phagocytic macrophages [193,200]. Yet, It remains to be determined exactly how TLR-mediated glycolytic metabolism influences cell size, granularity, and phagocytic capacity.

Interestingly, I found that an increase in mitochondrial-derived ATP is essential for persistent chemokine secretion following MPLA removal. Protein translation consumes more ATP than any other cellular process[339], and mTOR has been reported to induce mitochondrial biogenesis in order to promote ATP generation for this purpose[175,177]. The Sherwood lab has consistently reported that MPLA improves leukocyte recruitment to sites of infection[252], and in Chapter III, I found that MPLA-primed mice have an increase in tissue macrophages, but not neutrophils, that are

required for resistance to infection. These data suggest that tonic secretion of chemokines may support this process.

It is interesting that macrophage/monocyte chemokine secretion, but not pro-inflammatory cytokine or neutrophil chemokine secretion, is driven by a metabolic process that only up-regulates after TLR agonist clearance. For what physiologic reason would MPLA-primed macrophages do this? In addition to bacterial clearance, recruited monocytes and macrophages can also engage in wound healing and inflammation resolving processes that are necessary to recover from damage induced by TLR agonists[125]. Thus, it is tempting to speculate that MPLA-primed macrophages are tonically secreting chemokines to recruit macrophages that serve a dual purpose: resolving mild MPLA-induced inflammation while concurrently increasing the capacity to kill incoming pathogens, if needed. Supporting this suggestion, endotoxin tolerant macrophages have been demonstrated to have greater wound healing and antimicrobial capacity[329]. Additionally, endotoxin tolerant animals are more resistant to damage from ischemia through a mechanism dependent on macrophages[347-349]. Furthermore, monocytes from human septic patients have up-regulated both antimicrobial activity and wound healing processes[331]. While intriguing, additional functional roles of these recruited macrophages are unclear. Further, the impact of persistent metabolic reprogramming on macrophage wound healing and resolution functions remains to be determined.

mTOR is an essential regulator of metabolism in macrophages and other leukocytes. These studies demonstrate that mTOR activation is required for complete induction of the MPLA-primed macrophage phenotype, and is required for MPLA-

induced resistance to infection in mice. Rapamycin can induce immunosuppression via inhibition of T-cell proliferation. However, I previously demonstrated that MPLA successfully protects RAG2$^{-/-}$ mice, which have complete loss of the mature T- and B-cell compartment. Thus, my data suggest that rapamycin is inhibiting MPLA-mediated protection due to its impact on innate phagocytes. Many cells in the body utilize mTOR, so it is possible that rapamycin is impacting non-immune cells, however the role of mTOR in parenchymal tissue or endothelial cells during infection remains to be explored.

As antimicrobial resistance continues to decrease the efficacy of antibiotics, hospitalized patients will require novel prophylactic interventions to prevent nosocomial infection. This study explains how MPLA induced metabolic reprogramming drives persistent augmentation of macrophage antimicrobial processes and chemokine secretion without excessive cytokine toxicity. A temporary reorientation of the metabolic, transcriptional, and antimicrobial landscape of macrophages, such as that provided by MPLA, may significantly improve the immune response of hospitalized patients to antibiotic-resistant pathogens.

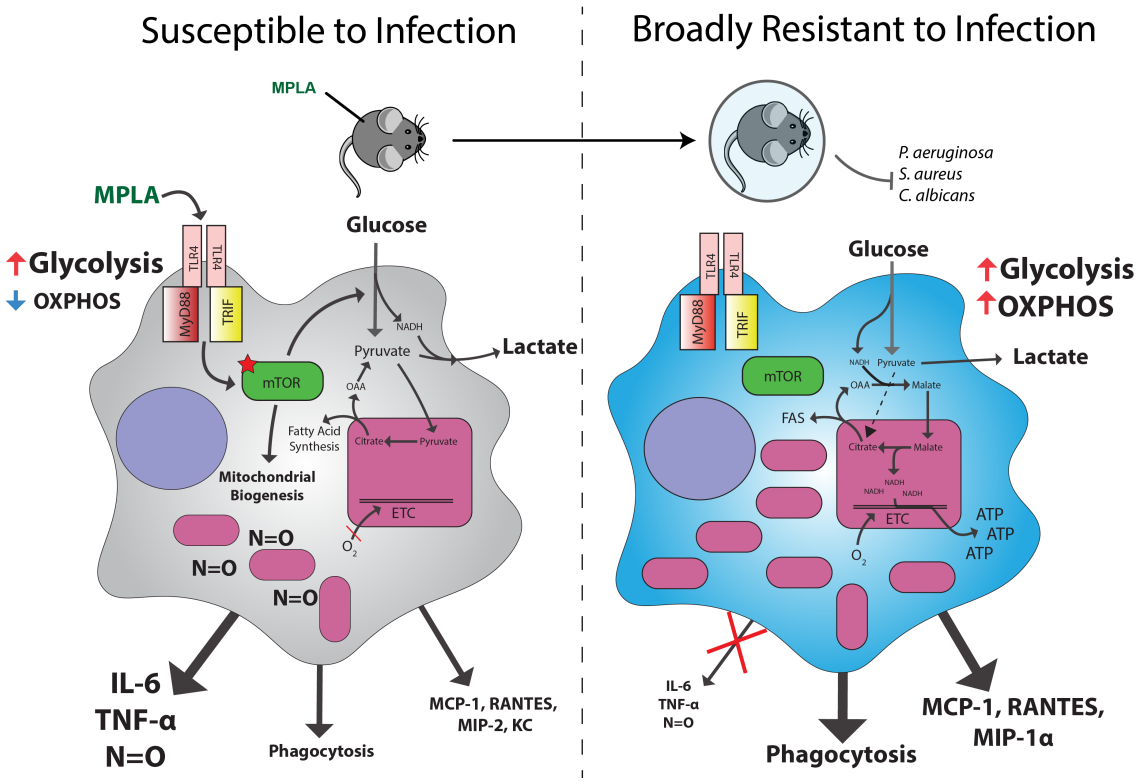


Figure V-6. Summary of findings from Chapters III-V

Central Findings:

- MPLA induces persistent broad resistance to infection
- MPLA induces sustained mTOR initiated glycolysis and mitochondrial biogenesis in macrophages
- Macrophages adapt to recovered mitochondrial function through malate shuttling and ATP production
- Metabolic reprogramming drives persistent phagocytosis and chemokine secretion
- mTOR-initiated metabolic reprogramming is required for resistance to infection

CHAPTER VI

DISCUSSION

The incidence of nosocomial infection remains a pressing clinical problem that will only increase with the development of antibiotic resistance. Furthermore, treatments for sepsis are stalled. Assessing and modulating the immune system offers a promising clinical strategy to reduce infection risk. In this dissertation, I clarified the predicative value of innate immune assessments, particularly the magnitude of the cytokine response to LPS, and suggested antimicrobial activity as a more accurate assessment. Additionally, I detailed the cellular mechanism of a potential clinical immunomodulatory strategy, the prophylactic administration of MPLA, which can be used to reduce the incidence of *S. aureus* bloodstream infection. Then, I uncovered a unique persistent metabolic phenotype in macrophages that occurs after TLR agonist exposure. This metabolic phenotype consisted of persistent glycolysis along with reactive mitochondrial biogenesis and an increase in malate driven NADH shuttling and ATP generation. I then demonstrated that this metabolic phenotype has functional consequences for the macrophage, namely persistently heightened phagocytic capacity and tonic monocyte/macrophage chemokine secretion. Finally, I demonstrated that metabolic reprogramming is a critical process underlying MPLA-induced resistance to infection, as inhibition of mTOR activity diminished the metabolic, functional, and host-protective consequences of MPLA priming.

While the phenomenon of immunoprophylaxis has been observed for nearly a century, this work provides one of the most molecular mechanistic insights into the phenomenon yet reported. Additionally, this work uncovers new fundamental biology

regarding the metabolic activity of macrophages after they have responded to a TLR ligand. In this chapter, I discuss the implications of the findings presented in this thesis, explore important questions raised by the work, consider the limitations of the method used thus far, and detail future directions for research. In sum, this work will support novel clinical and biologic investigations into the use of TLR agonists as immunomodulatory therapies for hospitalized patients and opens up new avenues for inquiry into the metabolism of macrophages.

Tolerance versus resistance: the clinical implications

Despite decades of work on endotoxin tolerance from both a mechanistic and clinical perspective, one central question remains unclear: Is endotoxin tolerance good or bad? One could provide a logical explanation for either answer. Indeed, pro-inflammatory cytokines are central to activating immune responses, and reduced pro-inflammatory responses to a bacterial product may indicate a defective immune system. On the other hand, sepsis is defined by immune over-activation and cytokine storm, so reduced pro-inflammatory cytokine responses may help protect the organism from damaging inflammation. Both explanations are plausible, but my work suggests that neither is correct. I believe this confusion stems from a fundamental misunderstanding of the difference between tolerance and resistance.

Schneider and Ayers define tolerance as "the ability to limit the health impact of a given pathogen burden" and resistance as "the ability to limit pathogen burden"[350]. Hence, the ability to tolerate pathogen stress does not imply that the burden of the stress will be reduced in some way. In fact, some mechanisms of resistance, such as the release of antimicrobial peptides in response to a pathogen, by definition reduce the ability to

tolerate the presence of a pathogen due to off target effects on host cells. TLR4 agonist-primed mice are tolerant to LPS, as they do not reduce the presence of LPS, but rather tolerate its negative health effects by inducing signaling proteins that limit pro-inflammatory cytokine secretion. Thus, TLR4 agonist-primed mice are appropriately called endotoxin tolerant. At the same time, TLR4 agonist-primed mice *do* mitigate the presence of diverse pathogens, as they clear invading pathogens more efficiently. Thus, TLR4 agonist-primed mice should also be referred to as infection resistant. When cellular antimicrobial processes are inhibited, TLR4 agonist-primed mice cannot resist infection and do not display significant evidence of tolerance to the pathogen[232]. Tolerance to endotoxin and resistance to infection, while both present in TLR4-agonist primed mice, are independent and dissociable processes[350]. As my experiments with CpG-ODN demonstrate clearly, it is possible to be endotoxin sensitive and pathogen resistant, so assessments of tolerance or sensitization to endotoxin do not inform whether the host is infection resistant.

The distinction between tolerance and resistance is particularly important when considering hospitalized patients. Patients may have varying degrees of tolerance to the early health effects of immunologic molecules found on pathogens, however, the presence of this tolerance is unrelated to the ability of the patient to eradicate the pathogen and resist eventual infection. Thus, measuring tolerance does provide information on how a patient will respond to an upcoming infection. Better techniques to measure infection resistance must be developed in order to assess patient infection susceptibility accurately. My studies suggest that cellular antimicrobial capacity is indicative of infection resistance. Host antimicrobial capacity is particularly difficult to

assess *ex vivo* due to the inability to recreate the infectious microenvironment outside the host. However, there are simple, currently available assays that can be used to probe infection resistance. Further, one can begin to develop more complex or physiologically relevant assays that may be useful in future clinical work.

Ex vivo antimicrobial assessments can be rapidly performed on patient blood. Simple *ex vivo* phagocytosis assays, involving fluorescent heat-killed or live bacteria can be performed on blood monocytes and neutrophils and assessed via flow cytometry or fluorescent plate reader. Additionally, DHR respiratory burst assays, such as those used in my studies, can be rapidly performed on blood monocytes and neutrophils. Further, *ex vivo* leukocyte chemotaxis assays, involving the migration of cells towards a chemokine gradient, may offer insight into leukocyte recruitment potential[351]. While these assays are easy to perform, the removal of cells from blood causes leukocytes to significantly change phenotype[352]. Moreover, it is not possible to assess macrophage antimicrobial capacity via *ex vivo* blood assessments. Thus, future studies should seek to perform *in vivo* antimicrobial assessments in patients, similarly to how a tuberculosis test is performed[353]. A small amount of fluorescent non-pathogenic, attenuated, or heat-killed bacteria could be injected underneath the skin and monitored via fluorescence imaging. These tests would essentially be a safe and controlled microinfection, and a more rapidly dimming fluorescent signal would indicate improved antimicrobial responses. The predictive value of these assays should be determined in future studies, but will likely prove much more valuable than measuring pro-inflammatory cytokine responses to LPS.

Overall, it is important for clinicians and scientists to understand the difference between infection tolerance and infection resistance, as they are typically independent.

Further, due to the immunocompromised status of many hospitalized patients and the severity of most nosocomial infections, infection resistance, not tolerance, should be assessed and used to stratify patients by infection risk.

Persistent macrophage responses to inflammatory stimuli

The persistent phenotypic state I observed after the removal of MPLA has broad implications for the physiologic macrophage response to inflammatory stimuli as well as pathologic conditions such as chronic inflammatory diseases. It has long been recognized that TLR-priming can induce a state of endotoxin tolerance for up to 2 weeks. The work presented here demonstrates that during this state metabolic processes are also changing, and that these metabolic processes support a variety of functions, underscoring the fact that endotoxin tolerant macrophages are not inert. Even more interestingly, this regulated temporal response of macrophages following TLR stimulation appears to mirror the response of macrophages over the course of a live bacterial infection. Thus, these data prompt the speculation that at least a portion of the macrophage response to infection is pre-programmed upon pathogen detection.

During infection, the earliest functional response of macrophages is to secrete pro-inflammatory cytokines such as TNF-α and neutrophil recruiting chemokines like KC (CXCL1) and MIP-2 (CXCL2). Following this, macrophages up-regulate machinery that increases phagocytic and respiratory burst capacity. As the infection progresses even though pathogen burden may still be high, pro-inflammatory cytokine secretion begins to decrease whereas chemokines that recruit both neutrophils and macrophages remain persistently elevated[333]. In fact, even during severe septic infections, serum TNF-α secretion can be missed if blood cytokines are measured too late[333,354]. As the infection

progresses over the course of days, phagocytic capacity, respiratory burst capacity, and monocyte-lymphocyte chemokine secretion persist, even as monocytes begin to up-regulate late wound healing processes[331,355]. Recruited monocytes are able to assist with bacterial clearance and also aid in inflammation resolution and wound healing[356].

In the studies presented here, I observed dynamic changes in macrophage metabolism and function that parallel this time course. Notably, early TLR-induced aerobic glycolysis supports the secretion of pro-inflammatory cytokines as well as antimicrobial molecules such as nitric oxide. However, this metabolic program eventually evolves to one of persistent glycolysis and mitochondrial biogenesis that drives increased oxygen consumption and ATP production. This late phase metabolism supports phagocytosis and the persistent secretion of monocyte/macrophage chemokines. These late outcomes reflect immunologic processes that occur in the later phase of infection, yet these macrophages have not been in the presence of a TLR agonist for over 3 days. Thus, the correlation between a live infection and transient exposure to a TLR agonist suggests that at least some of the processes observed during infection are pre-programmed. Administering MPLA prior to infection starts this time course early, which offers protection to animals once macrophage up-regulate persistent antimicrobial activity and have begun to recruit macrophages.

Outside of infection with a microorganism, a pre-programmed inflammatory response may also be instrumental in driving chronic inflammatory processes such as rheumatoid arthritis, coronary artery disease, or vasculitis. Indeed, macrophages found at sites of inflammation in rheumatoid arthritis are characterized as "over-indulging on glucose" resulting in an ATP^{high} ROS^{high} macrophage phenotype with elevated oxygen

consumption[328]. These macrophages are also seen in atherosclerosis and vasculitis[357]. Yet, because these macrophages do not need to phagocytose and kill bacteria, the excessive glucose consumption and ROS production results in increased oxidized intracellular proteins and persistent T-cell activation[358]. Similarly to MPLA-priming, macrophages found in these conditions secrete high levels of the chemokines CCL2 (MCP-1), CCL5 (RANTES), and CXC3L1, which facilitates monocyte recruitment to sites of inflammation and can worsen autoimmune conditions[359,360]. Further, TLR4 and MyD88 play essential roles in mediating atherosclerosis[361]. However, in contrast to MPLA-primed macrophages, macrophages in chronic inflammatory conditions secrete high levels of IL-6 and IL-1β [358]. This discrepancy may be due to the development of endotoxin tolerance in MPLA-primed macrophages, which limits the extent to which pro-inflammatory cytokines can be secreted. Alternatively, the persistent metabolic phenotype induced by TLR agonists may simply provide the cellular machinery for other non-canonical stimuli or T-cell derived pro-inflammatory cytokines to have a more pronounced effect[328]. Regardless, the metabolic pathways that drive these chronic inflammatory conditions may be the same as those induced by MPLA.

The identification that macrophages persistently respond to a stimulus also has implications for the development of potential therapeutics. It is likely that macrophages can respond in a persistent manner to stimuli other than TLR agonists. Thus, discovering the stimuli that induce persistent macrophage reprogramming, and characterizing the time course of phenotypic changes that occurs may open the door to single-dose or interval-dose therapies that improve disease outcomes. Macrophages are already targeted by therapeutics used for a wide range of diseases, including rheumatoid arthritis,

osteoporosis, and cancer. The goal of these therapeutics is to change the interactions between macrophages and their environment. For example, antibodies targeting RANKL, a protein that encourages development of osteoclasts, reduces the ability of osteoclasts to resorb bone[362]. Stimuli that induce persistent macrophage reprogramming may prove useful in diseases where an undesired macrophage phenotype is driving disease, such as in cancer or rheumatoid arthritis[363].

Finally, it is worth noting that even though pro-inflammatory, M1-polarizing, bacterial products initiate these persistent processes, it is clear that early markers of inflammation die off rather quickly. This suggests that iNOS or TNF-α, which are classically used to define pro-inflammatory macrophages, are inappropriate markers[364]. Rather, these markers simply identify macrophages that have very recently responded to a stimulus. While markers of chronic inflammation may be more appropriate to identify pro-inflammatory processes, they are not specific to macrophages. Rather, identification of late-phase proteins such as CCL2 and CCL5, in combination with phagocytosis proteins such as Fc gamma receptor 1 or the formyl peptide receptor (FPR) as well as TNF-α and iNOS may be more effective at identifying macrophages that are in all phases of the M1 response. Regardless, it is important to consider that macrophages engaged in a program initiated by a TLR agonist may not actively secrete pro-inflammatory cytokines or express early markers of activation.

In summary, the protective phenotypic and metabolic adaptations that MPLA-primed macrophages undergo as they execute a pre-programmed response to a stimulus mirrors an expected and desired response to a live infection. However, these very protective processes may be instrumental in the pathogenesis of autoimmune diseases or

neoplastic conditions. Clinical use of MPLA in vaccines strongly suggests that the compound can be used safely if given intermittently, and it is very unlikely that a single administration of MPLA will result in autoimmune conditions. However, further study of the persistent phenotypic and metabolic processes in TLR stimulated macrophages may uncover novel targets for therapeutics in chronic inflammatory diseases.

Dynamic metabolic reprogramming in macrophages

Macrophage metabolic reprogramming has only recently reemerged as an area of interest to immunology. This reemergence has occurred largely due to the development of new technologies that can dissect the specific metabolic pathways that mediate macrophage activation. Thus, targeting macrophage metabolism is now a viable therapeutic strategy for disease, but many of the specific metabolic pathways that macrophages utilize remain unknown.

The most surprising and important concept derived from my studies is that macrophage metabolic responses to TLR agonists are remarkably dynamic. Most previous studies on macrophage metabolism have focused on the first 24 hours after TLR stimulation. Indeed, it is appreciated that macrophage metabolism is dynamic even within the first 24 hours[365]. However, my studies demonstrate that macrophages engage in metabolic programming well after 24 hours and that this has far reaching effects on macrophage and host immune function.

Aerobic glycolysis is an ideal metabolic program for macrophages early in infection. Glycolysis upon activation provides rapid ATP and biosynthetic intermediates with which to generate immunologic and antimicrobial molecules. Additionally, an anticipatory increase in glycolysis prepares macrophages for hypoxic conditions such as

those that may be encountered at sites of infection[185]. Further, activated macrophages produce gaseous mediators like NO or antimicrobial molecules like itaconate that, in addition to inhibiting pathogen ETC function, can inhibit host cell ETC function[304,366]. However, aerobic glycolysis and dysregulated mitochondrial metabolism eventually come at a cost by limiting efficient ATP generation. Neutrophils, which are very short lived, exclusively run aerobic glycolysis[367].

Unlike neutrophils, glycolysis is not the only metabolic program that macrophages can utilize to generate ATP. For example, anti-inflammatory macrophages have high oxidative metabolism, suggesting that macrophages can engage elevated mitochondrial metabolism depending on the context. It is not clear why anti-inflammatory macrophages engage in this process, but there are significant metabolic and functional parallels between anti-inflammatory macrophages and the described endotoxin tolerant macrophages[329]. However, unlike anti-inflammatory macrophages, endotoxin tolerant macrophages have adapted to early glycolysis and mitochondrial dysfunction so many leverage the metabolic consequences of aerobic glycolysis into late mitochondrial activity. Notably, glycolysis generates significant NADH that can be utilized by mitochondria if the electrons from NADH can pass the mitochondrial membrane. In cells with a high glycolytic rate, NADH transport to the mitochondria not only increases ATP production, but also keeps the mitochondria healthy, and an increased cytoplasmic NADH redox potential is strongly associated with impaired mitochondrial metabolism[368].

Cytoplasmic NADH can account for over 30% of the NADH utilized by mitochondria for ATP generation[369]. The canonical mechanism used by cells to shuttle NADH into the mitochondria is the malate-aspartate shuttle[370]. In this shuttle, malate is

imported in exchange for aspartate, bringing the electrons from cytosolic NADH into the mitochondria. However, malate can enter the mitochondria in exchange for other metabolites, such as citrate, if they are in high enough concentration and their export can be withstood. In cells engaged in persistent or heavy fatty acid synthesis, citrate must be exported from the mitochondria for conversion to acetyl-coA by ATP citrate lyase. Mammalian cells encode a variety of malate/citrate antiporters that can support this exchange[316,371]. Activated macrophages generate significant NADH from glycolysis and engage in a high rate of fatty acid synthesis, making them ideal candidates for malate/citrate transport. Yet, early in activation, the TCA cycle and the ETC are disrupted, leaving no incentive for accelerated NADH transport. Clearance of the TLR agonist disinhibits the mitochondria, and in response, as my data in Chapter IV demonstrate, macrophages begin to shuttle NADH and generate ATP. It remains to be determined exactly which transporters are used by macrophages to increase malate transport, but it is likely a combination of malate-aspartate and malate-citrate antiporters.

Another process central to the reactive metabolic program observed here is mitochondrial biogenesis. As mentioned in chapter IV, mitochondrial biogenesis has also been observed in many other cell types following LPS stimulation[321,323]. Thus, while these other cell types engage in a similar metabolic phenotype, this phenotype may have markedly different functional consequences in each cell. For example, multiple studies have found that when TLR4 agonists are administered prior to ischemia or ischemia reperfusion, the tissue survives much better[347,372]. Some of the protective benefit for the priming is thought to be due to stability in cellular mitochondria brought about by persistent mitochondrial biogenesis as well as modulation of the glycolytic enzyme

hexokinasae-2[322,373]. Additionally, TLR4 agonist pretreatment can induce endotoxin tolerance in endothelial cells, and can change the ability of endothelial cells to adhere to blood leukocytes upon repeated LPS exposure[374-376]. Thus, my data suggest that reactive mitochondrial biogenesis is a universal metabolic response to TLR4 stimulation that is associated with prior TLR4 agonist exposure. However, the consequences of this biogenesis may vary depending on each cell's typical functional abilities. It remains to be determined whether malate shuttling also occurs after LPS exposure in other cell types.

Translating MPLA to clinical use in hospitalized patients

It is remarkable that the scientific and clinical community has known about the potential prophylactic benefit of microbial products for over 100 years but there has never been a randomized clinical trial to investigate their efficacy as prophylactics. Calls for clinical trials extend back to the 1950's, but none have been performed[217]. Why is this? The likely explanation is that for the first half of the 20th century there was rightful concern over the toxicity of the therapeutic. In a definitive review on the topic in the 1958, Drs. Bennet and Cluff stated that, "Despite many proprietary claims, there is no real evidence that any preparation to date can produce a therapeutic effect without the danger of undesirable and unpleasant reaction to the endotoxin"[217]. Following the 1950's, and with a progressive tightening of drug regulations, enthusiasm for endotoxin therapy waned precipitously. Despite the lack of clinical investigation, there is some evidence, largely from the vaccine literature, that microbial products can safely induce resistance to infection.

The Bacille Calmette-Guerin (BCG) vaccine, which is a tuberculosis vaccine consisting of weakened *Mycobacterium bovis*, has been administered worldwide for

decades. Epidemiologic studies following vaccine administration have noted that the BCG vaccine, when administered to infants, can reduce infant mortality in a manner unrelated to its effect on tuberculosis[377,378]. This improvement in mortality can be observed at a 12 month follow up after vaccination, suggesting that the non-specific effect of the vaccine is long-lived[378]. It has also been noted that the measles vaccine provides a similar benefit[379]. The mechanism of this resistance is not entirely clear, but it is thought that innate immunomodulation underlies protection[379,380]. There are generally no side effects from these vaccines. The very processes that induce protection in models of microbial product prophylaxis have also been implicated in this phenomenon, so these epidemiologic observations may indicate that non-specific immunomodulation can be induced effectively and safely in humans[381].

There is also good evidence that immunomodulatory doses of MPLA can be administered safely to humans. In the mid 1990's, the pharmaceutical company GlaxoSmithKline began patenting MPLA for use as a vaccine adjuvant[382]. Due to its ability to activate B-cells, likely in combination with its ability to induce durable monocyte and T-cell mobilization, MPLA has proven to be a very effective and safe vaccine adjuvant[383]. GlaxoSmithKline is continuing to develop MPLA for vaccines, and has not expressed interest in pursuing MPLA as a prophylactic alone. However, the difficulties in translating prophylactic MPLA to clinical settings are largely logistic and relate to the current use of the molecule as a vaccine adjuvant. If the translation of MPLA proves too logistically difficult, other MPLA-like analogs, such as synthetic, non-lipid A, monophosphoryl hexacyl disaccharides (PHADs), can be developed for clinical use[384].

While early pre-clinical data with these analogs indicate they are similarly efficacious as MPLA, clinical studies with these compounds have yet to be performed.

In order to prove efficacious, clinical trials with MPLA or PHADs should focus on reducing the incidence of nosocomial infection in a patient population with high susceptibility to infection for a definite period of time. One potential population is patients that are receiving bowel surgery. These patients have some of the highest incidence of SSI, likely due to the microbial contents of the bowel. MPLA has already been demonstrated to protect against the organisms that commonly infect bowel surgery patients. Around 5% of these patients acquire an SSI, thus a reduction of the incidence or severity of these infections would prove highly beneficial. Another population that may benefit from MPLA is military personnel on the battlefield. Military personnel occasionally engage in activities that put them are serious risk of traumatic injury and a resulting infection, further complicated by the high potential for wound contamination in combat areas, as well as prolonged field care and long evacuation times. Administration of MPLA to these soldiers prior to a risky mission or immediately following a survivable traumatic injury may decrease the incidence and severity of infection. If MPLA proves safe and effective in early studies, its use could be broadened to wider cohorts of at-risk patients, and MPLA, or PHAD, may be appropriate for anyone undergoing moderately invasive hospital care.

Potential toxicity aside, one concern for an MPLA or an MPLA-like clinical trial is an unexpected disturbance of homeostatic processes, such as healing processes. In addition to their roles in bacterial infections, macrophages play a major role in wound repair. Thus, it is possible that MPLA may change the normal mechanisms of wound

repair from a surgical insult. There is evidence that LPS, if present near a wound, can impair normal wound healing[385]. While this possibility should be explored clinically, there is some evidence that MPLA will not harm, and may actually benefit wound healing. Numerous studies on this issue have noted that MPLA and LPS preconditioning can improve the survival of ischemic tissue, suggesting that priming may help limit the extent of an injury[347,372,386]. Nevertheless, examining the impact of MPLA administration on normal wound healing should be considered. Despite these concerns, clinical and experimental data overwhelmingly suggest MPLA will be safe and effective in at-risk patients. Clinical trials evaluating MPLA's efficacy in reducing the incidence of nosocomial infection should be undertaken.

Beyond simple innate prophylaxis, MPLA's ability to reduce the incidence of infection via innate immunomodulation could be combined with its activity as a potent vaccine adjuvant. It is possible that MPLA could be administered alongside common bacterial antigens from *P. aeruginosa* or *A. baumanii* in a "nosocomial vaccine". Injection of this vaccine, when administered to a patient prior to surgery or immediately upon hospital arrival, may reduce the short term and long term risk of nosocomial infection in hospitalized patients. A nosocomial vaccine, relying on decades of supportive research, may offer promise to patients as antibiotic resistance continues to grow.

Limitations of the studies

It is important to consider the limitations of the studies presented in this dissertation. To begin, all the studies in this dissertation were conducted using the mouse. There are significant advantages to working with the mouse. Severe *in vivo* infections can be successfully modeled using mice. Moreover, bone marrow derived macrophages can be

readily isolated from mouse femurs. However, there is significant controversy regarding the relevance of mouse modeling for human studies, particularly with regard to the genomics of inflammatory responses[387,388]. Plainly, mice are not humans, and it will be important to determine whether these findings translate to humans.

There are a variety of ways in which these studies could be translated to assessments of human macrophages. Human monocyte derived macrophages can be easily generated from human peripheral blood. However, monocyte derived macrophages are not tissue macrophages, and I demonstrated in chapter III that monocytes, particularly the inflammatory subset, are not responsible for the beneficial effects of MPLA prophylaxis. Rather, future studies should aim to acquire human bone marrow for bone marrow-derived macrophage differentiation. Bone marrow is routinely extracted from healthy patients for bone marrow transplant or for clinical assessment, and this bone marrow could be used to generate human bone marrow derived macrophages in the presence of human M-CSF. Additionally, the spleen is not typically donated during organ donation, and mature human tissue macrophages could be isolated from these spleens, as has been done recently with dendritic cells[389].

Another major limitation of my macrophage studies is that they are conducted in pure macrophage cultures *in vitro*. In the host, macrophages are surrounded by additional cell types, both leukocytic and parenchymal, and these cells can significantly impact macrophage function. Macrophages have a different phenotype in each organ they are found, largely due to signals present in the tissue microenvironment[118]. Moreover, leukocytes like natural killer cells and T-cells can secrete pro-inflammatory cytokines that macrophages are unable to secrete, such as interferon-gamma[390,391]. These molecules

may appreciably change how macrophages respond to TLR agonists, or the ways in which macrophages sustain the effects of TLR agonists. Nevertheless, my studies establish that it is possible for macrophages to engage in persistent functional and metabolic reprogramming and, using rapamycin, provides some evidence that this occurs *in vivo*. These studies motivate additional work in determining how non-macrophage derived signals can alter persistent macrophage responses. Additionally, future studies should aim to develop new technologies that can assess leukocyte metabolism *in vivo*. The development of better metabolic probes, such as fluorescent metabolites or targeted intracellular antibodies, will one day make it possible to perform sophisticated leukocyte metabolism experiments *in vivo*.

While my experiments in Chapter V demonstrate that rapamycin inhibits the protective effect of MPLA, a major limitation of this study is that rapamycin is administered systemically, and can impact mTOR activity in all cells. Specific inhibition of macrophage mTOR activity could be achieved using conditional knockouts of mTORC1 components, such as with $LysM^{cre}$-$raptor^{fl/fl}$ mice, or by packaging inhibitors such as rapamycin inside of liposomes or polymersomes, which are preferentially phagocytosed by macrophages in the same way as clodronate-liposomes. Experiments using these approaches of mTOR inhibition will be essential to determine the specific contribution of macrophage mTOR activity for the protective effect of MPLA.

Overall, there are major limitations to these studies that must be considered. However, these studies still provide critical insights into the host response to infection and the biology of macrophages. Many of the limitations can and should be addressed

with future studies. In addition to addressing limitations, future studies can significantly expand on the findings presented in this dissertation.

Future Directions

The studies presented in this dissertation open up many new avenues of scientific inquiry. First, how do macrophages sustain a functional and metabolic phenotype over time? There are a variety of molecular hypotheses for how this might occur. One of the most likely explanations is that epigenetic modifications are induced during priming that persist following clearance of the TLR4 agonist. Epigenetic modifications change the interaction between DNA and histones, thereby causing a tightening or loosening of the DNA wound around specific histones. Epigenetic modifications are coordinated by tranferase proteins that add methyl, acetyl, or phospho groups on histones[392]. If certain gene promoters are loosely wound, the threshold for gene transcription of particular genes decreases. Importantly, these modifications have been demonstrated to be persistent over time[393]. Epigenetic modifications can be assessed by chromatin immunoprecipitation (ChIP) against histones that contain specific modifications of interest.

There is evidence that monocytes can sustain epigenetic modifications long after exposure to microbial products[272,394]. Moreover, TLR agonists like LPS are known to induce epigenetic modifications during priming, and some of these epigenetic changes have been shown to be persistent[273]. Epigenetic modifications are thought to be essential components of the suppression of cytokine genes as observed during endotoxin tolerance[395]. It is interesting to consider the possibility that targeted epigenetic modifications may underlie my observation of elevated production of CCL2, CCL3, and

CCL5, but not TNF-α or IL-6. Thus, it remains to be explored whether epigenetic modifications mediate the metabolic phenotype observed in Chapter IV or drive TLR agonist-induced resistance to infection.

My RNA-sequencing data presented in chapter V demonstrate that 3 days after MPLA clearance, many genes have returned to control levels, suggesting that epigenetic modifications may not be the complete explanation for phenotypic persistence. Moreover, my macrophages sustain a strikingly active phenotype even though mRNA is not strikingly elevated. Thus, an alternative explanation could be that post-translational mechanisms are sustaining the phenotype. In order for the macrophage to return to a control phenotype after MPLA exposure, the proteins generated during priming must be degraded. Proteins can be degraded by ubiquitin tagging and subsequent degradation by the 26S proteasome, or can be degraded in bulk by the lysosome through autophagocytic mechanisms[396,397]. These processes can be assessed by tracking protein ubiquitinylation over time, or through observing the formation of autophagosomes via immunofluorescent microscopy[398]. Additionally, most metabolic genes are not regulated at the level of transcription. Cellular localization of metabolic proteins, such as hexokinase-2 on the mitochondrial membrane or GLUT1 on the plasma membrane, significantly alters the function of these proteins[399]. The relevance of post-translational protein modifications in the persistence of TLR agonist induced metabolic reprogramming remains to be explored.

In addition to phagocytosis and respiratory burst functions explored in Chapter V, tissue macrophages have many additional functions that must be performed to maintain homeostasis. This prompts the question, how does TLR priming change the ability of macrophages to perform organ-specific homeostatic functions? There has been some

work on the immediate impact of LPS on homeostatic processes. For example, LPS has been demonstrated to alter microglial neuron pruning, a process that has significant developmental consequences[400]. LPS can also cause an up-regulation of osteoclast bone resorption while concurrently inhibiting osteoblast differentiation[401,402]. Future studies should investigate how TLR agonist priming impacts these, and other, homeostatic processes using controlled culture conditions in macrophage tissue sub-populations isolated from mice and stimulated with TLR agonists. Additional studies should aim to characterize tissue macrophage phenotypes and function *in vivo* to determine how different macrophages throughout the body respond to an event such as MPLA priming. These studies will also be instrumental in finding novel and potentially therapeutic ways to persistently modulate macrophage activity for therapeutic purposes.

There are many additional questions to explore in light of the data presented here. How does mTOR activate mitochondrial biogenesis in TLR-primed macrophages? What additional metabolic pathways, besides the TCA cycle, are altered by TLR agonists? However, the last question I would like to raise in order to motivate future work is, why are septic patients susceptible to secondary infection, while TLR agonist-primed animals are resistant?

It is clear that sepsis and critical illness predispose patients, and animals, to secondary infection[403,404]. Yet, the organisms that cause sepsis contain the very TLR agonists used in this dissertation to induce resistance to infection. Studies have shown that subclinical infections can also induce resistance to secondary infection, so it is unlikely that additional products or toxins within pathogens explain this discrepancy[272]. Rather, it is more likely that severe infection or inflammation tips patients over an

inflammatory threshold that places them into immunosuppression, whereas TLR agonist priming does not pass this threshold (Figure VI-1). Models where severe inflammation leads to persistent immunosuppression have been proposed[405], yet these models leave out the significant advantage that could be gained by a *mild* preceding inflammatory stimulus. Thus, many of the processes that these models assume are responsible for infection susceptibility, such as endotoxin tolerance, are also present in resistant animals. It will be essential to discover the processes that only occur in animals susceptible to infection.

This 'inflammatory tipping point' could be determined by administering successively larger doses of LPS, MPLA, or pathogen, prior to an infection and then observing at what dose animals begin to lose resistance to an upcoming infection. Then, the difference in physiologic consequence between the highest protective dose, and dose that induces susceptibility can be compared. Studies such as this will yield a deeper understanding of the tipping point between resistance and susceptibility, will allow for better patient risk assessments, and may uncover novel targets for therapeutics aimed at changing patient susceptibility.

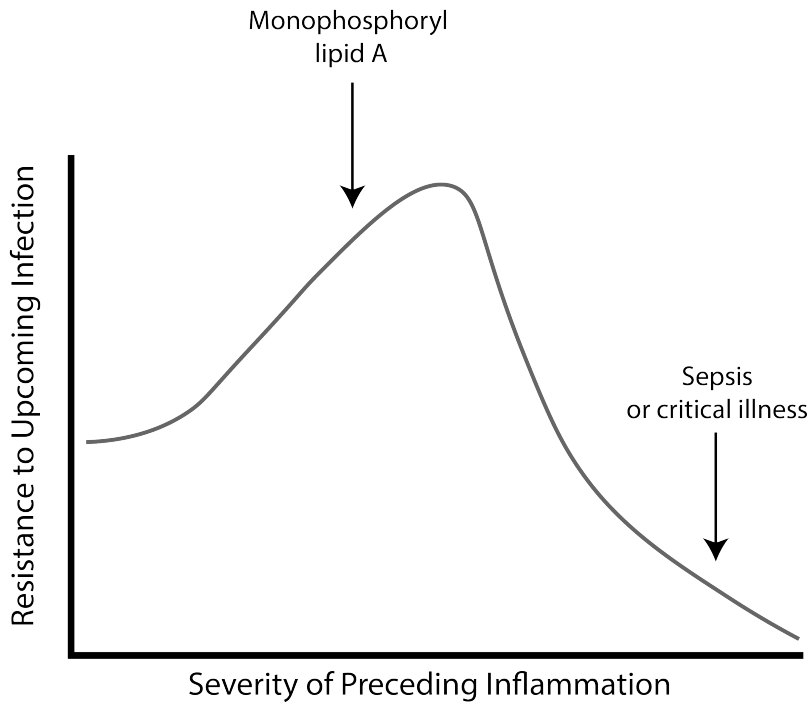


Figure VI-1. Schematic depicting how prior inflammation changes the response to an upcoming infection.

Conclusions

Successful prevention of nosocomial infection is paramount to the advancement of hospital care in the 21st century. This dissertation reveals the need for better predictive assessments of infection susceptibility, and suggests ways in which this could be achieved. Moreover, this dissertation reveals how TLR agonists can modulate macrophages to induce short-term but strong protective immunity against a broad-spectrum of pathogens. In doing so, this dissertation uncovers unique metabolic mechanisms by which macrophages support persistent responses to inflammatory stimuli. This work advances the blossoming scientific field of immunometabolism while concurrently offering real-world strategies for hospitalized patients. Overall, identifying the ways in which host immunity is altered during hospitalization, in combination with strategies that restore immune-competency, may one day prove life-saving for patients.

CHAPTER VII

MATERIALS AND METHODS

Animals

All animal procedures complied with the National Institutes of Health guidelines for the Care and Use of Experimental Animals, and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Male 10 to 12-week-old BALB/c mice were purchased from Envigo Laboratories (Indianapolis, IN). Male wild type, $RAG2^{-/-}$ C57/Bl6 and B6.129S4-$Ccr2^{tm1Ifc}$/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). WT C57/Bl6 mice were purchased from the Jackson Laboratories. $MyD88^{-/-}$ and $TRIF^{-/-}$ mice were purchased from the Jackson Laboratories. $LysM^{cre}$-HIF-$1\alpha^{fl/fl}$ mice were received as a gift from Dr. Holger Eltzschig at the University of Texas at Houston. Femurs from $TLR4^{-/-}$ mice were received as a gift from Dr. Brad Greuter and Daniel Kashima from Vanderbilt University. Femurs from $MyD88^{-/-}TRIF^{-/-}$ double knockout mice were received as a gift from Dr. Doug Golenbock at the University of Massachusetts Medical School.

Mouse Models of Infection

In Chapter II, *P. aeruginosa* intraperitoneal infection was used to study the host response to infection. *P. aeruginosa* was purchased from American Type Culture and Collection (Manassas, VA; ATCC 19660). The culture was grown in tryptic soy broth and resuspended in sterile saline. For infection, *P. aeruginosa* was diluted in lactated Ringers solution at a concentration of $2x10^8$ CFU/ml. Mice were inoculated with $1x10^8$ *P. aeruginosa* by injection into the peritoneal cavity. At 6 hours after inoculation, the

peritoneal cavity was lavaged with 2 ml of sterile phosphate buffered saline. Peritoneal lavage fluid was cultured on tryptic soy agar overnight and colonies were counted to determine CFU per ml. For characterization of peritoneal leukocytes, cells in the peritoneal lavage were counted, centrifuged (300 x g for 10 minutes at 4°C) and resuspended in PBS at desired cell concentrations for flow cytometry. Additionally. whole blood was harvested by carotid artery laceration under isoflurane anesthesia and collected in heparinized syringes for organ injury marker analysis, leukocyte analysis, and cytokine measurements.

In Chapter III, a *S. aureus* infection was used to study the prophylactic effects of MPLA. *S. aureus* was obtained from American Type Culture and Collection (Manassas, VA; ATCC 25923). The culture was grown in tryptic soy broth and resuspended in sterile saline solution prior to inoculation. For intravenous (i.v.) infection, *S. aureus* was diluted in lactated Ringers solution at a concentration of $2x10^8$. Mice were inoculated with $1x10^8$ cfu *S. aureus* diluted in lactated Ringers solution through the dorsal vein of the penis. For intraperitoneal (i.p.) *S. aureus* infection, mice were inoculated by i.p. injection with 1 x 10^8 cfu *S. aureus* diluted in lactated Ringers solution. Bacterial burden in the spleen, lungs, kidney, and peritoneal lavage fluid was quantified by performing serial dilutions of tissue homogenates or peritoneal lavage fluid, followed by culture on tryptic soy agar overnight (37ºC). For survival studies, mice were monitored daily for 15 days following i.v. inoculation with *S. aureus*.

In vivo Leukocyte Depletion Models

Leukocyte depletion experiments were carried out in Chapter III. For neutrophil depletion experiments, mice received i.v. injection with 100 μg of monoclonal anti-mouse 1A8-Ly6G purified blocking antibody or purified rat monoclonal IgG2a, kappa isotype control (eBioscience, San Diego, CA) 24 hours prior to the first MPLA injection. For macrophage depletion experiments, mice received i.v. injection with 0.2 ml clodronate liposomes or PBS-control liposomes (Liposoma, Amsterdam, The Netherlands) 24 hours prior to the first MPLA injection.

TLR Ligand Treatment

MPLA derived from *Salmonella enterica* serotype Minnesota Re 595, was purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Invivogen (San Diego, CA). For *in vivo* experiments, MPLA was solubilized in 0.2% triethylamine solution. For *in vitro* experiments, MPLA was solubilized in DMSO. For all experiments, ultrapure LPS derived from E. Coli 0111:B4, synthetic ODN 1826 (CpG-ODN), synthetic ODN 2138 (GpC-ODN), and high molecular weight (HMW) poly(I:C) were purchased from Invivogen (San Diego, CA) and solubilized in sterile saline. For *in vivo* intraperitoneal experiments as presented in chapter 2, TLR ligands were diluted in lactated Ringers solution (100 μg/ml) and administered by intraperitoneal injection at 20μg in 0.2 ml, except LPS which was given at 2μg in 0.2 ml. Vehicle treated mice received intraperitoneal injection of 0.2 ml lactated Ringers solution. For *in vivo* intravenous experiments, MPLA was diluted in lactated Ringers solution (100 μg/ml) and administered by intravenous injection via the dorsal vein of the penis at 20μg in 0.2 ml. Vehicle treated mice received intravenous injection of 0.2 ml lactated Ringers solution via the dorsal vein of the penis.

Organ injury markers and evaluation of leukocytes in blood

Whole blood was harvested by carotid artery laceration under isoflurane anesthesia and collected in heparinized syringes. Blood was collected in K3EDTA tubes (Greineer Biosciences, San Diego, CA) and stored on ice for complete blood count (CBC), analysis of organ injury markers, and cytokine measurements. CBC measurements were performed using a Forcyte veterinary hematology analyzer (Oxford Science, Oxford, CT). Remaining blood was centrifuged (4750 rpm for 15 minutes at 4°C) to collect plasma for organ injury marker analyses. Blood urea nitrogen (BUN) was measured using the Vet Axcel Chemistry Analyzer (Alfa Wassermann Diagnostic Technologies, LLC, West Caldwell, NJ). The CBC was analyzed by the Translational Pathology Shared Resource at Vanderbilt University Medical Center.

Flow Cytometry

For assessments throughout the dissertation, Leukocytes were suspended in cold PBS (1 x 10^7 cells/ml), incubated with anti-mouse CD 16/32 (eBioscience, San Diego, CA, 1 µl/ml) for 5 minutes to block nonspecific Fc receptor-mediated antibody binding, then with fluorochrome-conjugated antibodies or isotype control antibodies (0.5 µg/10^6 cells/0.1 ml) at 4°C for 30 minutes. Samples were washed and resuspended in 250 µl cold PBS and run immediately on an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using Accuri C6 software. Antibodies used for these studies included anti-F4/80-FITC (clone BM8, eBioscience, San Diego, CA), anti-Ly6G-PE (clone 1A8, BD Biosciences), anti-Ly6C and appropriate isotype controls (eBioscience and BD Biosciences). Neutrophils are identified as $Ly6G^+F4/80^-$ cells and macrophages are identified as $Ly6G^-F4/80^+$ cells.

Histological Imaging

Kidney, lung, and spleen were harvested from mice 3 days after intravenous infection with *S. aureus* and embedded in parrafin. Tissues were then sectioned in 5μm slices and stained with an immunohistochemical anti-neutrophil antibody or anti-F4/80. Tissues were sectioned and stained by the Translational Pathology Shared Resource at Vanderbilt University Medical Center.

Bone Marrow Derived Macrophages (BMDMs)

For bone marrow derived macrophages used throughout the dissertation, femurs and tibias of were harvested from male 8-16 week old C57/Bl6 mice, and flushed with 10-15 ml cold PBS to obtain bone marrow cells. Bone marrow cells were then incubated in RPMI 1640 with glutamine and 25mM HEPES (Gibco, Calsbad, CA) containing 10% certified FBS (Gibco, Calsbad, CA), 1% antibiotic-antimycotic (Gibco, Calsbad, CA), and 10ng/mL mouse recombinant macrophage colony stimulating factor (M-CSF, R&D Systems, Minneapolis, MN) for 7 days. At the initiation of the growth period, bone marrow cell concentration was $4x10^4$/mL, except for the assessments at the beginning of Chapter IV, as this has been found to yield a mature, pure population of macrophages[312]. After 7 days of incubation, macrophages were washed with PBS and incubated with media containing various TLR ligands for 24 hours. At 24 hours, macrophages were washed thoroughly with PBS and rested in media for 3 days before assessment. 24hr macrophages were stimulated with MPLA 24hrs prior to assessment.

Cytokine Measurements

For *P. aeruginosa* and LPS experiments presented chapter II, concentrations of IL-6, IFN-γ, IL-1β, and KC in plasma were measured using a Bio-Plex Multiplex Bead Array

and read with the Bio-Plex Magpix Multiplex Reader (Bio-Rad Laboratories, Hercules, CA). For ex vivo LPS blood stimulation experiments as presented in Chapter II, whole blood was mixed 1:1 with RPMI 1640 with glutamine (Gibco, Carlsbad, CA) containing 100ng/mL LPS. After 6 hours, cells were centrifuged and plasma was collected. TNF-α concentrations were measured using a Bio-Plex Bead Array and read with the Bio-Plex Magpix Multiplex Reader (Bio-Rad Laboratories, Hercules, CA).

For heat-killed *P. aeruginosa* experiments as presented in chapter II, BMDMs were incubated with $1x10^6$ CFU/ml of heat-killed *P. aeruginosa* for 24 hours. Prior to the assay, *P. aeruginosa* was heat killed by incubation at 65°C for one hour. IL-6 concentration in the media was determined by IL-6 ELISA (eBioscience, San Diego, CA).

For *in vivo S. aureus* experiments as presented in Chapter III, as well as bone marrow derived macrophage experiments throughout the dissertation, concentrations of IL-6 and TNF-α were measured by Ready-Set-Go ELISA (eBioscience, Inc., San Diego, CA). Concentrations of the cytokines CCL5, CCL2, CCL3, and CXCL2 (MIP-2) were detected by DuoSet ELISA kits from R&D Systems (Minneapolis, MN). All ELISAs were analyzed using a BioTek ELx800 (Biotek, Winooski, VT).

Western Blot

Cells were washed twice with ice-cold Hanks balanced salt solution (HBSS, Gibco, Calsbad, CA) and lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing PhosStop and complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Lysates were diluted 1:1 in Laemmli buffer and denatured at 95°C. Samples were then separated by gel electrophoresis on Mini Protean precast 4–20% Tris-glycine gels (Bio-

Rad Laboratories, Hercules, CA). Proteins were transferred to nitrocellulose membranes (Perkin-Elmer, Boston, MA, USA) in a Tris-Glycine solution (Bio-Rad Laboratories, Hercules, CA) containing 20% methanol. Membranes were blocked with 5% fraction V BSA (RPI Corp., Mount Prospect, IL, USA) diluted in tris-buffered saline with 0.05% w/v Tween-20 (TBST) (Sigma-Aldrich, St. Louis, MO). Membranes were then incubated with primary antibodies at 4°C overnight. Membranes were washed in TBST and incubated with HRP-conjugated secondary antibodies. Protein bands were detected by incubation with ECL reagent (Bio-Rad Laboratories, Hercules, CA), and film exposure. Films were scanned and analyzed using ImageJ software from the U.S. National Institutes of Health (Bethesda, MD, USA).

Phagocytosis Assay

For *in vivo* phagocytosis studies, mice were injected i.p. with 500 µg of pHrodo-tagged *S. aureus* bio-particles (Life Technologies, Carlsbad, CA). After 6 hours, peritoneal leukocytes were harvested by PBS peritoneal lavage. Following harvest, leukocytes were incubated with surface marker antibodies (anti-Ly6G-PE, anti-F4/80-FITC, and anti-Ly6C-PE-Cy5.5) and pHrodo mean fluorescence intensity (MFI) was determined by flow cytometry.

For BMDM phagocytosis experiments analyzed by flow cytometry as presented in Chapter III, BMDMs were incubated with pHrodo-tagged *E.coli* bio-particles (Life Technologies, Carlsbad, CA) and suspended in RPMI 1640 with glutamine and without phenol red (Gibco, Calsbad, CA), for 1.5 hours to allow for phagocytosis to occur. Following incubation, BMDMs were washed three times with PBS. Cells were then

detached using StemPro Accutase (Thermo Fisher Scientific, Waltham, MA) and pHrodo mean fluorescence intensity (MFI) was determined via flow cytometry.

For time course phagocytosis experiments analyzed by plate reader as presented in Chapter V, pHrodo-tagged S. aureus bioparticles particles (Life Technologies, Carlsbad, CA) were incubated with BMDMs for 2 hours. Starting at 15 minutes after the initiation of incubation, and recurring every 15 minutes following for 2 hours total, pHrodo MFI was determined by a BioTek Synergy MX plate reader (Biotek, Winooski, VT).

Respiratory Burst Assay

For *in vivo* assessments of peritoneal leukocytes as presented in chapter III, peritoneal leukocytes were obtained by peritoneal lavage with 2 ml cold PBS and assessed via the Respiratory Burst Assay (Cayman Chemical Company, Ann Arbor, MI). Following isolation, cells were incubated with dihydrorodamine-123, which fluoresces in the presence of reactive oxygen species, for 15 minutes and ROS were elicited by 45 minutes of 200nM phorbol 12-myristate 13-acetate (PMA) stimulation. After PMA stimulation, rhodamine MFI was determined via flow cytometry. Neutrophils, macrophages, and monocytes were determined by FSC-A versus SSC-A.

For assessments of BMDMs, cells were incubated with dihydrorodamine-123. ROS were then elicited by incubation with 200nM PMA for 45 minutes. After stimulation, cells were washed with PBS, detached with StemPro Accutase (Thermo Fisher Scientific, Waltham, MA) and rhodamine MFI was determined via flow cytometry.

Seahorse Assay

Cells were plated in a 96-well Seahorse assay plate at $4x10^4$ cells/well in Seahorse Assay Media (Agilent Technologies, Santa Clara, CA) and assessed on the Seahorse XF^e96 Extracellular Flux Analyzer (Agilent Technologies). For the glycolysis stress test, cells were sequentially treated with 10mM glucose (RPI, Mount Prospect, IL), 1μM oligomycin (Agilent Technologies), and 50mM 2-deoxyglucose (Sigma-Aldrich, St. Louis, MO). For the mitochondrial stress test, Assay media was supplemented with 10mM glucose and cells were sequentially treated with 1μM oligomycin (Agilent Technologies), 1μM FCCP (Agilent Technologies), and 0.5 μM of antimycin A and rotenone (Agilent Technologies).

Gross measurements of cellular metabolism

For ATP assays as presented in Chapter IV, BMDMs plated at $5x10^5$/mL and were lysed with Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI). ATP concentration in the lysate was the incubated with firefly luciferase along with the luminescent ATP Determination Kit (Invitrogen, Carlsbad, CA). Luminescence was then determined by the BioTek Synergy MX plate reader (Biotek, Winooski, VT).

For glucose and lactate measurements as presented in Chapter IV, glucose and lactate in the media were measured by the YSI 2300 Stat Plus Glucose & Lactate Analyzer (YSI, Yellow Springs, OH).

For nitric oxide secretion assays as presented in Chapter IV, nitrites in the media were detected by Griess Reagent Kit (Thermo Fisher Scientific, Waltham, MA). Media was incubated with a 1:1 mixture of N-(1-naphthyl)ethylenediamine and sulfanilic acid for 30 minutes at room temperature and were assessed by spectrophotometric analysis on the . BioTek Synergy MX plate reader (Biotek, Winooski, VT).

For Mito Tracker staining as presented in Chapter IV, Mito Tracker Green was purchased from Thermo Fisher (Waltham, MA). BMDMs were incubated in MitoTracker green for 45 minutes and then assessed via flow cytometry.

Mitochondrial DNA/ Nuclear DNA Measurement

Mitochondrial DNA (mtDNA) and nuclear DNA (nucDNA) were simultaneously isolated using a DNeasy Blood & Tissue Kit (Qiagen). DNA concentration and quality were verified with a Thermo Scientific NanoDrop 2000 spectrophotometer. Presence of the mitochondrial gene *MT-CYB* and nuclear gene *ACTB* were analyzed by Real-Time PCR with the SsoFast EvaGreen Supermix Kit (Bio-Rad, Hercules, CA). Primer sequences for *MT-CYB*: Forward: GCCACCTTGACCCGATTCT; Reverse: TTCCTAGGGCCGCGATAAT. For *ACTB* Forward: AGCCATGTACGTAGCCATCCA; Reverse: TCTCCGGAGTCCATCACAATG. Real-Time PCR reactions were run in quadruplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and quantification of gene expression was determined by the comparative ΔΔCt method.

Metabolite extraction and gas chromatography - mass spectrometry (GC-MS) analysis of ^{13}C-glucose labeling

BMDMs were placed in glucose-free complete media supplemented with 11mM U-^{13}C6-glucose (Cambridge Isotopes, Tewksbury, MA) for 24 hours. BMDMs were then harvested as described in Noguchi et al (2009)[406]. Cell metabolism was quenched by precooled -80^{o}C methanol. A biphasic extraction was then used to separate polar metabolites into an aqueous phase and non-polar lipid metabolites into an organic phase. The extraction results in mixing of free metabolites from separate subcellular

compartments. Polar metabolites were converted to their tertbutylsilyl derivatives using MBTSTFA + 1% TBDMCS (Thermo Fisher Scientific, Waltham, MA)[407]. Nonpolar extracts were converted to fatty acid methyl esters as described in Young et al (2014)[407]. For GC-MS analysis, 1 μl of each derivatized sample was injected into a GC-MS (Agilent 7890A/5975C) equipped with a 30 m HP-5 MS capillary column for analysis of isotopic enrichment. Raw mass isotopolog distributions were corrected for natural isotope abundance as described by Fernandez et al. (1996)[408]. ISA was used to calculate fractional enrichment of acetyl-CoA and fractional lipid synthesis based on palmitate labeling measurements, as described by Ahn and Antoniewicz (2013)[409].

^{13}C Metabolic Flux Analysis

An isotopomer model was constructed using the isotopomer network compartmental analysis (INCA) software package[410]. The model provides a detailed description of the TCA cycle and anaplerotic pathways but does not attempt to accurately resolve fluxes in glycolysis due to the limited number of metabolites measured by GC-MS. Metabolic fluxes were estimated by incorporating pmole/cell/day glucose consumption and pmole/cell/day oxygen consumption as determined by basal oxygen consumption rate in the Seahorse Xf^{e}96. All model fits were over-determined by 94 measurements. Flux estimation was repeated a minimum of 10 times from random initial values to ensure a global minimum was obtained. All results were subjected to a $\chi 2$ statistical test to assess goodness of fit, and accurate 95% confidence intervals were computed for all flux parameters by evaluating the sensitivity of the sum-of squared residuals to parameter variations.

RNA-sequencing

Total RNA was isolated under an RNase-free environment, using the RNeasy Mini Kit (Qiagen). RNA concentration and quality were verified with a Thermo Scientific NanoDrop 2000 spectrophotometer. Total RNA quality was then re-assessed using the 2100 Bioanalyzer (Agilent Technologies). At least 200ng of DNase-treated total RNA with a RNA integrity number greater than 6 was used to generate polyA (mRNA) enriched libraries using TruSeq Stranded mRNA sample kits with indexed adaptors (Illumina). Library quality was assessed using the 2100 Bioanalyzer (Agilent Technologies) and libraries were quantitated using KAPA Library Quantification Kits (KAPA Biosystems). Pooled libraries were subjected to 75 bp paired-end sequencing according to the manufacturer's protocol (Illumina HiSeq3000). Bcl2fastq2 Conversion Software (Illumina) was used to generate de-multiplexed Fastq files.created by a polyA-selected build.

In vivo and *in vitro* Rapamycin Treatment

For in vitro studies, rapamycin (Cayman Chemical Company) was reconstituted in DMSO and added to BMDM culture media at 100nM 1 hour prior to the addition of MPLA. Rapamycin was then removed along with MPLA.

For in vivo studies, Rapamycin (Cayman Chemical Company) was reconstituted in 100% ethanol and diluted in a vehicle containing90% distilled H_2O, 5% Tween 80 (Sigma-Aldrich, St. Louis, MO), and 5% polyethylene glycol 400 (Sigma-Aldrich, St. Louis, MO). Rapamycin or vehicle was then administered at 3mg/kg to mice via intraperitoneal injection 3 hours before intravenous injection of MPLA.

Statistics

All data were analyzed with GraphPad Prism 7 (La Jolla, CA). Data from multiple group experiments were analyzed using one-way ANOVA followed by Dunnett's or Tukey multiple-comparison post-hoc test. Due to significant heteroscedasticity intrinsic to the Bioplex cytokine data as identified by a Levene's test of equality of variances with $p<0.0001$, all *in vivo* cytokine data derived from the Bioplex as presented in Chapter II were log-transformed prior to statistical comparison. All data values are presented as mean ± SEM, except for bacterial counts and body temperature, for which mean values alone are designated. A *p* value of less than or equal to 0.05 was considered statistically significant. All data values are presented as mean ± SEM, except for bacterial counts and body temperature, for which mean values alone are designated. A *p* value of less than or equal to 0.05 was considered statistically significant. For RNA-sequencing studies, Fastq files were analyzed by DESeq. An adjusted p values of 0.05 was used as a cutoff for statistical significance.

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