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Mechanisms of Immuno-Modulation With The TLR4 Agonist Monophosphoryl Lipid A in the Treatment of Sepsis

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Mechanisms of Immuno-Modulation With The TLR4 Agonist Monophosphoryl Lipid A in the Treatment of Sepsis

by

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Dedication

For Nicole, my explanation for the sunrise.

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Mechanisms of Immuno-Modulation With The TLR4 Agonist Monophosphoryl Lipid A in the Treatment of Sepsis

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Monophosphoryl lipid A (MPLA) is a Toll-like receptor 4 agonist that has been approved for use as a vaccine adjuvant in humans. In these studies, we evaluated the effect of MPLA treatment on the innate immune response to systemic infection in mice with emphasis on bacterial clearance, leukocyte recruitment and function, as well as potential signaling pathways involved in TLR4 immmuno-modulation. Mice received either intravenous or intraperitoneal treatment with MPLA prior to induction of bacterial peritonitis by cecal ligation and puncture (CLP), intraperitoneal or intravenous infection with *Pseudomonas aeruginosa*, or burn wound infection. In each model, MPLA treated mice showed significantly improved survival compared to vehicle-treated controls. MPLA treatment prevented infection-associated hypothermia, and enhanced bacterial clearance despite causing attenuation of pro-inflammatory cytokine production. When MPLA was administered after a CLP core temperature was not preserved, but bacterial clearance was still enhanced. Concentrations of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6, that are regulated through the MyD88-dependent signaling pathway, were markedly lower in the plasma of MPLA-treated mice whereas plasma concentrations of Trif-dependent gene products such as G-CSF, MCP-1 and RANTES were largely unaffected. Treatment with MPLA increased the numbers of granulocytes, undifferentiated myeloid cells, and macrophages at infection sites. MPLA treatment also increased the percentage and total numbers of myeloid cells mediating phagocytosis of bacteria. Depletion of monocytes did not eliminate the enhanced bacterial clearance induced by MPLA, but depletion of Granulocytes did. MPLA treatment also increased the recruitment of immature myeloid cells expressing myeloid derived suppressor cell markers. While TLR4 deficient mice were un-affected by pretreatment with MPLA, Trif Deficient mice were still tolerized by MPLA treatment prior to CLP. Cytokine analysis further supports the theory that MPLA exerts its effects primarily through the MyD88

independent pathway. In conclusion, these studies show that MPLA treatment significantly augments the innate immune response to bacterial infection by enhancing bacterial clearance despite attenuation of pro-inflammatory cytokine production. The enhanced bacterial clearance is mediated, in part, by increased numbers of myeloid cells with effective phagocytic functions at sites of infection and this is dependent upon activation of TLR4.

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(Interpretation of original by Raphael)

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Sepsis: historical perspective

While the practice of medicine has dealt with many afflictions, few have been as persistently enigmatic and confounding as the entity of sepsis. Sepsis, or septicemia, is defined as the presence of infective agents or their toxins in the bloodstream; it is characterized by elevated body temperature, chills, weakness, and if left untreated circulatory collapse and death.[1] Machiavelli described this state deftly in The Prince when he stated, "…realizing the physicians tell us of hectic fever that in its beginning it is easy to cure but hard to recognize whereas after a time not having been detected and treated at the first it becomes easy to recognize but impossible to cure."[2] In fact the concept of sepsis and the origins of the term can be traced back to Hippocrates.[3] Both early Greek and ancient Egyptian medicine recognized that the gut was a major reservoir of infectious agents that could spread systemically and cause disease.[4] Aristotle took this notion a step further and hypothesized that sepsis was associated with the generation of microscopic creatures.[4] Severe infections and the resultant immunological dysregulation they trigger remained shrouded in mystery until germ theory began to reveal the potential causes of sepsis in the mid-1800s.

It was with the association made by Dr. Ignaz Semmelweis in Vienna between cadaveric contamination of physician hands and high rates of postpartum infections that medicine gained its first formal insight into microbial causes of disease.[5] Referred to at the time as "childbed fever," mortality from postpartum sepsis declined from 10-20% to 1-2% after Dr. Semmelweis instituted chlorinated hand washing in his practice. Unfortunately at the time such practices were still shunned by the mainstream medical establishment in Europe. Contemporaneous with the work of Dr. Semmelweis were the

efforts of Dr. Joseph Lister to develop antiseptics that would eventually transform surgery. The carbolic acid employed by Dr. Lister dramatically reduced the rate of surgical wound infections and ushered in the modern era of infection control.[6] While microbial contamination was the main target of this early work, Dr. Lister himself even recognized the innate inflammatory properties of damaged tissue.

It was Dr. Louis Pasture who formalized the causation of microbial contamination and infection; and it was from his work in sterilization and vaccination that Leeuwenhoek's observations were ultimately transformed into the formal discipline of microbiology.[7] After the establishment of Koch's Postulates, particular pathogens became implicated with their related diseases, and science began to hone in on targeting the microbes involved in severe infections.[8] Once bacterial (and later viral) causes of sepsis were identified, interest flourished in understanding the immune response to such pathogens. The undisputed father of this field was Elie Metchnikoff, whose contributions include the observation of phagocytosis, defining the inflammatory state as necessary in the resolution of infection, clearance of damaged cells by macrophages, and the discovery of many innate immune functions such as lytic agents, cell migration, and leukocyte recruitment.[9, 10] Most relevant to this dissertation is the observation made by Metchnikoff that phagocytosis was enhanced by prior exposure to non-specific bacterial products, in essence the first account of immuno-modulation.[9]

At the dawn of the 20th century Paul Ehrlich (co-recipient of the Nobel Prize with Metchnikoff) began the development of modern antibiotics, and the elusive quest for defeating severe infections began.[11] Dr. Gerhard Domagk, driven by his experience with the high mortality rate from battle wounds during WWI, would develop sulphanilamide derivative antibiotics, broadening the arsenal against sepsis.[12] The search for better antibiotics reached a tipping point when Dr. Alexander Fleming

discovered Penicillin, that was later isolated and mass produced during WWII.[13] Almost as soon as modern antibiotics were put into use resistant strains of bacteria began to emerge, setting the stage for our current war of attrition.[14]

Sepsis: clinical significance

Sepsis has dramatically shaped human history, accounting for the demise of roughly 20 million people during the bubonic plague in Europe, and contributed to the population crash of the Americas after European colonization.[15, 16] However public sanitation efforts, improvements in medical care, and the use of antibiotics have accounted for massive improvements in public health over the past century. Life expectancy has increased significantly, rising from the average life expectancy of 58 years in 1900 to 77.8 years by 2004.[17] During this time infectious diseases shifted from the number one cause of mortality to number eight.[18, 19] Despite the long history and continued effort to adequately manage severe infections, sepsis persists as a major health issue to this day; and is still one of the top 10 causes of mortality in the United States.[19]

A disturbing trend has emerged in that the incidence of sepsis has increased by roughly 8% since 1979, and while the fatality rate has decreased, the number of annual deaths from sepsis has tripled.[20] The International Sepsis Forum estimates that on average 750,000 people develop sepsis each year in North America; mirroring the results of a previous study demonstrating that 751,000 cases of severe sepsis occurred in the United States in 1995; half of which developed fulminate septic shock.[21, 22] An equivalent number of cases occur annually in Europe.[22] Mortality from septic shock has been shown to range from 20 to 80% depending upon the age and baseline health status of the patient. [23] This issue becomes highly important for industrialized societies

with aging populations who are predisposed to severe infections and tend to have poor outcomes.[24] Our success in treating serious conditions such as cancer and HIV, coupled with the advancement in life saving treatments such as organ transplantation also has increased the number of patients predisposed to major infections; this point is illustrated by the fact that sepsis is now the leading cause of mortality for patients undergoing curative surgery for cancer.[25, 26] Patients who survive sepsis have serious long-term sequelae, including significantly higher mortality rates and decreased quality of life years after their initial disease.[27] In addition to the major health burden, sepsis is an increasingly expensive condition to treat, with an estimated annual cost of $17 billion in the United States alone.[21]

Causes of sepsis

The causative agents responsible for septicemia have shifted in response to our population's health status and our use of antibiotics. Since the inception of modern antibiotic therapy, bacterial pathogens have been developing resistance.[28, 29] As the use of antibiotics has increased, so has the multitude of resistance mechanisms, resulting in a growing number of infections that do not respond to any available antibiotics.[30] The development of new antibiotics to combat multi-drug resistant infections has not kept pace with this growing public health crisis.[31] The widespread use of antibiotics beginning in the 1940s resulted in a shift from Gram positive to Gram negative organisms as the predominant cause of sepsis.[32] This trend remained until the 1980s when the primary cause of sepsis reverted back to Gram positive organisms.[20] While bacteria are still the dominant organisms implicated in sepsis, fungal etiologies are on the rise with a relative increase of close to 10% annually.[33] Early introduction of broad spectrum antibiotics has proven to be beneficial; however, mortality can still be as high

as 56% in patients receiving adequate antibiotic therapy.[34, 35] Disturbingly, the cause of sepsis is undetermined in over 80% of septic patients, and over 30% of patients in fulminate septic shock.[33] The leading nidus of infection for sepsis is the respiratory system (for both community acquired and nosocomial cases), other important sources include primary circulatory infections, urinary infection (primarily in women), gastrointestinal sources, and device related or soft tissue wounds.[21]

Defining sepsis, the origin of Systemic Inflammatory Response Syndrome

Along with the evolution of the causative agents responsible for sepsis the definition and treatment regiments have also changed over time. The first attempt to formally define the clinical entity of the "sepsis syndrome" occurred in 1989 lead by Dr. Robert Bone.[36] The definition criteria consisted of hypothermia (<96ºF) or hyperthermia (>101ºF), tachycardia (>90 beats/min), tachypnea (>20 breaths/min), clinical evidence of an infection site, and at least one end organ with inadequate perfusion or demonstration of dysfunction (cerebral dysfunction, hypoxemia, elevated plasma lactate, or oliguria). This definition proved insufficient and in 1991 the American College of Chest Physicians and the Society of Critical Care Medicine convened a consensus conference that resulted in the creation of the term systemic inflammatory response syndrome (SIRS), that is still commonly used in the clinical literature.[37]

The term SIRS was developed in recognition of the fact that many non-infections insults such as pancreatitis, trauma, burns, and ischemia/reperfusion injury could result in a state resembling that of septic shock.[38] This observation validated the long held notion that septic shock resulted from an over activation of the immune system in response to infection. The SIRS criteria was defined by the 1991 consensus conference as 2 or more of the following: body temperature < 36°C or > 38°C, heart rate >90

beats/Min, respiratory rate >20 breaths/Min (or hyperventilation with a PaCO2 <32 mmHg), white blood cell count >12,000 cells / mm^3 or <4,000 cells / mm^3, or immature neutrophils >10%.[37] Sepsis was determined to fall under this SIRS definition; however this guideline for diagnosis was too sensitive, lacked sufficient specificity, and was an inadequate predictor of outcome.[38]

To address the need for better defining criteria for sepsis and SIRS, an international committee convened in 2001.[39] This 28 member coalition of experts concluded that the guidelines developed during the 1991 consensus conference should remain in place for lack of sufficient evidence in the existing literature to develop an improved diagnostic paradigm. The 2001 conference summary statement even acknowledged that the lack of an improved definition highlights the dearth in current knowledge on the topic of sepsis and SIRS, underscoring the need for further research in the field. In 2002 the European Society of Intensive Care, the Society of Critical Care Medicine, and the International Sepsis Forum, all participants in the previous year's conference, assembled to produce the "Barcelona Declaration." This document called on critical care providers, governmental and health agencies, along with the general public to join the fight against sepsis and reduce the mortality rate by 25% in 5 years.[40] The Barcelona Declaration would evolve into the Surviving Sepsis Campaign (SSC), an international effort consisting of 11 different organizations aimed at providing guidelines to health care providers to accomplish the initial goal of significant mortality reductions in sepsis.

Treatment of sepsis

The Surviving Sepsis Campaign (SSC) developed a series of evidence based intervention bundles derived from the current literature in order to standardize the

management of patients with sepsis and septic shock.[41] The first approach outlined is to initiate fluid resuscitation within the first 6 hours of recognition of sepsis or septic shock, to maintain adequate central venous pressure (between 8 – 10 mmHg), a mean arteriole pressure > 65 mmHg, urine output > 0.5L / hr, and a mixed venous oxygen saturation of > 70%. If these criteria are not met with standard fluids then packed red cells should be administered judiciously in response to the patient's hematocrit. The next step in treatment is to draw cultures for a proper diagnosis, the administration of appropriate antibiotics, and the removal of any nidus of infection.

The SSC guidelines call for the administration of both vasopressors and inotropic agents to maintain adequate cardiac output and end organ perfusion. Interestingly, the guidelines also call for the administration of corticosteroids in the case of septic shock for patients who require vasopressors in spite of adequate fluid resuscitation. The primary goal of corticosteroid therapy is to treat adrenal insufficiency and increase patient responsiveness to vasopressors, however these agents also have the potential to suppress immune function. This harkens to the notion that much of the pathology seen in sepsis and septic shock is the result of an overactive immune response to infection or microbial products. Another advent in modern sepsis management is an emphasis on glucose control; at the suggestion of SSC, glucose should be maintained at a level < 150 mg/dL in patients after hemodynamic stabilization. The use of blood products should be limited to patients with a hemoglobin < 7 g / dL who have received proper fluids. The SSC care bundle also calls for the use of recombinant activated human protein C in patients who exhibit a high risk for mortality.

Despite the numerous experts and entities involved in the process, and the extensive literature review conducted to formulate these guidelines, many of the suggestions have received a relatively low confidence rating even by the SSC. However,

a follow-up study performed to assess the effectiveness of the SSC guidelines concluded that many of the care bundle components were of definite benefit.[42] A study investigating the effectiveness of the SSC guidelines was conducted over a two year period at over 160 study sites in 30 countries and involved over 15,000 patients. During this period compliance with the entire management bundle increased from 18.4% to 36.1% and absolute mortality odds ratio decreased by 5.4%.[43] While the results from the Surviving Sepsis Campaign are important, they fall short of the 25% reduction in mortality originally hoped for, and mortality remained as high as 63% in patients with multiple organ dysfunction in this study. The treatment approaches recommended by SSC are supportive and, with the exception of treatment with activated protein C, do not directly attack the underlying immunologic mechanisms that are thought to cause the pathophysiology of sepsis. These results illustrate the fact that we still don't fully understand the pathophysiology of sepsis, nor have we developed an adequate treatment paradigm.

Sepsis pathophysiology

Concurrent with the early drive to generate antibiotics to treat microbial infections was a growing sense of awareness that bacterial subunits were capable of inducing states mimicking sepsis in the absence of live infections. Dr. Richard Pfeiffer, while working with Koch in Berlin, discovered endotoxin from studying *Vibrio cholera* infections.[44] Endotoxin, or lipopolysaccharide (LPS), proved to be one of the most potent inducers of sterile septic shock; but it would take nearly a century to begin understanding the effect of this molecule on the immune system.

As scientific understanding of the causes of sepsis and the ensuing immune response grew, so did the acceptance that it was the host response that played an

important role in the deleterious outcomes. Sir William Osler states in The Evolution of Modern Medicine that, “Except on few occasions, the patient appears to die from the body's response to infection rather than from it.”[45] This deft observation harkens back to the notion purported by Machiavelli that once fully engaged, the state of septic shock is both remarkable and unrelenting.

Sepsis can result from bacterial, fungal, viral, and even parasitic infections. In addition endotoxin (LPS), excreted exotoxins acting as super-antigens, and other microbial subunits are capable of eliciting a septic shock like state without any infectious agent.[46-48] Regardless of the inciting entity, the resultant inflammatory response is often similar and can range from local inflammation to a systemic hyper-inflammatory state with multi-organ dysfunction. The inflammatory process is beneficial and often sufficient to resolve mild, well localized infections in a healthy host; however the systemic inflammatory response to uncontrolled infections or blood-borne pathogens is truly life-threatening.

Cytokines have long been implicated in the hyper-inflammatory state of sepsis. Interleukin-1β (IL-1β), produced in large quantities by monocytes and endothelial cells, has been detected in circulation in up to 90% of septic patients surveyed, and has been shown to correlate with poorer outcomes in meningococcemia associated sepsis.[48, 49] When administered to animals, IL-1 alone triggers a high cardiac output hypotensive response with associated leucopenia, thrombocytopenia, hemorrhage, and pulmonary edema.[50] The other main inflammatory cytokine implicated in sepsis is Tumor Necrosis Factor-α (TNF-α).[48, 51] TNF-α is also produced in large part by macrophages and neutrophils in response to endotoxin and other inflammatory mediators.[52] TNF-α can elicit many of the pathological changes associated with septic shock in the absence of infection such as hemodynamic instability, fever, metabolic

acidosis, coagulation cascade activation, hypoglycemia, and even impaired renal function.[48] An ever growing number of secreted soluble factors from a variety of cell types have been discovered to contribute to the state of septic shock, often acting in synergy with one another. These include IL-2, IL-6, IL-8, IL-12, IL-18, Interferon-γ (IFN-γ), and granulocyte-macrophage colony stimulating factor (GM-CSF), just to name a few.[48]

Changes in the vasculature are a common and serious issue in septic patients. Endothelial and leukocyte activation (in particular neutrophils) during sepsis results in capillary leakage, damage to the endothelium, and leukocyte obstruction of capillary blood flow.[53-55] These effects can be triggered directly by microbial product-induced activation of the cells involved, or by the cytokines commonly associated with sepsis.[48, 51] The coagulation pathways are activated through a variety of different means during sepsis, but most notably through the extrinsic pathway, leading to a hyper-coagulable state resulting in the excessive formation of thrombi, as well as the depletion of coagulation factors, commonly referred to as disseminated intravascular coagulation (DIC).[56, 57]

TNF-α has been shown to activate the coagulation cascade, and IL-1 induces a pro-coagulant state while inhibiting anticoagulant mechanisms.[58, 59] Endothelial damage and dysfunction further activates the coagulation cascade in sepsis, and also results in impaired anti-coagulant activity.[60, 61] The damage to blood vessels, numerous thrombi formed, and large leukocyte infiltration results in end-organ dysfunction and tissue necrosis. In addition, thrombin produced by sepsis-induced activation of the coagulation cascade not only induces clot formation but also causes activation of leukocytes through binding and activation of specific receptors. Activated

protein C was developed to target the pro-thrombotic and pro-inflammatory mechanisms associated with activation of the coagulation system during sepsis.

The vascular and circulatory insufficiency seen in sepsis, and particular septic shock, is also mediated by nitric oxide (NO). NO has been linked to TNF-α and endotoxin-associated hypotension and considerable evidence shows that it is the primary factor that contributes to systemic vasodilation during sepsis.[62, 63] NO also contributes to sepsis-induced organ injury through the generation of peroxynitrite, which causes cellular injury and death. While NO levels have correlated with some pathological changes seen in septic shock, it also may play a protective role by preventing platelet and leukocyte adhesion.[51] Furthermore, NO-derived reactive nitrogen species are an important mechanism used by phagocytes to kill engulfed bacteria. Several nitric oxide synthase (NOS) inhibitors have been employed in clinical trials to treat sepsis without significant benefit, and in some cases were detrimental.[64]

Through a variety of mechanisms the over-activation of the immune system in sepsis can eventually lead to multi-organ dysfunction and failure. Myocardial depression seen in sepsis has been attributed to inflammatory cytokines (such as TNF-α, IL-1β, and IL-6), as well as other soluble inflammatory mediators such as lysozyme, leukotrienes, prostaglandins, and NO.[65] Serum from septic patients has been shown to impair myocyte contractility in vitro.[66] Bacterial DNA has also been demonstrated to have a myocardial depressive effect, probably mediated through toll-like receptor signaling.[67] Pulmonary dysfunction is another hallmark of sepsis and a long-held diagnostic criterion. Many of the same inflammatory mediators are associated with pulmonary dysfunction as well as cardiac dysfunction. Acute respiratory distress syndrome resulting from sepsis or other systemic inflammatory disorders is mediated largely by the infiltration of inflammatory myeloid cells and congestion of airways and blood vessels within the

lung.[68] Iatrogenic damage is also a concern with ventilated septic patients, a problem further compounded by diaphragmatic dysfunction resulting from both disuse and oxidative stress.[65] Renal impairment has historically been seen as secondary to ischemia but recently has been linked to local cytokine production, reactive oxygen species production, and fibrin deposition.[69] The use of norepinephrine to treat circulatory insufficiency in sepsis can also exacerbate renal failure because of its constrictive effect on glomerular afferent arterioles, illustrating the difficulties in managing patients with severe sepsis.[70, 71] Nearly every organ system studied has been shown to suffer from dysfunctions during sepsis, culminating in a high mortality rate and management difficulties that still plague the field. In addition, the breakdown byproducts resulting from tissue destruction are pro-inflammatory and further contribute to the immunological dysfunction seen in sepsis.[72]

Much of the pathology involved in sepsis is the result of over activation of the host immune response via toll-like receptors (TLRs) and other innate pathogen sensing mechanisms.[73] The co-evolution of microbes and the multi-cellular organisms they colonize and infect has driven the development of our immune system which is capable of detecting and eradicating a wide array of potential pathogens; both previously encountered and completely novel to the host. The ability of the innate immune system to detect and respond to such a varied barrage is in large part the result of sensing mechanisms for conserved pathogen associated molecular patterns (PAMPS). Despite the vast heterogeneity found in the potential microbial pathogens, there are a large number of conserved molecules, that the host is capable or recognizing as “foreign” through pattern recognition receptors (PRRs).[74]

The functions of PRRs elucidated thus far include the activation of complement and coagulation cascades, triggering pro-inflammatory signaling pathways, initiating

apoptosis, opsonization, and phagocytosis. PRRs are both secreted by and expressed on or within cells. Mannan-binding lectin (MBL), C-reactive protein (CRP), and serum amyloid protein (SAP) are acute phase reactants secreted by the liver that function as PRRs. CRP and SAP opsonize bacteria by binding phosphorylcholine and activate the classical complement pathway.[75, 76] MBL activates the lectin complement pathway after attaching to terminal mannose residues found on viruses, fungi, parasites, as well as Gram-positive and negative bacteria.[77] MBL also recognizes damaged and apoptotic cells both exacerbating inflammatory states, and facilitating the clearance of damaged or neoplastic tissue. Both LPS binding protein (LBP) and soluble CD14 are capable of binding LPS and catalyzing the activation of myeloid cells by transferring LPS to the surface associated CD14, MD-2, TLR4 signaling complex. LBP is also capable of binding glycolipids and the lipoproteins from spirochetes, mycobacterium, and mycoplasma.[78] Interestingly, while low concentrations of LBP enhance the cellular responsiveness to LPS, at high concentrations LBP can inhibit LPS induced inflammation.[79] This illustrates how even simple carrier proteins of the innate immune system can play an important regulatory role in the inflammatory response to infection.

In addition to the secreted PRRs many are expressed intra and extra-cellularly on a wide array of cell types. Examples include macrophage mannose receptor (MMR), macrophage scavenger receptor (MSR), macrophage receptor with collagenous structure (MARCO), and DEC205 found on dendritic cells (DCs).[74, 80] These surface associated receptors are involved in the binding and phagocytosis of a wide range of potential pathogens. Intracellular PRRs include the NOD-like receptors (NLRs) and RIG-like helicases (RLHs). NLRs are capable of sensing peptidoglycan (PGN) degradation products such as meso-diaminopimelic acid and muramyl dipeptide (MDP), bacterial RNA (muramyl dipeptide), flagellin, and viral RNA.[81, 82] Signaling through

the various NLRs can activate NF-kB, and / or Caspase 1, triggering inflammatory and apoptotic cellular events. The RLHs are capable of detecting viral PAMPs, leading to the activation of NF-kB and interferon regulator factors (IRF), triggering an anti-viral response.[83]

Probably the most well studied of all the PRRs discovered to date are the Toll-Like Receptors (TLRs). TLRs are capable of sensing both internal and external threats from a wide range of sources. TLRs have become the quintessential PRR since the discovery of human TLR4 in 1997 and the identification of it's responsiveness to LPS the following year.[84] Soon after the discovery of the developmental protein "Toll" in *Drosophila*, its homology to human IL-1 receptor was established along with its role in NF-kB signaling.[85] Thus far 11 human TLRs have been discovered. They are capable of sensing a vast array of substrates summarized in figure 1.1.[86, 87]

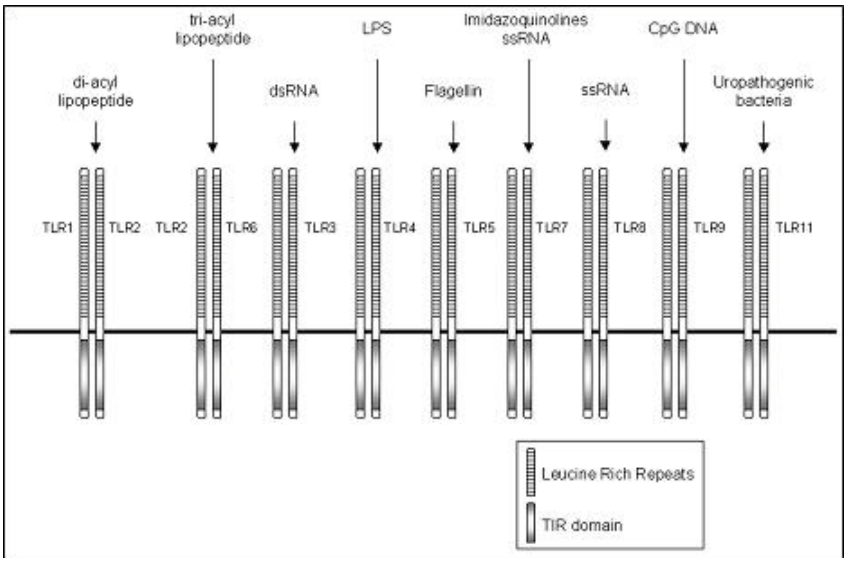


Figure 1.1 The known human TLRs and their prototypic ligands.
Adopted from: Kawai et al. 2005.

An ever expanding list of tissues and cell types express the PRRs responsible for triggering the hyper-inflammation associated with sepsis and septic shock. Epithelial cells from the cornea to the gastrointestinal track express TLRs enabling them to detect

potential threats.[88, 89] Endothelial cells express NLRs, TLRs, and RLHs enabling the vascular lining to play an important role in the inflammatory process.[90] Even neurons from the CNS and peripheral nervous system have been shown to express TLRs and respond to their agonists.[91, 92] The dominant cell type associated with detecting and responding to invading pathogens are leukocytes such as neutrophils, macrophages, and dendritic cells; which express the greatest variety of PRRs and have long been considered to drive the inflammatory response of sepsis. Once a microbe or its associated PAMPs have been detected by innate immune sensing mechanisms, a complex cascade of responses are triggered.

TLR4: the endotoxin centurion

TLR4 is one of the best characterized TLRs and is critical for the innate immune response to Gram-negative bacteria, as well as pure LPS. Lipopolysaccharides, or LPS, are amphipathic molecules located on the outer portion of Gram-negative bacteria and function as a barrier to both polar and non-polar molecules.[93] The LPS macromolecule consists of a hydrophilic polysaccharide portion composed of the highly variable O-specific chain and core oligosaccharide linked to a hydrophobic portion termed lipid A.[94] LPS is released by the lysis of bacteria triggered by innate immune mechanisms, or during the natural division and death of Gram-negative organisms.[95] Significant amounts of LPS can also be released in-vivo after treating Gram-negative infections with antibiotics, that may have deleterious effects on septic patients.[96] LPS can form aggregates in solution that are dissolved and rapidly bound by LPS binding protein (LBP), that facilitates the binding of LPS to surface associated and soluble forms of CD14.[97, 98] Interestingly, lipoproteins have been shown to bind LBP-LPS complexes and may play a role in LPS clearance and inflammatory control.[99] This is supported by

the finding that an inverse relationship exists between HDL levels and the risk of developing severe sepsis in hospitalized patients.[100] CD14 was considered to be the LPS receptor responsible for cellular recognition and signal initiation, but it was eventually shown to be a glycosylphosphatidylinositol (GPI)-anchored protein lacking the necessary trans-membrane and intracellular components to relay signaling events after binding to LPS.[101] Inhibition of CD14 has been shown to only partially attenuate the effect of LPS, indicating that it is involved, but not critical for LPS recognition.[102] Mice deficient in CD14 are resistant to both endotoxin and Gram-negative bacterial infection induced sepsis.[103] It is now widely accepted that CD14 facilitates the transfer of LPS to the TLR4 receptor complex, however CD14 also facilitates the recognition of lipopeptides, lipoteichoic acid (LTA), peptidoglycan, and dsRNA by other TLRs.[98, 104]

Mice with mutations in TLR4 helped solidify the importance of this receptor in the recognition of LPS.[73, 105] Despite a reduction in nitric oxide generation and a delay in cytokine production TLR4 deficient mice (C3H/HeJ) mortality was increased after live Gram-negative infection due to uncontrolled bacteria proliferation.[106] It was also demonstrated that humans with mutations in TLR4 were hypo-responsive to LPS inhalation challenge; and that this could be reversed ex-vivo with supplementation of wild-type TLR4.[107] TLR4 is responsive to a variety of substrates other than LPS including lipoteichoic acid, *Mycobacteria spp*., *Treponema* glycolipids, respiratory syncytial virus Protein F, and endogenous ligands such as heat shock protein (HSP) 60.[73, 108] Like many surface associated immune receptors, the LPS recognition complex has been shown to consist of numerous molecules and to differ in composition by cell type.[101] For example, LPS receptor binding has been shown to associate with HSP 70 & 90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5

(GDF5) on monocytes.[109] While Triantafilou and colleagues demonstrated that antibody blockade of these molecules abrogated LPS induced cytokine production, they admit that this phenomenon is most likely attributable to steric inhibition of TLR4 receptor activity.[101] The membrane associated signaling complex also participates in the regulation of TLR4 mediated inflammation. For example surfaced expressed RP105/MD-1 inhibits TLR4 activation and regulate the inflammatory response to LPS.[110] In addition to TLR4, the surface receptor MD-2 (Ly96, or ESOP-1) has been shown to be critical for LPS recognition and signal transduction.[111] MD-2 binds LPS and complexes with TLR4 to confer the proper conformational changes in TLR4 structure for intracellular signaling transduction to transpire. TLR4 / MD-2 / LPS complex dimerization is also required for signaling initiation.[112] Figure 1.2 depicts the critical steps in extracellular LPS binding and recognition for TLR4 dependent endotoxin recognition to occur.

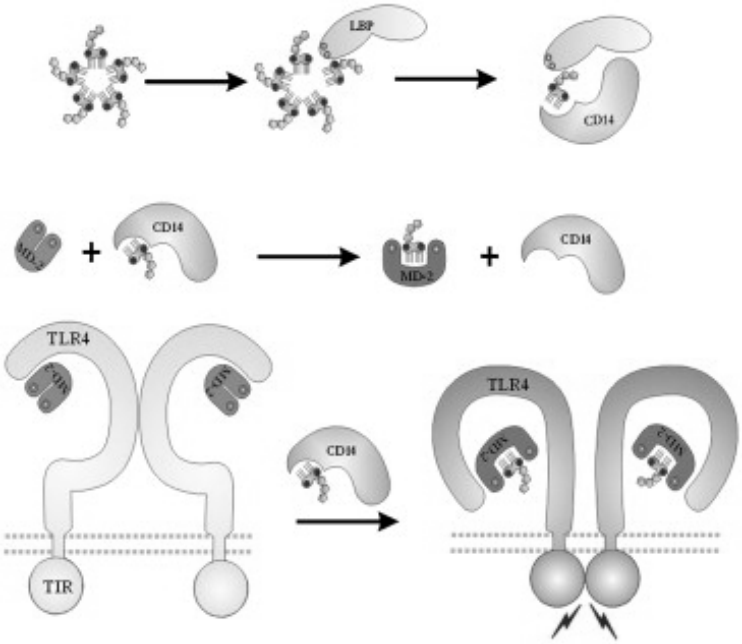


Figure 1.2 LPS aggregates are disrupted by LBP and transferred to soluble or surface associated CD-14. Adopted from Jerala 2007.

Toll like receptors are type 1 trans-membrane glycoproteins structurally composed of an extracellular LRR (leucine-rich repeat) domain responsible for ligand

binding and an intracellular TIR (Toll/IL-1 receptor) domain necessary for signal transduction.[113] The LRR region of TLR4 form a “horseshoe” structure composed of an N-terminal, central, and C-terminal region. The central region of TLR4 does not possess the stabilizing asparagine ladder sequence found in other LRRs which may account for the greater diversity of ligand recognition attributed to TLR4.[114] Ligand / MD-2 / TLR4 complex formation triggers dimerization of TLR4, causing close association of the LRR C-terminal region and allowing for adapter molecule recruitment and phosphorylation events to occur at the intracellular TIR domain of TLR4.[113] TLR4 is unique in that it signals through both of the two pathways utilized by TLRs, the MyD88-dependent and -independent pathways outlined in figure 1.3.

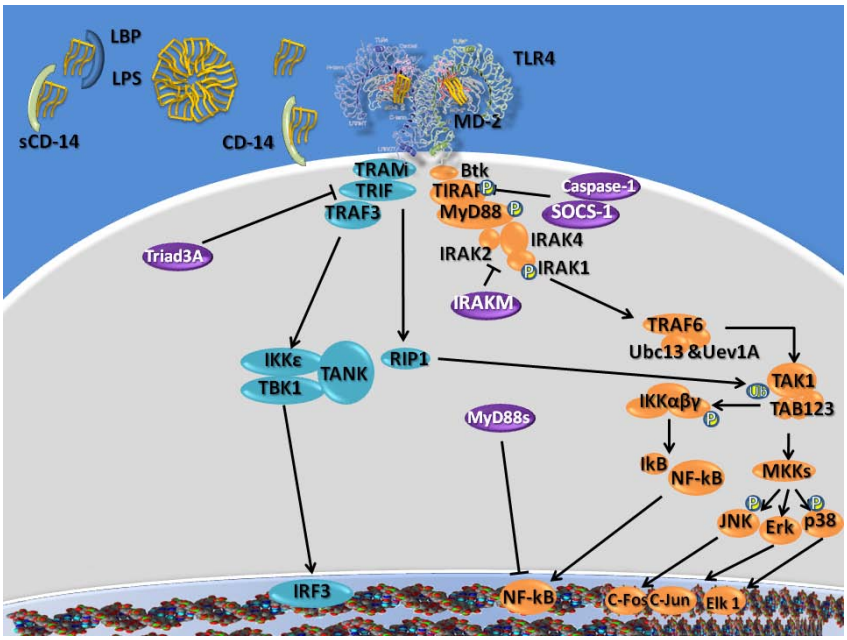


Figure 1.3 The TLR4-associated MyD88-dependent and TRIF-dependent signaling pathways. TLR4 Crystal structure adapted from Park 2009.

The recruitment of adaptor molecules to the TIR region of TLR4 also affords another opportunity for negative regulation. The intracellular proteins ST2L and SIGIRR are capable of binding and sequestering the adaptor molecules recruited by the TIR

domain of TLR4.[115, 116] For initiation of the myeloid differentiation primary response protein 88 (MyD88) dependent pathway the TIR cytosolic region of activated TLR4 is bound by MyD88, and is dependent on association with the TIR domain-containing adapter protein (TIRAP); also referred to as Mal.[117] TIRAP is phosphorylated by Burton's Tyrosine Kinase (Btk) which is a necessary step for recruitment and activation of MyD88.[118] Interestingly, the TIRAP 180L mutation appeared during human colonization of Eurasia, conferring a greater cytokine response to infection upon heterozygous individuals, but a greater susceptibility to hyper-inflammatory septic shock in homozygous individuals.[119]

TIRAP is susceptible to regulation by caspase-1 mediated cleavage and ubiquitination by SOCS-1 (Suppressor of Cytokine signaling 1), inhibiting downstream activation of the MyD88 dependent pathway.[120, 121] Janssens and colleagues demonstrated that the inducible splice variant MyD88(S) can prevent TLR mediated activation of NF-kB but not the activation of AP-1, representing another potential mechanism of immune regulation.[122] MyD88 can recruit and activate the p85 subunit of phosphoinositide 3-Kinase (PI3K), leading to the activation of Akt which in turn exerts a multitude of effects.[123] MyD88 association with the TLR4 signaling complex recruits a number of interleukin-1 receptor associated kinases (IRAK), including IRAK1, IRAK2 and IRAK4.[124] Post-phosphorylation IRAK1 dissociates from the TLR4 signaling complexes and binds tumor necrosis factor receptor associated factor 6 (TRAF6).[125] IRAK1 phosphorylation can be inhibited by the IRAKM splice variant produced in response to LPS exposure, acting as another method of inflammatory control.[126] Activated TRAF6 complexes with Ubc13 and Uev1A to form an ubiqitination complex.[127] This heterometric complex mediates the activation of membrane associated TGF-β activated Kinase (TAK1) and TAK1-binding proteins

TAB1, TAB2, & TAB3.[128, 129] The TAK1 complex activates IkB Kinases (IKK) through phosphorylation, leading to the liberation of NF-kB sequestered in the cytoplasm.[130] TAK1 also activates the MAP Kinases MKK3 and MKK6 leading to activation of JNK and p38.[130]

TLR4 also activates the MyD88-independent pathway which is associated with recruitment of TICAM-1 (TIR domain-containing adapter molecule 1, also known as TRIF) to the TIR domain of TLR4.[131] This process is dependent upon TRIF-related adaptor molecule (TRAM) recruitment of TRIF to the membrane. Recent work by Tanimura and colleagues has shown that LPS activation of TLR4 triggers endosome / lysosome trafficking of the TLR-TRAM-TRIF signaling complex followed by TRAF3 recruitment and activation.[132] These findings support the previous studies done by Husebye and colleagues that demonstrated TLR4 was internalized and trafficked to the endosome after activation.[133] The TLR4-TRIF signaling complex is regulated in part by ubiquitination mediated though Triad3A.[134] TRIF is bound by the serine / threonine kinase RIP1 (receptor interacting protein 1), that has been shown to be an essential step for activation of NF-kB but not IRF3.[135] RIP1 activation leads to NF-kB and MAPK signaling events via activation of the TAK1 signaling complex.[136] TRIF also recruits and activates TRAF3, that in turn binds and activates the proteins TANK (TRAF family member associated NF-kB activator), TBK1 (Tank Binding Kinase 1), and IKKi.[137] This signaling complex activates the transcription factor Interferon regulatory Factor 3 (IRF3).[138]

The primary end result of TLR4 cascade initiation is the activation of transcription factors responsible for the production of the pro-inflammatory response associated with LPS exposure. IRF3 activation through the Trif-dependent pathway, triggers the production of interferon-inducible protein 10 (IP10, also known as CXCL10),

as well as IFNβ.[139] The liberation and nuclear translocation of NF-kB after TLR4 stimulation is responsible for the generation of the classical pro-inflammatory response (production of IL-1, TNFα, and IL-6) associated with LPS exposure.[140, 141] Interestingly NF-kB was first discovered by Baltimore and colleagues while investigating the effects of LPS on pre-B cells.[142] The kinetics of NF-kB signaling have been shown to be dependent upon the dose and source of TLR4 ligand, with high dose LPS triggering maximal nuclear translocation in monocytes within 15 minutes, whereas live *E. coli* took 20 minutes produce half the level of activation.[143] The response to LPS is also dependent upon the cell type and co-stimulatory events. For example the MyD88-independent pathway has been shown to be activated in human neutrophils after stimulation with LPS and interferon, but not when stimulated with LPS alone unlike monocytes.[144, 145]

Significant synergy and inhibition occurs between the various innate immune signaling pathways. Immuno-receptor tyrosine-based activation motifs (ITAM) pathways are activated by Fc receptors, chemokine receptors, and adhesion molecules.[146] ITAM activation can either inhibit or enhance TLR signaling events.[147] Likewise Nod2 activation by bacterial MDP increases cellular IRAK-M, inhibiting TLR4 mediated inflammation.[148] TLRs also synergistically impact each other as demonstrated cytokine production enhancement resulting from TLR8 and TLR3/4 co-stimulation in DCs and macrophages.[149] The extensive communication which occurs between various signaling cascades, and even between the two arms of TLR4 signaling pathways, emphasize the complexity of innate immunity and the difficulty in elucidating ways to control the immune response.

Endotoxin Tolerance: suppression vs. modulation

While Gram-negative organisms account for only 40% of sepsis, patients with Gram-negative bacteremia have a 50-60% chance of developing fulminate septic shock; whereas patients suffering from fungal or gram positive bacteremia have only a 5-10% chance of progressing to septic shock.[150] The enhanced inflammatory effect of Gram-negative pathogens is indicative of the dramatic stimulatory effect of TLR4 agonists. While integrated regulatory mechanisms exist to curb to activation of TLR4 and the resultant inflammatory response, large scale activation of TLR4 is capable of overwhelming these suppressive functions. However, non-fatal endotoxemia or sepsis is often followed by a period of hypo-inflammation believed to result from unbalanced immune regulatory mechanisms. The power of innate anti-inflammatory systems was noted by Dr. Pasteur in his description of sepsis survivors, attributing their recovery to "*natura medicatrix*."[151] This state of immune anergy following a massive inflammatory insult was given the name compensatory anti-inflammatory response syndrome (CARS) by Dr. Bone in 1996, one of his final gifts to the field of critical care medicine before passing the following year.[152]

The dichotomy of endotoxin has been a well known and a long confounding phenomenon in the field of immunology. Following the discovery of endotoxin by Koch and Pfeiffer, Dr. Centanni demonstrated in 1894 that subcutaneous administration of endotoxin would result in severe local inflammation and leukotaxis in volunteers.[153] Dr. Shwartzman discovered in 1928 that rabbits injected subcutaneously with sterile bacterial culture filtrate containing endotoxin followed by intravenous injection with the same filtrate would result in a focal necrotizing lesion at the initial injection site.[154] The Shwartzman reaction could also be elicited systemically with two doses of intravenous endotoxin, creating a state similar to septic shock with associated DIC. The injection interval proved to be critical for both models, with an 18-24 hour interval

between exposures eliciting the greatest response. Injection regimens of shorter or longer intervals were not capable of creating the lesions or septic like state. These results indicated that priming with LPS was capable of both enhancing and suppressing the response to subsequent endotoxin exposures.

Long before any mechanism of action was elucidated, endotoxin was used as a therapeutic agent for a vast array of conditions. In 1887, Dr. Von Jauregg observed a correlation between secondary bacterial infections and improvement in neuro-syphilis patients.[155] The first therapeutic use of endotoxin most likely occurred in 1893 when heat killed Gram-negative bacteria was used to treat Typhus. These early experiences spurred on the development of "fever therapy", where endotoxin and other microbial pyrogens were used to treat everything from cancer to schizophrenia. The occasional success, such as Wittich's experience with treating chronic asthma, may be attributable to the immune-modulatory effects of TLR agonists.[156] During the years of fever therapy, the phenomenon of endotoxin tolerance was discovered. It was noted that patients undergoing treatment regimens with endotoxin would require larger and larger doses over time to elicit a pyrogenic response.[157] This mimicked observations that patients recovering from typhoid fever and malaria were tolerant to endotoxin, as were "volunteers" after experimental typhoid infections.[158] Experimental endotoxin tolerance was fully developed by Dr. Beeson in the 1940s by repeated injection of rabbits with vaccine strains of Gram-negative bacteria.[157] Like many studies before and since, Dr. Beeson demonstrated that exposure to one strain of bacteria would tolerize an animal to unrelated strains and species.

Endotoxin tolerance has historically been viewed as a hypo-responsive state of immune-suppression similar to the compensatory anti-inflammatory response syndrome (CARS). Clinical manifestations of CARS can occur as a result of trauma as well as

sepsis and other SIRS related conditions. CARS is associated with a decline in the total number of T-cells, a decrease in monocyte secretion of soluble mediators and activation marker expression, as well as decreased production of pro-inflammatory cytokines such as TNFα, IFN-γ, and IL-1.[159-161] Patients suffering from this hypo-inflammatory state are at increased risk for developing secondary infections.[162] In addition to a decline in the production of inflammatory mediators, CARS is associated with an increase in the production of anti-inflammatory mediators such as soluble TNFα receptor (sTNFR), IL-1 receptor antagonist (IL-1Ra), and regulatory cytokines such as IL-10.[163] Relatively high levels of soluble CD14 and MD-2 has been measured in the plasma of septic patients which could inhibit TLR4 activation in myeloid cells.

The serum from septic and burn patients has been shown to be immune-suppressive, a phenomenon attributable to the numerous soluble anti-inflammatory agents recovered from such patients.[151, 164, 165] The effect of SIRS and CARS on myeloid cell function has proven at times to be contradictory. Von Knethen and colleagues described reduced macrophage oxidative bust activity in a cell culture model of sepsis.[166] However in a recent study by Hiroshi and colleagues, it was shown that neutrophils recovered from trauma patients with and without secondary infections demonstrated enhanced oxidative burst activity when stimulated *ex-vivo* with fMLP.[167] Early theories portrayed CARS to follow SIRS in a Newtonian fashion of action and reaction; there is a growing consensus however that SIRS and CARS temporally overlap to create a state of massive immune-dysregulation.

Much like CARS endotoxin tolerance has been shown to correlate with decreased production of pro-inflammatory cytokines such as TNFα, IL-6, and IFN-γ in response to subsequent endotoxin challenge or infection.[168] While CARS is viewed as a negative predictor of outcome in the severely ill, experimental models of endotoxin tolerance are

not necessarily deleterious. LPS pre-treatment has been shown to protect against fatal endotoxemia in mice.[169] LPS tolerance has been shown to protect against live infections with *Cryptococcus neoformans, Salmonella enteritica,* as well as poly-microbial sepsis induced by cecal ligation and puncture.[170-172] This enhanced anti-microbial response occurred even with a decline in pro-inflammatory cytokine production. Our research group has shown that priming with LPS protects against infection with *Staphylococcus aureus*.[173] We have also demonstrated that LPS priming before a peritoneal infection with *Pseudomonas aeruginosa* enhanced bacterial clearance, reduced mortality, and attenuated the production of IFNγ and IL-12.[174] We found that the priming effect occurred independent of IFNγ; since IFNγ knockout mice were also resistant to infection and pretreatment with IFNγ did not affect cytokine production or bacterial clearance. In this study it was also demonstrated that LPS tolerant mice exhibited greater expression of the regulatory cytokine IL-10 after infection. Subsequent studies by our research group have demonstrated that endotoxin tolerance does occur in IL-10 deficient mice and that this regulatory cytokine is not necessary for the enhanced bacterial clearance effect.[175]

One purported mechanism of enhanced bacterial clearance in LPS-primed mice is a reduction in neutrophil apoptosis.[176] While Feterowski and colleagues observed reduced apoptosis in neutrophils from LPS-primed mice, the neutrophils did not exhibit enhanced reactive oxygen species (ROS) production; indicating that the improved bacterial clearance was mediated more by surviving phagocytes rather than enhanced per cell function. By contrast, one recent study demonstrated enhanced phagocytic activity in LPS-primed human macrophages; however this study was conducted using samples from cystic fibrosis patients and may not be easily extrapolated to healthy patients.[177] In-vitro treatment of human macrophages with LPS reduced the cellular HIV infection rate

by the down regulation of the CCR5 receptor, indicating that endotoxin priming may protect against infection with some viruses.[178] LPS pretreatment has also been shown to protect against hemorrhagic shock, thermal injury, and ischemia reperfusion injury [179-183] Interestingly, Maung and colleagues have shown thermal injury itself can confer a state mimicking endotoxin tolerance, where survival from bacterial infection was enhanced on day 7 post burn.[184]

With the multitude of immune regulatory mechanisms described thus far, it seems that there are endless possible mechanisms by which endotoxin tolerance could be induced. The immune modulating effects of LPS are not specific for a particular pathogen or insult. In-vivo treatment of humans with LPS can tolerize recovered leukocytes to the inflammatory effects of ligands for TLR2, 3, 5, and 7.[185] Likewise, lipoteichoic acid (LTA) from Gram-positive bacteria induced tolerance to both LTA and LPS in a TLR2 dependent fashion, and this effect was not dependent upon soluble mediators.[186] LTA pre-treatment has been shown to reduce the inflammatory response to LPS by inhibiting phosphorylation of ERK, JNK, p38, and the degradation of IkBα/β.[187] Other processes and ligands exhibit tolerizing effects similar to LPS. Therefore, it appears that the immune-modulating effects of LPS may result from a pathway common to many ligands. The regulatory cytokine IL-10 was believed to be the mediator of endotoxin tolerance and its production is enhanced by both TLR4 and TLR2 priming; however the necessity of IL-10 for tolerance induction has been refuted by our lab and others. [188-190] TNFα production is diminished in LPS tolerance and blockade of TNFα in mice has proven beneficial in both Gram-positive and -negative bacteremia; however in clinical trials anti-TNFα antibodies have not proven effective in the treatment of sepsis.[51] The effect of LPS tolerance on IL-10 and TNFα production occurs at the

transcriptional level; the transcription of IL-6, IL-12, IL-1β, CXCL10, and TGFβ are also altered by endotoxin tolerance.[191]

Many of the changes seen by endotoxin priming have been associated with alterations in the TLR4 signaling cascades. While the surface expression levels of TLR4 are altered by LPS exposure and Gram-negative infection there has been no firm association between surface TLR4 quantities and the induction of endotoxin tolerance. The surface associated receptor complex RP105/MD-1 has been shown to inhibit TLR4 signaling but it has not proven to be necessary for tolerance induction.[110, 192] LPS pre-treatment inhibits phosphorylation of the intracellular TIR domain of TLR4 as well as the Mal adapter molecule, thus inhibiting MyD88 downstream signaling.[193, 194]

Toll interacting protein (tollip) negatively regulates the intracellular TLR4 signaling complex and has been shown to increase in activity after LPS treatment.[195] The dominant negative splice variant MyD88s is up-regulated after exposure to endotoxin and is known to inhibit TLR4 signaling, but its role in endotoxin tolerance has not been fully investigated.[196] Tolerance induction has also been shown to inhibit TLR4 / TRIF / TBK1 signaling complex formation, thus inhibiting the MyD88-independent pathway as well.[197] IRAK-M is induced by TLR4 activation, and appears to be necessary for endotoxin tolerance to occur in mice.[198] SOCS1, an inhibitor of cytokine signaling pathways, is activated by LPS exposure and is associated with endotoxin tolerance in Kupffer cells and RAW264.7 cells.[199] LPS tolerance induction has been correlated with impaired NF-kB signaling, depletion of p65 and p50 isoforms, and a relative increase in the nuclear translocation of inactive p50 homodimers.[200, 201]

With this vast array of potential Endotoxin tolerance mechanisms it is unclear which factors are truly necessary for LPS tolerance induction in-vivo. Tolerance

induction can occur from both MyD88-dependent and -independent pathway activation, with the stimulation of both simultaneously through TLR4 producing a synergistic effect.[202]

Lipid A, considered to be the TLR4 stimulating portion of LPS, has been shown to selectively down-regulate the MyD88 dependent pathway when used as a tolerizing agent.[203] Foster and colleagues demonstrated, quite elegantly, that the tolerant state is the result of differential chromatin modification of targets downstream of TLR induced transcription factors.[204] They showed that there were two classes of genes, those that remained easily inducible by a second dose of LPS (non-tolerizable), and genes that were responsive to an initial exposure but not subsequent doses of LPS (tolerizable). Several hundred genes proved to be tolerizable and non-tolerizable; and as expected pro-inflammatory genes like IL-6 were silenced but antimicrobial genes such as Cnlp (Camp cathelicidin antimicrobial peptide) were not. Transcription of some non-tolerizable genes was enhanced during subsequent LPS exposures. This process was dependent upon histone acetylation levels and not upon secreted factors acting in a positive feedback fashion.

Through a vast array of signaling events, endotoxin tolerance is capable of dramatically augmenting the immune response to subsequent inflammatory challenges. Although LPS tolerance was once thought to represent a state of immuno-suppression, LPS-priming has been shown to enhance some beneficial immune functions such as bacterial clearance and leukocyte survival. Therefore, endotoxin tolerance exerts a more immune-modulatory effect as compared to CARS, which is viewed almost exclusively as immune-suppressive. The state of endotoxin tolerance appears to be mediated by the selective reprogramming of the immune response and has the potential of protecting the host from infectious and other inflammatory insults. Early researchers used endotoxin

quite liberally in humans, but given its high level of toxicity and narrow therapeutic index LPS has very little clinical applicability.[205] LPS alone can be fatal and a documented case of intentionally self-administered endotoxin (1 mg bolus i.v., over 3000X the normal experimental dose) resulted in septic shock which was survived only through aggressive treatment over the course of 8 days in intensive care.[206]

Given the large health burden of severe infection and growing problem of antibacterial resistance there is a real need for alternative treatments aimed at regulating the innate immune response to improve host resistance to infection. Endotoxin tolerance has a rich history. However, safer and more reliable agonists are needed instead of LPS if immune-modulation is to become a viable therapeutic option.

Monophosphoryl Lipid A

Monophosphoryl Lipid A (MPLA) was first developed by Dr. Edgar Ribi and colleagues while trying to decouple the inflammatory effects of LPS from its beneficial immune-stimulatory properties.[207] MPLA is derived from the LPS of *Salmonella minnesota* R595 by successive acid and base hydrolysis; yielding a mixture of 3-0-desacyl-4'-MLA consisting of a conserved disaccharide backbone phosphorylated at the 4' position with 3-6 fatty acid side chains (Figure 1.4).[208]

It was soon discovered that MPLA did not elicit as dramatic an inflammatory response as the parent molecule LPS, but was capable of activating myeloid cells and increasing T / B cell interactions.[209] MPLA increased the ability of antigen presenting cells such as monocytes and B-cells to sensitize naïve T cells, inducing the development of Th1 and Th2 phenotypes.[210] MPLA also enhanced dendritic cell (DC) redistribution to the T cell zone of the spleen, potentially increasing their antigen presentation activity. MPLA exposure triggered maturation of monocytes-derived DCs

but required a higher dose than LPS to attain this effect.[211] These properties lead to the development of MPLA into a vaccine adjuvant and it is currently used in a hepatitis B virus vaccine formulation.[212] Numerous derivations of MPLA have since been developed in hopes of creating mucosal vaccines capable of stimulating immediate innate immunity as well as generating long lasting acquired immunity.[213-215] MPLA has proven to have a very good safety profile and can be administered in humans at doses 10,000 times that of LPS. In addition, MPLA treatment tolerized volunteers to the inflammatory effects of subsequent LPS exposure.[216] This included a significant reduction in IL-6, TNF-α and IL-8 production as well as decreased subjective discomfort from secondary LPS exposure.

In mice, MPLA is capable of conferring protection against fatal endotoxemia and CLP induced peritonitis, however this protection is not as significant as the protection conferred by LPS when administered at the same dose.[217] This is probably attributable to LPS being

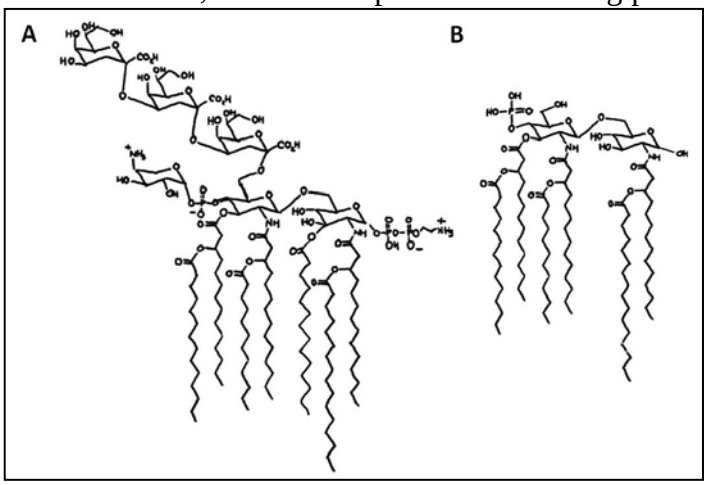


Figure 1.4 the structure of LPS and MPLA. (A) native LPS compared to (B) MPLA derived from *Salmonella minnesota* R595. Adapted from Casella et al. 2008

10,000X more potent as an immuno-stimulant. Much like LPS, MPLA has been shown to protect rodents from myocardial ischemia injury and endothelial dysfunction, indicating that MPLA may be applicable in treatment of a variety of insults.[218] When administered at equivalent doses, MPLA induced the production of less TNF, IL-6, and

IFN-γ as compared to LPS.[219] Both LPS and MPLA elicited the up-regulation in TNFα, IL-1β, TNF-receptor, and IP-10 gene transcription after a single administration, however TNFγ gene expression returned to baseline faster in the MPLA treated group.[220] The cytokine response elicited by subsequent LPS exposures was similar in LPS- and MPLA-primed mice. Subsequent exposures to LPS resulted in blunted expression of TNFα and IL-1β; but TNF receptor mRNA levels in MPLA- and LPS-primed macrophages were not suppressed. Vogel and colleagues have shown that LPS induces higher expression of IL-12p35, and IFNγ mRNA compared to MPLA, but MPLA induces greater expression of IL-10 mRNA.[221] Other researchers have shown that MPLA enhanced the secretion of TNFα, but unlike Lipid A, IL-1β secretion was not induced by MPLA treatment.[222] This appeared to result from the inability of MPLA to activate caspase-1, which cleaves pre IL-1β into its active secreted form.

TLR4 interacts with CD14 and MD-2 to form the endotoxin receptor complex, which is the major receptor engaged by both LPS and MPLA to initiate signaling.[214] Ohto and colleagues were able to show, through x-ray crystallography, the binding of MD-2 and heptacylated lipid A, a TLR4 agonist similar in structure to MPLA.[223] MPLA has also been shown to bind TLR2 on human monocytes and induce NF-kB activation and TNFα production.[224] Blockade of both TLR4 and TLR2 on human monocytes decreased NF-kB p65 nuclear translocation in response to MPLA.[224] While blockade of TLR4 inhibits NF-kB activation more so than TLR2 inhibition, the relative contribution of each receptor to MPLA-induced immuno-modulation is yet to be established. In fact, one study showed that the enhanced bacterial clearance induced by MPLA priming occurs independently of both TLR4 and CD14.[225] Furthermore, recent studies indicate that lipid A derivatives, such as MPLA, may primarily activate TRIF-associated signaling pathways.[203] TRIF based signaling favors the production of IFN-

β and IFNβ-induced factors such as CXCL10 .[226] The MyD88-dependent pathway is a strong activator of the nuclear transcription factor NF-kB, MAP kinases and AP-1, that are responsible for the production of pro-inflammatory factors such as TNF-α, iNOS and IL-6.[227] However, the signaling mechanisms that induce the state of immuno-modulation caused by MPLA priming are currently unknown.

The phenomenon of endotoxin tolerance and MPLA-mediated immuno-modulation may be a regular occurrence in normal physiology. It has long been known that the gut is a major reservoir of commensal bacteria, capable of both eliciting disease and contributing to the health of the host. This massive load of bacterial PAMPs are in constant contact with the epithelial surface of the GI mucosa, and normally do not trigger an inflammatory response. Some TLR polymorphisms predispose patients to inflammatory bowel disease by increasing their sensitivity to endogenous flora.[228] GI tolerance has recently been shown to result from intestinal alkaline phosphatase, which dephosphorylates LPS rendering a molecule quite similar to MPLA.[229] The mammalian enzyme acyloxyacyl hydrolase also cleaves acyl chains from LPS rendering it unrecognizable by TLR4. Animal models of intestinal alkaline phosphatase deficiency have shown enhanced sensitivity to LPS and increased intestinal inflammation.[230] Alkaline phosphatase is also expressed in the liver which can further detoxify any LPS absorbed into circulation from the GI tract.[231] Administration of bovine intestinal alkaline phosphatase prior to or shortly after CLP has been shown to reduce cytokine production as well as protect against hepatic and pulmonary injury.[232] Thus it appears endogenous methods of tolerance induction exist, that may depend upon the ability to generate detoxified LPS to modulate the TLR4 response.

Objectives of this dissertation

Immuno-modulation is a promising field of research for the development of novel methods to combat infectious diseases and hyper-inflammatory states such as SIRS and sepsis. While endotoxin tolerance induction with LPS is not a clinically viable option, MPLA affords a method of immuno-modulation which may be practical for use in patients. As noted above, MPLA is a potent immuno-modulator that causes minimal toxicity and is being used clinically as a vaccine adjuvant. To date, the mechanism(s) of endotoxin tolerance induction has yet be fully clarified. While alterations in macrophage function are believed to play a large role in endotoxin tolerance, their involvement is still not fully understood. Nevertheless, it is relatively clear that the anti-inflammatory effects induced by LPS exposure are mediated through reprogramming of pro-inflammatory signaling pathways in macrophages. However, the mechanisms by which TLR4 agonist treatment improves the host response to infection are essentially unknown. The state of immuno-modulation caused by LPS exposure was previously viewed as a potentially deleterious suppression of the immune response.[233] However, work from our laboratory has clearly shown that treatment with LPS will enhance the ability of the host to respond to a bacterial infection. A major goal of this project is to determine whether treatment with MPLA will also afford improved host resistance to infection.

LPS-induced enhancement of leukocyte survival might explain, in part, improved bacterial clearance but similar cellular survival results in MPLA-treated mice has yet to be demonstrated. A global assessment of myeloid cell functions *in vivo* following MPLA priming and infection is needed to better understand the cellular immunology responsible for the immune-enhanced phenotype. In addition, this project will further explain the intracellular mechanisms involved in MPLA-induced immuno-modulation. The literature to date has produced some ambiguity as to the exact mechanism of cellular recognition of

MPLA and induction of the immuno-modulated state, which will be further clarified through these studies. While progress has been made in the realm of understanding the mechanisms behind LPS tolerance induction, this is not necessarily analogous to MPLA-induced immuno-modulation. MPLA has been shown to differentially activate the TLR4 receptor mediated pathways in comparison to LPS. A clearer understanding of the receptor and intracellular signaling involved in MPLA priming is needed. Much of the mechanistic research done to date in the field of endotoxin tolerance has been conducted using cell lines or primary cells in culture. This does not replicate the complex interactions which occur in vivo. The research described herein was done to better understand the systemic effect of MPLA immune-modulation in various sepsis models, as well as which cell types, leukocyte functions, and signaling events are involved in MPLA mediated immune enhancement. With better mechanistic understanding of the effects that MPLA has on the innate immune system, it may be possible one day to develop immune-modulating strategies to combat major infections and inflammation.

Chapter 2: Methods

Mice

C57BL/6J, C3H/HeJ, C3H/HeOUJ, and C57BL/6J-Ticam1^{LPS2}/J mice age 8-10 weeks, were obtained from Jackson laboratory (Bar Harbor, ME). C57BL/6J-Myd88^{poc}/Mmcd mice were obtained from MMRRC (Davis, CA). Male mice were used for all intravenous administration studies and female mice were used for the intraperitoneal administration studies. Mice were housed and cared for in the AAALAC-accredited animal facility located in the Shriners Hospital for Children (Galveston, TX). All procedures were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee and meet NIH Guidelines for the Care and Use of Experimental Animals.

MPLA treatment

Monophosphoryl Lipid A (MPLA) derived from *Salmonella enterica* serotype Minnesota Re 595 was purchased from Sigma-Aldrich Corporation (St. Louis, MO). MPLA was dissolved in 0.2% triethylamine (1 mg/ml), heated to 60°C, sonicated for 30 minutes, and then diluted in phosphate buffered saline (100 µg/ml) prior to administration. For intraperitoneal or intravenous administration, MPLA was injected (20 µg in 0.2 ml) once daily for two consecutive days (total of 40 µg/mouse). All studies in Chapter 5 were conducted using ultra-pure MPLA pre-dissolved in pyogen free H_20 from Enzo Life Sciences (Plymouth Meeting, PA). Studies using ultra-pure MPLA were conducted using the normal administration regimen of 20 µg once daily for two consecutive days (total of 40 µg/mouse).

Cecal Ligation and Puncture

Vehicle- and MPLA-treated mice were anesthetized with 2% isoflurane in oxygen via facemask and presented to the surgeon in a blinded fashion to minimize experimental bias. A 1-2 cm midline incision was made through the abdominal wall. The cecum was identified and ligated 0.7 cm from the tip with a 3-0 silk tie. A double puncture of the cecum was performed using a 20-gauge needle and cecal contents were expressed from the puncture. Great care was taken to avoid ligation-induced obstruction of flow between the ileum and colon. The cecum was returned to the abdominal cavity and the incision was closed with surgiclips. All mice received 0.1 mg/kg buprenorphine subcutaneously immediately after CLP and twice daily thereafter. Control mice did not receive surgical manipulation. Experimental samples were harvested and temperature was measured 16 hours after surgery.

***Pseudomonas aeruginosa* challenge**

Pseudomonas aeruginosa (strain 15692, American Type Culture Collection, Rockville, MD) was propagated in tryptic soy broth overnight in a shaker incubator (37°C). Bacteria were washed twice in 30 mL of sterile 0.9% normal saline (NS), then re-suspended in 4 mL of normal saline and stored at 4°C until use. The concentration of viable colony forming units (CFUs) was determined by plating serial dilutions on tryptic soy agar. Plates were incubated overnight at 37°C. Mice received intraperitoneal injection with 1 x 10^8 CFU of *P. aeruginosa* or vehicle (0.2 ml normal saline) at 48 hours after receiving their last dose of MPLA or vehicle. For intravenous challenge, mice received 1 x 10^8 CFUs of *P. aeruginosa* or vehicle (0.2 ml normal saline) via the dorsal vein of the penis at 48 hours after receiving their last dose of MPLA or vehicle.

Burn wound infection

A cutaneous burn wound was induced as previously described [234]. Briefly, mice were anesthestized with 2-3% isofluane, the backs were shaved and covered with a Zetex cloth containing a rectangular opening corresponding to 15% of the mouse total body surface area. Normal saline was injected subcutaneously in the burn target area to prevent injury to underlying tissues. A full thickness flame burn was achieved using a Bunsen burner applied to the exposed skin for approximately 10 seconds. Fluid resuscitation was administered immediately by intraperitoneal injection of lactated Ringer's solution (3 ml) followed by an additional injection (1 ml) 24 hours later. Buprenorphine (0.1 mg/kg, subcutaneously) was given for analgesia prior to initiation of the burn injury and twice daily thereafter. At days 3 and 4 after burn injury, mice received intraperitoneal treatment with MPLA (20 μg) or vehicle. *P. aeruginosa* (1×10^8 CFU) was applied to the caudad portion of the wound on day 5 post-burn. Wound samples were harvested at 48 and 72 hours after inoculation with bacteria and homogenized for measurement of bacterial colony forming units. Lung tissue was also harvested at 72 hours after infection for measurement of bacterial burden.

Survival studies

Mice pre-treated with MPLA or vehicle alone underwent CLP as described above 24 hours after their last dose of MPLA or vehicle. Immediately after surgery the mice received a single dose of imipenem / cilastatin (Primaxin, 25 mg/kg in 1ml lactated Ringer's solution). Primaxin was used to extend the survival time. To measure mortality from burn wound infections MPLA and vehicle pretreated mice underwent thermal injury and wound infection as described above. In both survival study models mice received pain management twice daily with Buprenorphine (0.1 mg/kg, subcutaneously). The mice were monitored for 14 days after CLP or burn wound infection for mortality.

Cytokine analysis

At 6 hours after live bacterial challenge or 16 hours after cecal ligation and puncture, mice were anesthetized (2% isoflurane) and core temperature was immediately measured using a rectal probe. Blood was collected by laceration of the internal carotid artery and plasma was isolated after centrifugation of heparinized blood. Mice were then euthanized by cervical dislocation under isoflurane anesthesia and peritoneal fluid was collected by lavage with 7 ml of RPMI-1640 media. Cytokine levels in plasma and peritoneal fluid were measured using a Bio-Plex mouse 23-Plex panel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Heparinized blood was obtained by carotid laceration in mice anesthetized with 2% isoflurane and plasma was harvested from centrifuged blood (1200 XG for 10 minutes). Supernatant recovered from cell cultures was centrifuged at 1,500 RPM for 10 minutes at 4°C, any pellet was discarded and resultant culture media was stored at -20°C until use. IL-6, CXCL10, and IFN-β concentrations in plasma or cell culture medium were measured using an ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA).

Measurement of bacterial clearance

Arterial blood was aseptically collected by laceration of the internal carotid artery. Peritoneal lavage was performed with 7 ml of sterile RPMI-1640 media using aseptic technique. Liver, lung, and skin tissue was dispersed using a glass tissue homogeneizer and re-suspended in normal saline to a standard wet weight to volume ratio under aseptic conditions. Serial dilutions of whole blood, peritoneal lavage fluid and tissue

homogenates were plated onto tryptic soy agar and incubated overnight at 37°C. Isolate colonies were counted to determine bacterial burden.

Flow cytometry

Leukocytes were harvested from the peritoneum by lavage with 7 ml of RPMI-1640 media. The recovered fluid was then passed through a 70 micron mesh filter followed by incubation with anti-mouse CD16/32 (eBioscience Inc., San Diego, Ca) at a concentration of 1 µg per million cells for 30 minutes at 4°C to block non-specific binding of subsequent antibodies to Fc receptors. Two million leukocytes were transferred into polystyrene tubes containing isotype control or labeling antibodies (0.5 µg / tube) and incubated for 30 minutes at 4°C. Labeled cells were then washed with 2 ml of cold PBS and re-suspended in 0.5 ml of 1% paraformaldehyde fixative. The resultant samples were analyzed in the UTMB Flow Cytometry Core Facility using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Fluorochrome-conjugated antibodies against F4/80 (Clone BM8), CD11b (Clone M1/70), Gr1 (Clone RB6-8C5) as well as isotype controls were purchased from eBiosciences (San Diego, CA). Fluorochrome-conjugated antibodies against Ly6G (Clone 1A8) were purchased from BD Biosciences (San Jose, CA). To assess apoptosis and necrosis MPLA primed and unprimed mice were subjected to intraperitoneal infection as described above. 3 hours after infection peritoneal cells were harvested, Fc receptor was blocked, followed treatment with cytofix / cytoperm according to the manufacturer's instructions (BD Biosciences San Diego, CA). Following permeablization peritoneal cells were stained with FITC conjugated anti-Annexin V and 7AAD. Cells were assayed for staining by flow cytometry within 30 minutes.

For multispectral imaging flow cytometric analysis of nuclear morphology, peritoneal leukocytes were harvested and prepared as described above. Cells were surface labeled with fluorochrome-conjugated antibodies against F4-80, Gr1, and/or CD11b (eBioscience, San Diego, CA). The cells were fixed in 1% paraformaldehyde and shipped overnight to Amnis Corporation in Seattle, WA. Upon arrival the cells were stained with propidium Iodide and images were collected on more than 10,000 cells per sample using the ImageStream Flow Cytometer. Single color controls were collected and used to calculate a spectral crosstalk compensation matrix to compensate the imagery. Using algorithms in the IDEAS image analysis software (Amnis Inc., Seattle, WA, USA) the cells were immunophenotyped and the nuclei were analyzed for morphological char-acteristics.

In vivo phagocytosis assay

P. aeruginosa was propagated as described above and heat killed by incubation in a water bath for 1 hour at 56° C. The heat killed bacteria were labeled with Fluorescein isothiocyanate (FITC) purchased from Sigma-Aldrich Corporation (St. Louis, MO) as previously described [235]. The labeled bacteria were re-suspended in normal saline and stored at -80°C. MPLA treated and untreated mice received intraperitoneal injection with 1 x 10^8 CFU of heat-killed, FITC-labeled *P. aeruginoas* or vehicle (0.2 ml NS). Peritoneal leukocytes were collected 3 hours after injection of bacteria, labeled with antibodies against F4/80 and Ly6G and fixed in 1% paraformaldehyde. The samples were analyzed in the UTMB Flow Cytometry Core Facility using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Measurement of peripheral blood leukocyte counts

Mice receiving intravenous treatment with MPLA (20 μg in 0.2 ml LR) or vehicle (0.2 ml LR) received intraperitoneal challenge with 10^8 CFU *P. aeruginosa* or vehicle (0.2 ml NS) at 48 hours after receiving their last dose of MPLA or vehicle. Four hours after bacterial challenge mice were placed under general anesthesia and arterial blood was aseptically collected by laceration of the internal carotid artery. The cellular profile was analyzed within an hour using the Hemavet 950FS (Drew Scientific group, Waterbury, CT).

Monocyte depletion

To deplete monocytes / macrophages 200 μl of clodrinate liposomes (obtained from Dr. Nico van Rooijen: Vrije Universiteit, VUMC, Department of Molecular Cell Biology, Amsterdam, Netherlands; Cl_2MDP (or clodronate) was a gift of Roche Diagnostics GmbH, Mannheim, Germany) were administered two days prior to intra-peritoneal MPLA or vehicle administration. MPLA and vehicle were delivered as described above over two consecutive days. 200 μl clodronate liposomes were re-administered intraperitoneally one day after the final does of MPLA or vehicle. 24 hours after the final dose of clodronate mice underwent CLP as described above. 16 hours after CLP temperature was measured, peritoneal fluid and blood was collected for bacterial concentration and IL-6 level analysis. Control mice received liposomes without clodronate following this same regiment. To verify the effectiveness of monocyte / macrophage depletion mice primed with MPLA or vehicle which underwent liposome treatment were infected i.p. with 10^8 CFUs of *P. aeruginosa* 24 hours after the final dose of liposomes. 3 hours after infection peritoneal cells were harvested and assessed for myeloid surface marker (F4/80, Ly6G) expression by flow cytometry.

Neutrophil depletion

Mice were primed intraperitoneally with MPLA or vehicle as described above. 24 hours after their final dose of MPLA or vehicle they were injected intraperitoneally with 100 μg Ly6G specific antibody (Clone 1A8, BD Biosciences San Diego, CA). The following day mice underwent CLP as described herein. 16 hours after CLP temperature was measured, peritoneal fluid and blood was collected for bacterial concentration and IL-6 level analysis by ELISA. To verify neutrophil depletion mice treated with MPLA or vehicle which had received Ly6G antibodies for depletion were infected with 10^8 CFUs of *P. aeruginoas* 24 hours after antibody administration. 1.5 hours after infection peritoneal cells were harvested and assessed for myeloid surface marker (F4/80, Ly6G) expression by flow cytometry.

Myeloperoxidase (MPO) analysis

Mice were treated intraperitoneally with MPLA or vehicle, 48 hours after their final dose they were infected i.p. with 10^8 CFUs of *P. aeruginosa*. Within 3 hours of infection peritoneal leukocytes were collected, and after blocking Fc receptor, were stained for Ly6G and F4/80 surface marker expression. The cells were then washed and treated with cytofix / cytoperm according to the manufacturer's instructions (BD Biosciences San Diego, CA). After fixation and permeablization the cells were labeled with a FITC-conjugated MPO-specific antibody (Clone 8F4, HyCult Biotech, Netherlands) and analyzed by flow cytometry.

Complement mediated erythrocyte lysis assay

The complement activity of serum collected from MPLA treated and control mice, both before and after infection, was assessed as previously described [236, 237]. Briefly, rabbit erythrocytes were incubated with goat anti-rabbit serum and then with

serial dilutions of mouse serum samples. The absorbance of the resultant supernatant was measured at 540 nm. Sensitized erythrocytes were incubated in red cell lysis buffer (Sigma-Aldrich Corporation St. Louis, MO) and the resultant supernatant was serially diluted to generate a standard curve for determining % lysis.

T-cell suppression co-culture assay

4 mice were primed with MPLA intraperitoneally as described above. MPLA-primed peritoneal leukocytes were harvested and separated based upon Ly6G surface marker expression by MACS magnetic column according to the manufacturer's instructions (Miltenyi Biotec, Germany). Both the positive and negative peritoneal cell fractions were then incubated with mitomycin C at a concentration of 10 μg / ml for 3 hours at 37°C to inhibit proliferation. Concurrently CD4+ T cells were isolated from the spleen of an OT-II mouse by column separation (R&D systems, Minneapolis, MN). The $Ly6G^+$ and $Ly6G^-$ peritoneal cells were separately co-cultured with isolated OT-II $CD4^+$ T cells at varying ratios (1:1, 1:2, 1:4, & 1:8; T cell : myeloid cells). These co-cultures were incubated for up to 4 days in the presence of $OVA_{323-356}$ peptide, after which time T cell proliferation was assessed using a modified MTT assay.[238]

Statistics

Data were analyzed using GraphPad Prism version 4 software (GraphPad Software, Inc., La Jolla, CA). Cytokine measurements and flow cytometry data were analyzed using one way ANOVA and Tukey's Multiple Comparison Test or Mann-Whitney U test. Bacterial CFU data were analyzed using the Mann-Whitney U test or Tukey's multiple comparison test. Peripheral blood leukocyte counts were analyzed using the unpaired T-test. Experimental groups in survival studies were evaluated by Chi square analysis. A value of $p<0.05$ was considered statistically significant.

Chapter 3: The TLR4 Agonist Monophosphoryl Lipid A Augments Innate Host Resistance to Systemic Bacterial Infection

Introduction

The use of immuno-modulatory strategies aimed at improving resistance to bacterial infections could be beneficial in a variety of clinical scenarios in which the host is predisposed to infectious complications. Among those are patients with severe burns, major trauma or that have undergone major surgical procedures or have received immunosuppressive therapies for cancer or organ transplantation. The attractiveness of interventions that can improve the host response to infection is further enhanced by the increasing incidence of antibiotic resistance among bacteria, especially those that commonly cause nosocomial infections.

Bacterial lipopolysaccharide (LPS, endotoxin) is a component of the Gram negative bacterial cell wall that has known immuno-modulatory properties [239]. LPS is recognized by Toll-Like receptor 4 (TLR4), which is expressed on a variety of leukocytes and activates both TRIF- and MyD88-dependent signaling pathways [240-247]. Activation of TLR4 signaling induces the production of numerous pro-inflammatory mediators such as cytokines, chemokines and nitric oxide that facilitate the cardinal features of inflammation such as increased vascular permeability, edema formation and leukocyte recruitment. Interestingly, prior exposure to LPS induces a state in which a subsequent challenge with LPS or bacteria results in markedly decreased production of pro-inflammatory mediators [248]. The altered immunological phenotype that is elicited by priming with LPS has historically been referred to as endotoxin tolerance [249]. The induction of endotoxin tolerance has been shown to be highly effective in reducing both

morbidity and mortality associated with subsequent challenge with a normally lethal dose of LPS [173].

Because LPS priming attenuates pro-inflammatory cytokine production in response to LPS or bacterial challenge, many investigators previously characterized LPS tolerance as a state of immuno-suppression. However, few studies have examined the effects of LPS treatment on the host response to live bacterial infections. Studies from our laboratory, and others, have recently demonstrated that mice primed with LPS are more resistant to bacterial infections than control mice [173, 250, 251]. However, the clinical applicability of LPS as a therapeutic or prophylactic agent is precluded due to toxicity and a narrow therapeutic index in humans. Furthermore, the mechanisms by which LPS exposure lead to improved antimicrobial functions are currently unknown.

Monophosphoryl Lipid A (MPLA) is an endotoxin derivative that has been employed as a vaccine adjuvant in humans and has cleared regulatory approval for use in the United States [252]. MPLA is produced by hydrolysis of native diphosphoryl lipid A, the component of LPS that is recognized by TLR4, resulting in removal of a single phosphate group and varying degrees of de-acylation [253]. These structural alterations decrease systemic toxicity by greater than 99% compared to native lipid A resulting in an immuno-modulatory agent with greater potential for clinical use [217, 254]. The attenuated toxicity associated with MPLA administration is believed to result from reduced induction of pro-inflammatory cytokines such as TNFα, IL-1β and IFN-γ during initial exposure [219, 221]. Yet, MPLA retains significant immuno-modulatory activity and increases survival after otherwise lethal exposure to endotoxin in animal models [250, 255, 256]. However, the effect of MPLA on the innate response to clinically relevant models of infection has not been determined. Furthermore, the cellular and molecular mechanisms underlying the beneficial effects of MPLA treatment on innate

antimicrobial immunity have not been characterized. Therefore, further understanding of the immuno-modulatory properties of MPLA is required for its development as a therapeutic agent. In the present study, we evaluated the effect of MPLA treatment on leukocyte recruitment, cytokine secretion and bacterial clearance mechanisms in clinically relevant models of acute infection.

MPLA priming prior to infection has shown benefit in our models of sepsis. This effect was mediated though various routes of administrations and proved protective to multiple infection models, ranging from burn wound colonization to severe CLP induced sepsis. It has been previously reported that the effect of MPLA immuno-modulation can last up 14 days with multiple administrations, however the rapidity of effect has yet to be clarified.[208] While prophylactic applications do exists for immuno-modulatory agents in the prevention of sepsis (such as ICU patients with long hospital stay durations, burn patients, or the immuno-compromised) there is also a need to develop agents which can effect rapid protection even after a septic insult has occurred. The protective effect of MPLA immuno-modulation was assessed after CLP to determine if any benefits seen in the pre-infection model could be conferred after an insult.

Results

MPLA treatment prevents hypothermia and improves survival in mice with systemic infection

Core body temperature was measured in control mice and in mice receiving MPLA treatment during systemic infection. MPLA treatment prevented sepsis-induced hypothermia (Figure 3.1). In one set of studies, mice received intra-peritoneal treatment with either vehicle or MPLA prior to CLP or challenge with *Pseudomonas aeruginosa*. Vehicle-treated mice showed a significant decrease in core temperature at 24 hours after CLP or *Pseudomonas aeruginosa* infection as indicated by significantly decreased rectal

temperature compared to control, non-infected mice (Figure 3.1A). Mice that were treated with MPLA did not show a significant decrease in core temperature compared to control mice and their core temperature was significantly ($p<0.05$) higher compared to vehicle-treated and infected mice (Figure 3.1A). Further studies were undertaken to assess the effects of intravenous MPLA treatment on CLP-induced hypothermia (Figure 3.1B). Rectal temperature was significantly lower in vehicle-treated mice compared to control mice at 24 hours after CLP. Rectal temperature was not significantly different in MPLA-treated mice compared to non-infected mice and was significantly higher than in vehicle-treated mice after CLP (Figure 3.1B).

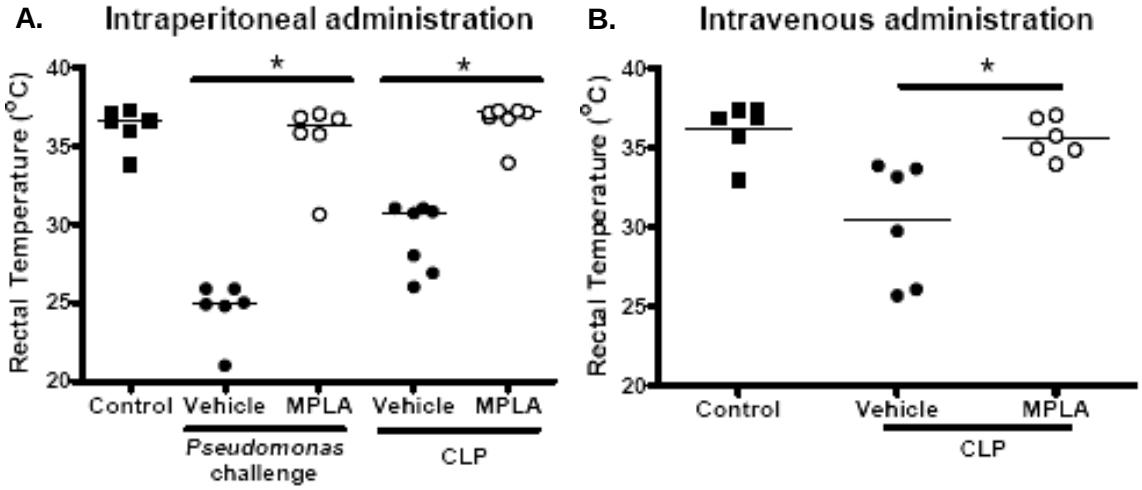
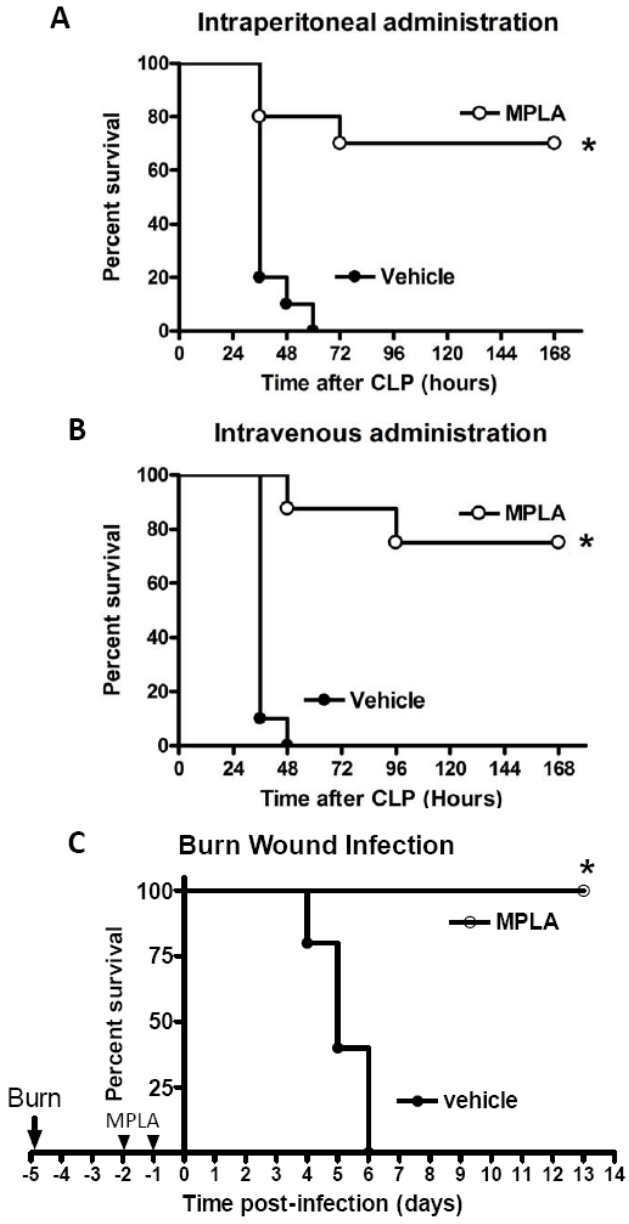


Figure 3.1 MPLA treatment attenuated infection-associated hypothermia. Mice received either intra-peritoneal (A), or intravenous (B) injection with MPLA (20 μg in 0.2 ml of lactated Ringer's solution) or lactated Ringer's solution alone (vehicle) on days 0 and 1 followed by intra-peritoneal challenge with 8 log CFU of Pseudomonas aeruginosa or CLP on day 3. At 6 hours after Pseudomonas infection or 16 hours after CLP, core temperature was measured with a rectal thermometer. *$p<0.05$ compared to vehicle, n=6-8 mice per group.

The effects of MPLA treatment on survival in mice with systemic infection were also determined. Intra-peritoneal treatment of mice with MPLA significantly improved survival during sepsis caused by CLP compared to mice treated with vehicle (Figure

3.2A). Vehicle-treated mice showed 100% mortality at 60 hours after CLP whereas 70% long-term survival was observed in mice receiving MPLA treatment. Improved survival was also seen after CLP in mice receiving intravenous administration of MPLA (Figure 3.2B). Vehicle-treated mice showed 100% mortality at 48 hours after CLP whereas 75% long-term survival was observed in mice receiving MPLA treatment.



In further studies, MPLA treatment improved survival in a model of *Pseudomonas* burn wound infection (Figure 3.2C). Mice with a 15% total body surface area burn were treated with MPLA on days 3 and 4 post-burn followed by inoculation of the wound with *Pseudomonas aeruginosa* on day 5. Mice receiving MPLA treatment

Figure 3.2 MPLA treatment improved survival during sepsis caused by cecal ligation and puncture or *Pseudomonas aeruginosa* burn wound infection. Mice received either intraperitoneal (A), or intravenous (B) injection with MPLA (20 μg) or vehicle on days 0 and 1 followed by CLP on day 3. Mice received a single dose of imipenem/cilistatin (Primaxin, 25 mg/kg in 1 ml of lactated Ringer's solution) immediately following surgery. The mice were monitored for 14 days following CLP. (C) Mice underwent a cutaneous burn followed by intraperitoneal treatment with MPLA or vehicle on days 3 and 4 post-burn. Wounds were infected with *Pseudomonas aeruginosa* (8 log CFU) on day 5 p ost-burn. *p<0.05 compared to vehicle, n=10 mice per group.

showed 100% survival whereas 100% mortality was observed in vehicle-treated mice at 6 days after wound inoculation (Figure 3.2C). In all experiments, mice that survived beyond 7 days after CLP or burn wound infection were still viable and showed normal activity levels at 2 weeks post-infection.

MPLA treatment decreased bacterial burden in infected mice

The efficacy of MPLA treatment for decreasing bacterial counts at the site of infection as well as distant locations was determined. Treatment of mice with MPLA resulted in significantly ($p<0.05$) reduced bacterial counts in peritoneal fluid and blood compared to vehicle-treated mice at 16 hours after CLP (Figure 3.3). Intraperitoneal bacteria were decreased by greater than one order of magnitude whereas blood bacteria were decreased significantly in mice receiving intravenous MPLA treatment compared to vehicle-treated controls (Figure 3.3). A similar reduction in blood and peritoneal bacterial counts was seen in mice receiving intra-peritoneal treatment with MPLA (Figure 3.3). Vehicle-treated mice had approximately 1 x 108 bacterial CFU in peritoneal fluid, which was decreased by 3 orders of magnitude in MPLA-treated mice. Similarly, blood

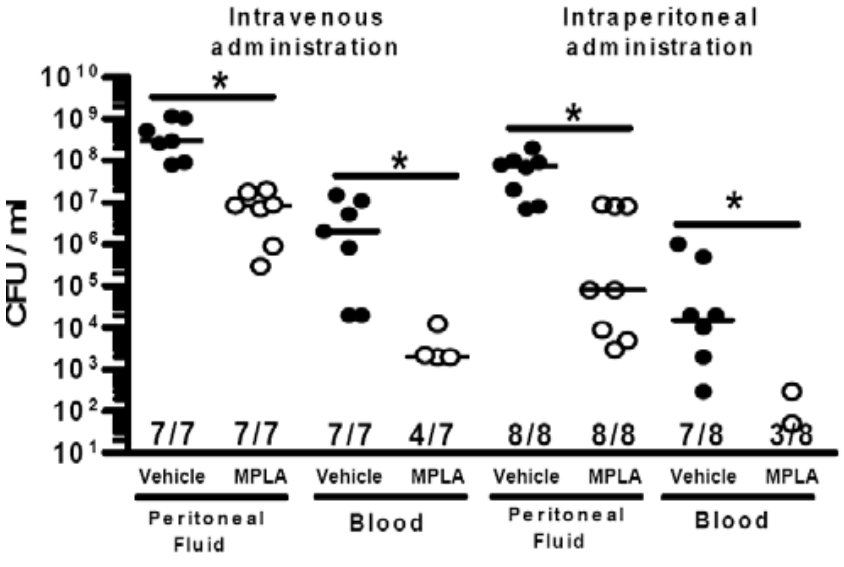


Figure 3.3 MPLA treatment enhanced local bacterial clearance and decreased the systemic dissemination of bacteria during sepsis caused by cecal ligation and puncture. Peritoneal fluid and whole blood were collected 16 hours after CLP from mice receiving intravenous or intraperitoneal treatment with vehicle or MPLA. Bacterial CFU in peritoneal lavage fluid and blood were determined after culture on tryptic soy agar. *$p<0.05$ compared to vehicle, n= 7-8 mice per group.

bacterial CFU were decreased by greater than 3 orders of magnitude in mice receiving intra-peritoneal MPLA treatment compared to vehicle-treated controls (Figure 3.3).

In further studies, MPLA treatment decreased the local and systemic dissemination of bacteria in a model of Pseudomonas aeruginosa burn wound infection (Figure 3.4). To assess dissemination of bacteria within the burn wound, the uniformly circumscribed wound was divided into equal thirds (Figure 3.4A). The wound was inoculated at area-3. At 48 hours after wound inoculation, the wound was excised, divided into the 3 segments as defined in Figure 4A and bacterial counts in homogenized skin segments was determined. At the inoculation site (Area-3), the median number of organisms was decreased in MPLA-treated mice compared to vehicle-treated controls but the difference was not statistically significant. However, Pseudomonas aeruginosa CFU in area-2 and area-1 were significantly lower in MPLA-treated mice compared to vehicle-treated controls (Figure 3.4A). In further studies, the entire burn wound was excised and lungs were harvested at 72 hours after inoculation of the burn wound with Pseudomonas aeruginosa and bacterial counts were determined (Figure 3.4B). Total wound colonization was decreased by greater than 3 orders of magnitude in MPLA-treated mice compared to vehicle-treated controls (Figure 3.4B). Examination of lungs showed that all mice in the vehicle-treated group grew Pseudomonas aeruginosa with a median CFU count of 4 x 107 CFU/g. Only 1 of 5 mice in the MPLA-treated group grew Pseudomonas from the lungs (Figure 3.4B).

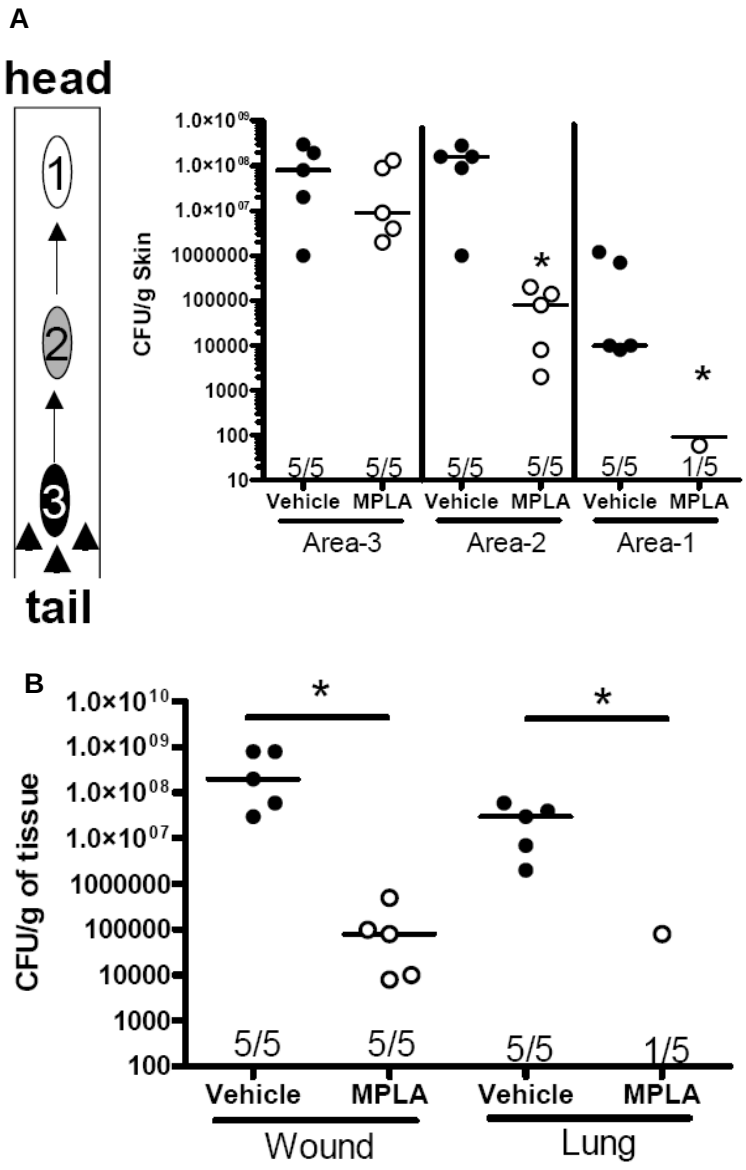


Figure 3.4. MPLA treatment enhanced local bacterial clearance and decreased the systemic dissemination of bacteria in a model of burn wound infection. Mice underwent a 15% total body surface area cutaneous burn followed by treatment with MPLA or vehicle on days 3 and 4 post-burn. The burn wound was inoculated with Pseudomonas *aeruginosa* (8 log CFU) in area-3 on day 5 post-burn. (A) The burn wound was excised at 48 hours after bacterial inoculation and divided into 3 equal segments as shown. The wound tissue was weighed, homogenized and bacterial counts were determined by serial dilution and plating on tryptic soy agar. (B) The entire burn wound was excised, weighed, homogenized and cultured at 72 hours after bacterial inoculation. Lungs were also excised from the same mice, weighed, homogenized and cultured. *$p<0.05$ compared to vehicle, n= 5 mice per group.

The effect of MPLA treatment on the dissemination of Pseudomonas aeruginosa after systemic challenge was also determined. In one study, mice received intra-peritoneal treatment with MPLA followed two days later by intra-peritoneal Pseudomonas aeruginosa challenge (Figure 3.5A). Intra-peritoneal MPLA treatment enhanced bacterial clearance at the sight of inoculation and decreased the systemic dissemination of Pseudomonas aeruginosa into blood at 6 hours after intra-peritoneal challenge (Figure 5A). The median bacterial colony forming units (CFU) in peritoneal lavage fluid and blood from MPLA-treated mice were significantly ($p<0.05$) lower than in vehicle-treated controls (Figure 3.5A). Intravenous treatment with MPLA also reduced the bacterial counts in mice receiving intravenous challenge with Pseudomonas

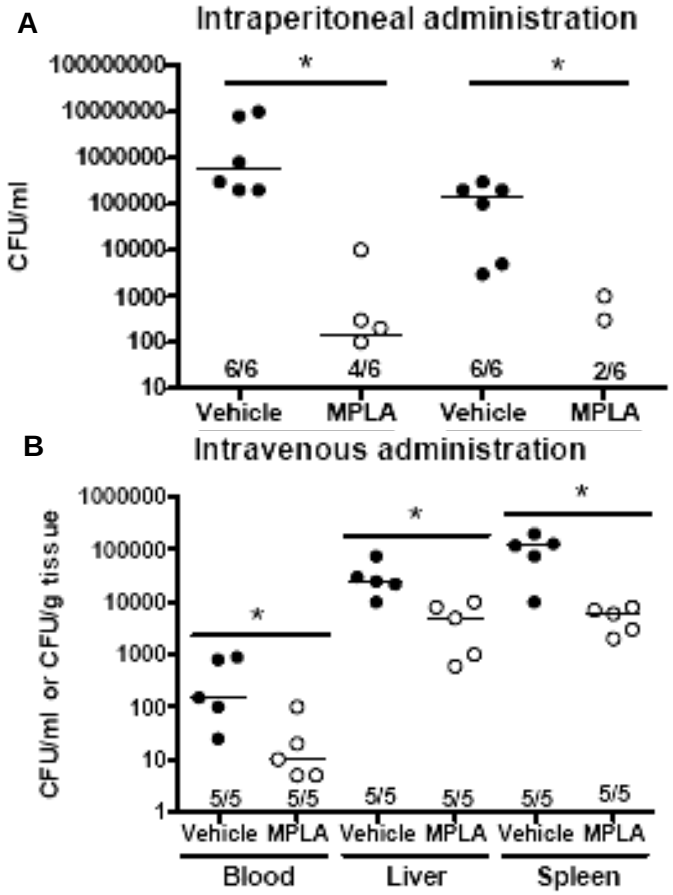


Figure 3.5 MPLA treatment enhanced local bacterial clearance and decreased systemic dissemination of bacteria after *Pseudomonas aeruginosa* challenge. **(**A) Mice received 2 intraperitoneal injections of MPLA (20 ug) or vehicle over two consecutive days. At 48 hours after vehicle or MPLA treatment, mice received intraperitoneal challenge with 1×10^8 CFU of *Pseudomonas aeruginosa*. At 6 hours after infection, bacterial burden in blood and peritoneal fluid was determined by plating samples on tryptic soy agar and performing colony counts. (B) Mice received 2 intravenous injections of MPLA (20 ug) or vehicle over two consecutive days. At 48 hours after vehicle or MPLA treatment, mice received intravenous challenge with 8 log CFU of *Pseudomonas aeruginosa*. At 6 hours after infection, bacterial burden in blood, spleen and liver was determined by plating samples on tryptic soy agar and performing colony counts. *$p<0.05$ compared to vehicle, n= 5-6 mice per group.

aeruginosa (Figure 3.5B). MPLA treatment significantly ($p<0.05$) reduced the bacterial burden in the blood, spleen and liver compared to vehicle-treated mice (Figure 3.5B).

MPLA treatment attenuated sepsis-induced pro-inflammatory cytokine production

Examination of plasma cytokine and chemokine concentrations showed that MPLA treatment significantly attenuated infection-induced production of many cytokines and chemokines after systemic *Pseudomonas aeruginosa* challenge or CLP (Table 1). Plasma cytokine and chemokine concentrations were not significantly different between vehicle- and MPLA-treated mice at 48 hours after MPLA or vehicle treatment and prior to *Pseudomonas* infection or CLP (control, no infection, Table 1). Concentrations of all measured cytokines and chemokines were significantly ($p<0.05$) increased at 6 hours after *Pseudomonas aeruginosa* challenge in vehicle-treated mice compared to non-infected controls (+ *Pseudomonas*, Table 1). Concentrations of most plasma cytokines and chemokines were significantly lower in MPLA-treated mice compared to vehicle-treated mice after *Pseudomonas aeruginosa* challenge (Table 1).

Detailed analysis revealed that the plasma concentrations of most pro-inflammatory cytokines, anti-inflammatory cytokines, hematopoietic factors and chemokines were significantly lower in MPLA-treated mice compared to vehicle-treated controls after *Pseudomonas aeruginosa* challenge (Figure 3.6). However, factors that are produced predominantly through Trif-dependent signaling such as G-CSF, MCP-1 (CCL-2) and RANTES (CCL-5) were not significantly different in MPLA-treated mice compared to vehicle-treated mice (Table 1).

MPLA treatment also significantly attenuated the production of certain cytokines after CLP (see Table 1, Figure 3.6). All measured cytokine and chemokine concentrations were significantly ($p<0.05$) increased in plasma from vehicle-treated mice at 16 hours after CLP compared to control mice (+ CLP, Table 1). Treatment with MPLA

	Control (no infection)		*+ Pseudomonas*		*+ CLP*	
	Vehicle	**MPL A**	**Vehicle**	**MPLA**	**Vehicle**	**MPLA**
Pro-inflammatory and Th1 cytokines						
IL-1α	33 ±5	20 ±3	962 ±230	47 ±2*	94 ±16	50 ±8*
IL-1β	90 ±17	96 ±17	4884 ±1702	322 ±32*	812 ±118*	465 ±85*
IL-2	14 ±2	nd	17 ±4	8 ±1	55 ±2	43 ±7
IL-6	17 ±3	21 ±4	41797 ±30301	718 ±370*	60437 ±22160	15926 ±8658*
IL-12 (p40)	182 ±334	165 ±23	6199 ±1661	708 ±93*	1900 ±641	628 ±191*
IL-12 (p70)	65 ±18	80 ±21	188 ±36	89 ±14*	nd	nd
IFN-γ	14 ±2	22 ±8	244 ±103	27 ±6*	443 ±32	534 ±78
TNF-α	39 ±6	43 ±4	310 ±65	124 ±44*	1060 ±225	564 ±96*
Anti-inflammatory and Th2 cytokines						
IL-4	nd	nd	nd	nd	nd	nd
IL-10	31 ±7	33 ±6	3061 ±846	180 ±21*	9396 ±2279	5153 ±1915
IL-13	175 ±22	145 ±18	347 ±52	176 ±25*	1874 ±151	1760 ±134
Hematopoietic regulating cytokines						
IL-3	nd	nd	15 ±3	30 ±4	34 ±6	32 ±3
IL-5	12 ±2	10 ±1	696 ±134	50 ±18*	93 ±21	43 ±7*
IL-9	165 ±48	100 ±24	913 ±178	445 ±64*	492 ±27	340 ±44
IL-17	3.07	2.93	179 ±83	13 ±1*	283 ± 80	206 ± 73
GM-CSF	10 ±1	27 ±5	140 ±23	45 ±6*	197 ± 12	135 ± 46
G-CSF	167 ±97	73 ±10	67641 ±12220	40162 ±2079	52130 ± 22790	90430 ± 27810
Chemokines						
Eotaxin	827 ±190	567 ±135	15187 ±3649	2514 ±899*	11630 ± 3823	3158 ± 992*
KC	52 ±7	11 ±1	12883 ±3656	205 ±58*	50050 ± 4198	11390 ± 4050*
MCP-1	66 ±23	47 ±5	35376 ±10562	11841 ±1675	22810 ± 4894	12420 ± 4901
MIP1-α	141 ±29	135 ±22	1481 ±400	191 ±18*	2805 ±399	1878 ±160*
MIP1-β	15 ±3	18 ±3	4757 ±1502	234 ±52*	1511 ±467	457 ±152*
RANTES	112 ±22	107 ±13	5708 ±1301	3377 ±522	252 ±41†	149 ±35

nd= below level of detection

Table I: Effect of intra-peritoneal MPLA treatment on plasma cytokine and chemokine concentrations in response to infection.
Mice received intraperitoneal injection with MPLA (20 μg in 0.2 ml of lactated Ringer's solution) or lactated Ringer's solution alone (vehicle) on days 0 and 1 followed by intraperitoneal challenge with 8 log CFU of *Pseudomonas aeruginosa* or CLP on day 3. At 6 hours after *Pseudomonas* infection or 16 hours after CLP, plasma was harvested and cytokine concentrations were measured using a bead array. *$p<0.05$ compared to vehicle, n=6-8 mice per group.

significantly attenuated CLP-induced production of the measured pro-inflammatory cytokines as well as the chemokines eotaxin, KC, MIP-1α and MIP-1β (Table 1). However, plasma concentrations of the anti-inflammatory cytokine IL-10, Th2 cytokine IL-13 and all measured hematopoietic factors were preserved as was production of the TRIF-associated chemokines RANTES (CCL5) and MCP-1 (CCL2) (Table 1). Production of the Th1 cytokine IFNγ was also preserved in MPLA-treated mice compared to vehicle-treated controls as was IL-17.

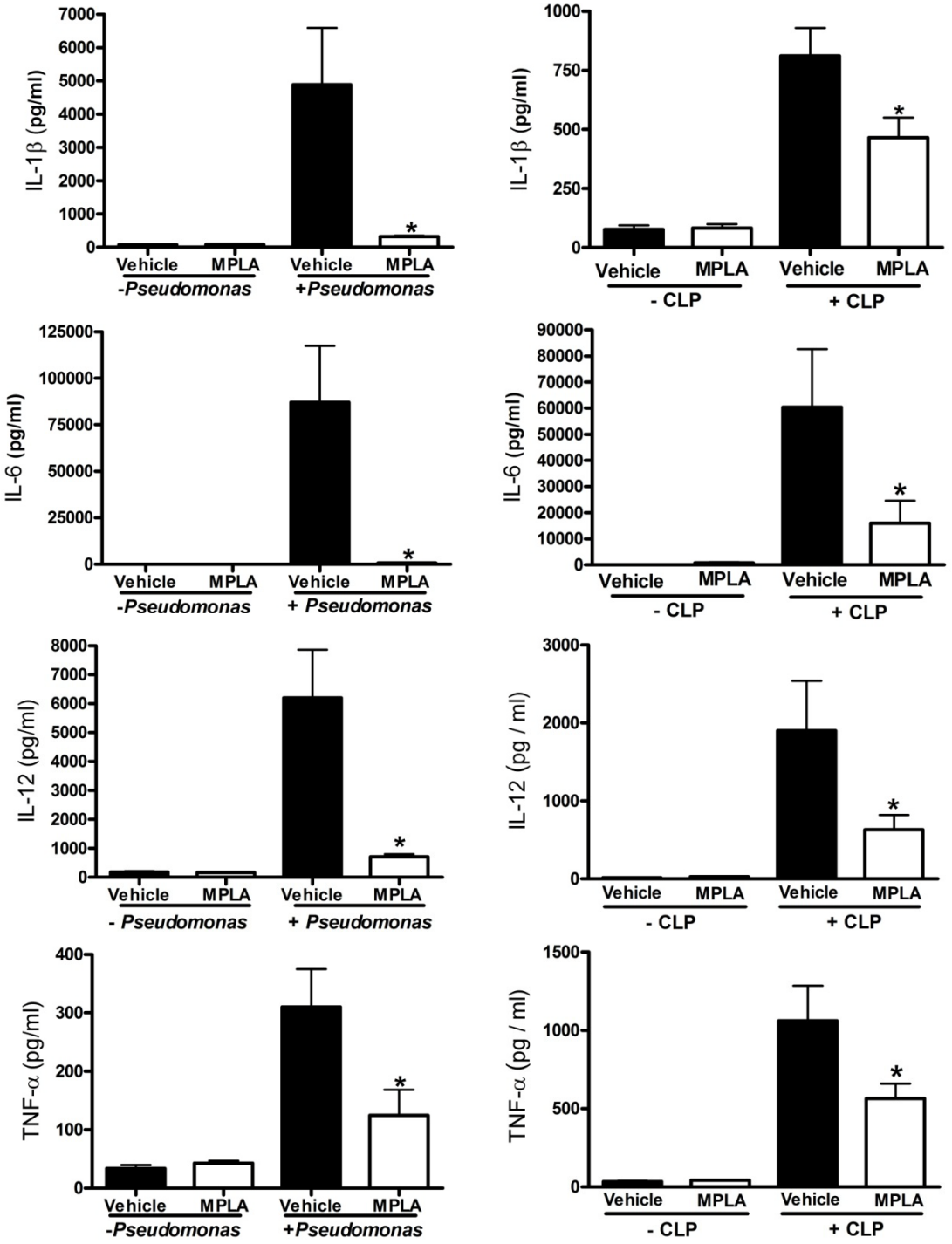


Figure 3.6 MPLA attenuated the production of inflammatory cytokines in response to i.p. *pseudomonas aeruginosa* infection and CLP. Vehicle treated mice had significant increased plasma pro-inflammatory cytokine levels after infection or CLP, whereas MPLA priming reduced the production of inflammatory cytokines after infection and did not increase production in the absence of infection. *P<0.05, n=6-8 mice per group.

Intra-peritoneal MPLA treatment increased the number of phagocytic myeloid cells at sites of infection

Studies were undertaken to assess the effect of MPLA treatment on the recruitment of cells to the primary site of infection. In one study, mice received intra-peritoneal treatment with MPLA followed 2 days later by intra-peritoneal challenge with *Pseudomonas aeruginosa.* Peritoneal leukocytes were harvested from mice and labeled with fluorochrome-conjugated antibodies against the myeloid cell biomarkers Ly6G (neutrophils) and F4/80 (macrophages) at 6 hours after *Pseudomonas aeruginosa* challenge (Figure 3.7A). Approximately 45% of peritoneal leukocytes isolated from control (vehicle-treated, non-infected) mice expressed the macrophage marker F4/80 and were negative for Ly6G, a profile typical of resident peritoneal macrophages (Figure 3.7A). The remainder of peritoneal cells from control (vehicle-treated, non-infected) mice were predominantly negative for Ly6G and F4/80 (Figure 3.7A). Analysis of these non-myeloid cell populations showed that they are composed primarily of B and T lymphocytes. Treatment with MPLA increased the numbers of leukocytes in the peritoneal cavity compared to vehicle treated mice ($1.9 \times 10^{7} \pm 4.4 \times 10^{6}$, versus $4.8 \times 10^{6} \pm 1.2 \times 10^{6}$). Peritoneal myeloid cells from MPLA-treated mice were composed primarily of macrophages ($F4/80^{+}Ly6G^{-}$) and neutrophils ($F4/80^{-}Ly6G^{+}$) cells (Figure 3.7A). Intra-peritoneal infection of vehicle-treated mice with *Pseudomonas* caused an increase in the percentage of neutrophils and undifferentiated myeloid cells that co-express both F4/80 and Ly6G as well as a decrease in the numbers of resident macrophages (Figure 3.7A). MPLA-treated mice that were challenged with *Pseudomonas* had significantly ($p<0.05$) increased percentages and total numbers of neutrophils ($6.4 \pm 0.8 \times 10^{6}$ vs $2.8 \pm 0.7 \times 10^{6}$) and undifferentiated myeloid cells ($7.9 \pm 1.1 \times 10^{6}$ vs $1.6 \pm 0.5 \times 10^{6}$) in peritoneal lavage samples compared to vehicle-treated and infected mice.

The phagocytic function of recruited myeloid cells from MPLA- and vehicle-treated mice was evaluated after intra-peritoneal challenge with FITC-labeled *Pseudomonas aeruginosa* (Figure 3.7B). MPLA pretreatment significantly ($p<0.05$) increased the percentage and total numbers of macrophages ($F4/80^{+}Ly6G^{-}$), undifferentiated myeloid cells ($F4/80^{+}Ly6G^{+}$) and neutrophils ($F4/80^{-}Ly6C^{+}$) that phagocytosed FITC-labeled *Pseudomonas aeruginosa* bacteria (Figure 3.7B).

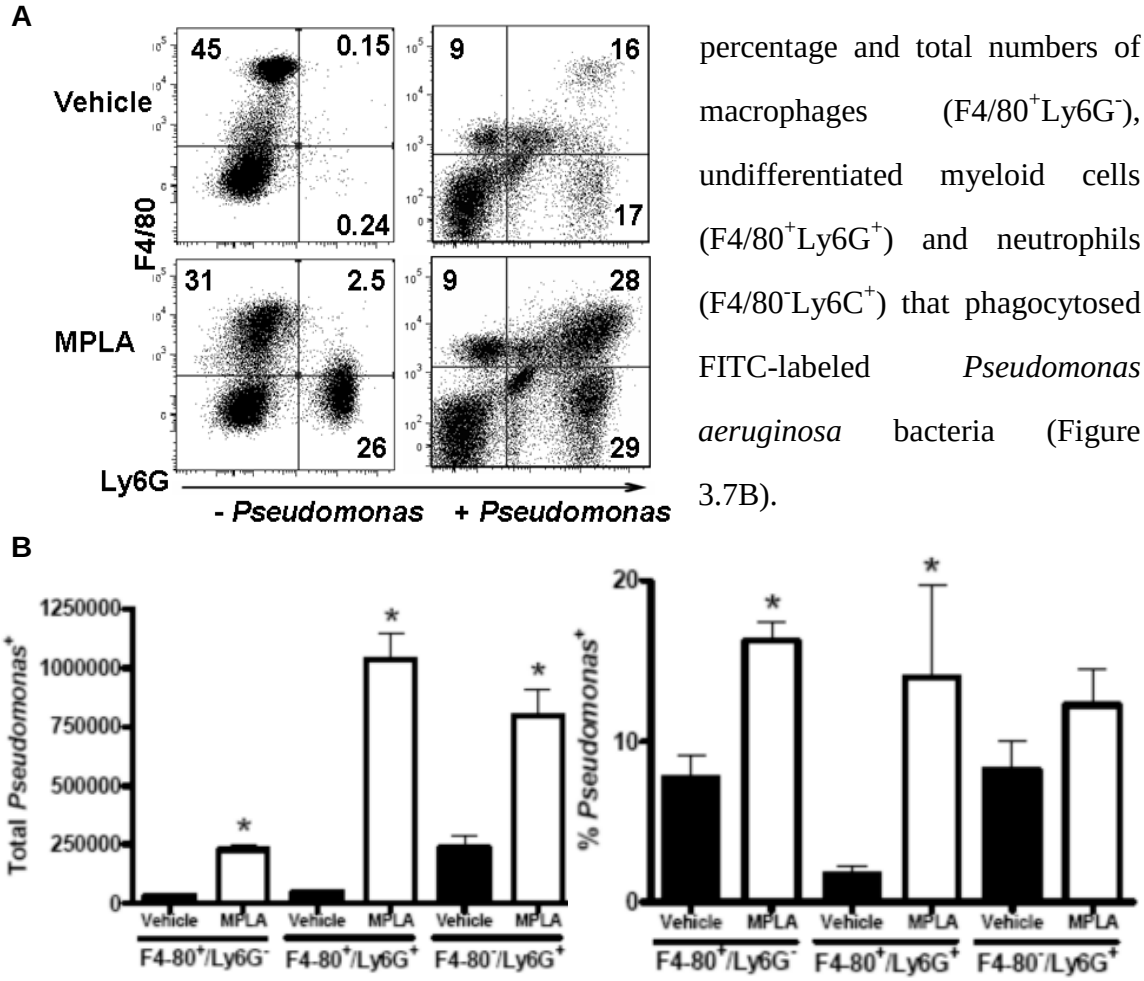


Figure 3.7 Characterization of peritoneal myeloid cell composition and phagocytic function from MPLA-treated mice. Mice received 2 intraperitoneal injections of MPLA (20 ug in 0.2 ml LR) or vehicle (0.2 ml LR) over two consecutive days. At 48 hours after vehicle or MPLA treatment, mice received intraperitoneal challenge with 1 x 10^{8} CFU of *Pseudomonas aeruginosa*. At 6 hours after bacterial infection, peritoneal leukocytes were recovered for analysis. For comparison, peritoneal leukocytes were harvested from mice challenged with aseptic lactated Ringer's solution (*-Pseudomonas aeruginosa)*. (A) Leukocyte expression of F4/80 and Ly6G was determined by antibody labeling and flow cytometry. The results are representative of those obtained in 3 separate experiments. (B) Mice receiving intraperitoneal treatment with MLPA- (20 μg in 0.2 ml LR) or vehicle- (0.2 ml LR) received intraperitoneal challenge with FITC-labeled and heat-killed *Pseudomonas aeruginosa* (8 log CFU). Peritoneal leucocytes were harvested, labeled with antibodies against F4-80 and Ly6G followed by analysis using flow cytometry. *$p<0.05$ compared to vehicle-treated mice, n= 4 mice per group.

The FITC mean fluorescent intensity, an indicator of the total number of *Pseudomonas aeruginosa* engulfed per cell, was not significantly different compared to controls. Analysis with an ImageStream flow cytometer (Amnis Corporation, Seattle, WA) showed that 77% of FITC-labeled *Pseudomonas aeruginosa bacteria* that were detected in this analysis were intracellular and not bound to the cell surface of leukocytes (Figure 3.8).

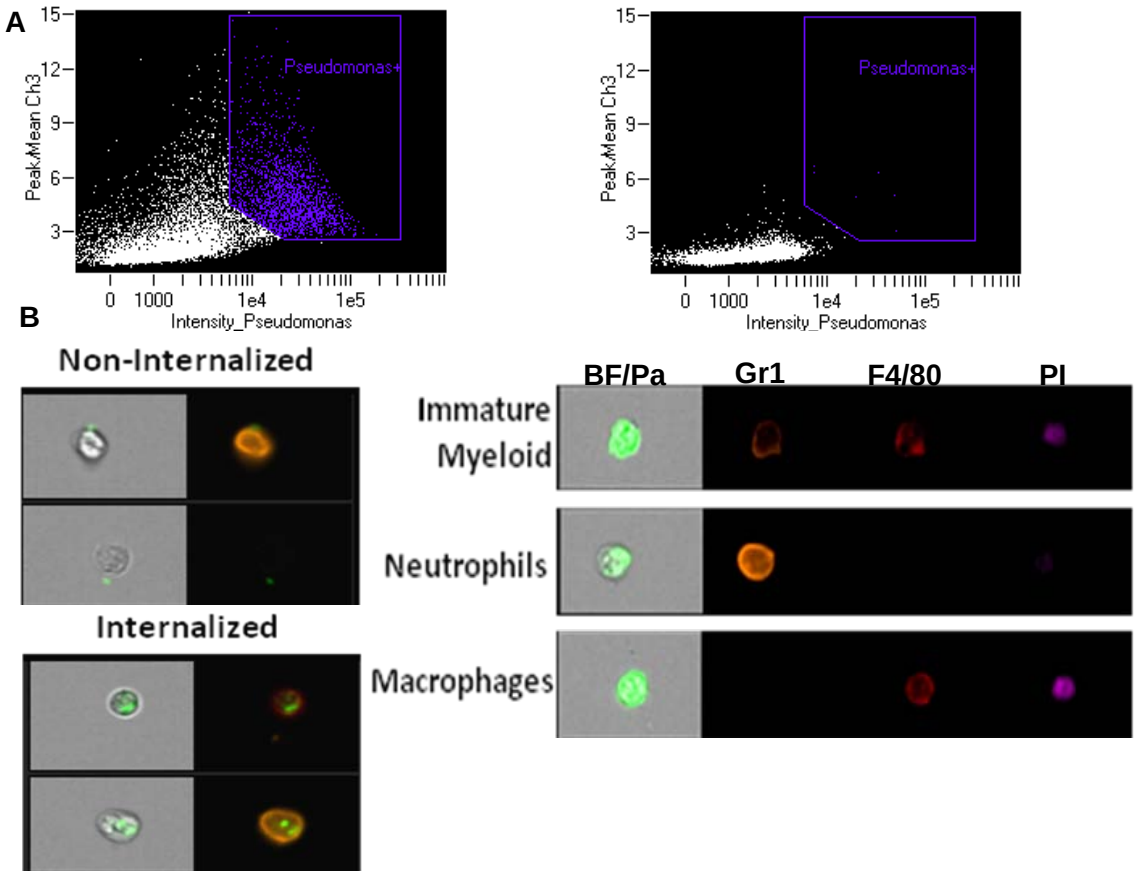


Figure 3.8 Amnis ImageStream analysis. Imaging flow Cytometry was capable of detective pseudomonas positive cells (A), discriminating internalized bacteria, and positively indentifying the cells type by surface marker (B).

The effect of MPLA priming on the recruitment of phagocytic myeloid cells to the peritoneum was not lost with age

Interestingly, aged mice (14-16 months old) responded similarly to intraperitoneal treatment with MPLA as young mice, exhibiting enhanced monocyte recruitment to the peritoneum from treatment alone and increased recruitment of a mixed myeloid population after infection compared to vehicle treated controls (Figure 3.9A). The total number of monocytes (F4/80+, Gr1-) that stained positive for the uptake of FITC labeled Pseudomonas aeruginosa was increased in the MPLA treated group, as well as in the immature myeloid population (F4/80+, Gr1+). There was only a small increase in phagocytosis seen in the neutrophil population (Gr1+, F4/80-) that was not statistically significant (Figure 3.9B).

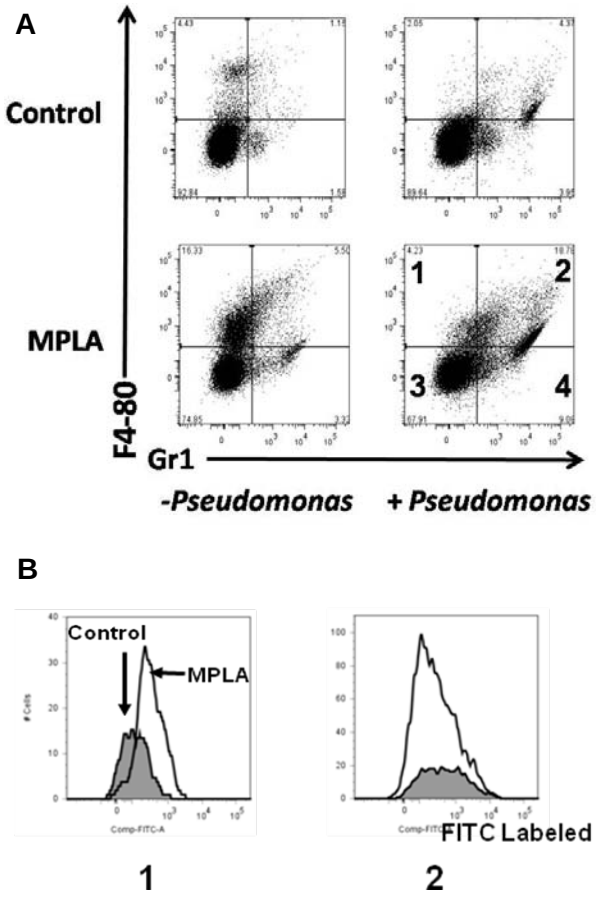


Figure 3.9 MPLA recruited a mixed phagocytic myeloid population to the peritoneum in aged mice. **A**, the resident myeloid population in control mice is composed primarily of pure monocytes (F4-80+, Gr1-), and neutrophils (Gr1+, F4-80-), with a small double positive population. After infection there is an expansion of the double positive population and neutrophils, coupled with a relative decline in pure F4-80 monocytes. MPLA expanded all three myeloid populations. Infected MPLA treated mice had an even larger increase in the Gr1+, F4-80+ double positive myeloid population. **B**, Mice primed with MPLA or vehicle were infected with heat killed, FITC-labeled *Pseudomonas aeruginosa*. Histograms of FITC expression levels from the 4 quadrants are compared between the two groups. Vehicle treated groups are depicted in shaded histograms, MPLA data is depicted by clear histograms.

MPLA did not increase the per cell expression of Fc receptor (FCR) but increased the total number of FcR+ peritoneal monocytes

One possible mechanism responsible for the enhanced bacterial clearance seen in MPLA-primed animals is up-regulated expression of Fc receptor. It has been demonstrated that macrophage expression of FcR is increased during sepsis.[257] A decline in Fc receptor expression has been shown to correlate with untreated intra-abdominal sepsis.[258] Arend and colleagues demonstrated that LPS and IL-1 are capable of increasing the expression of FcR on monocytes.[259] To determine if MPLA priming increased expression of FcR, peritoneal cells from MPLA-treated and untreated mice were stained for FcR and myeloid surface marker expression. The mean fluorescence intensity for FcR expression was not significantly different between monocytes from primed and un-primed mice (Figure 3.10). While there was not a statistically significant difference in the percentage of cell expressing FcR, there was a significantly higher total number of FcR+ monocytes recovered from MPLA primed mice.

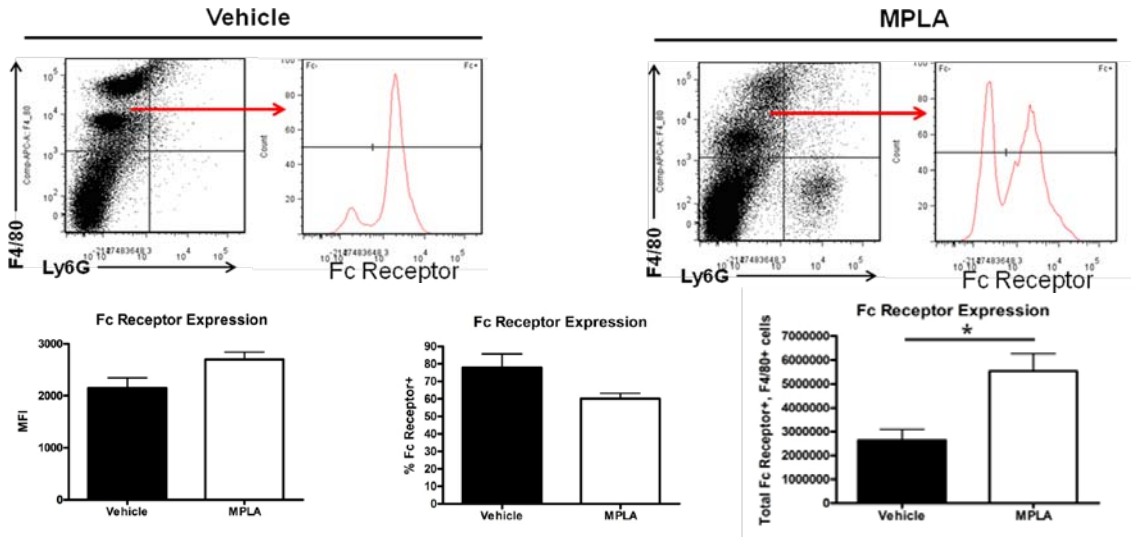


Figure 3.10 MPLA increased the number, but not MFI, of FcR expression. Peritoneal leukocytes from MPLA primed and unprimed mice were stained for FcR and surface marker expression. MPLA increased the number but not per cell expression of FcR. *p < 0.05, n=5 mice per group.

Intravenous MPLA treatment increased the numbers of myeloid cells at the site of infection

The effect of intravenous MPLA treatment on the recruitment of leukocytes to the primary site of infection was determined by harvesting intra-peritoneal leukocytes at 6 hours after intra-peritoneal challenge with *Pseudomonas aeruginosa* (Figure 3.11). As compared to vehicle treated controls, intravenous MPLA treatment did not significantly affect the numbers or percentages of myeloid cell populations within the peritoneum in the absence of *Pseudomonas* infection (Figure 3.11). In both groups, the predominant

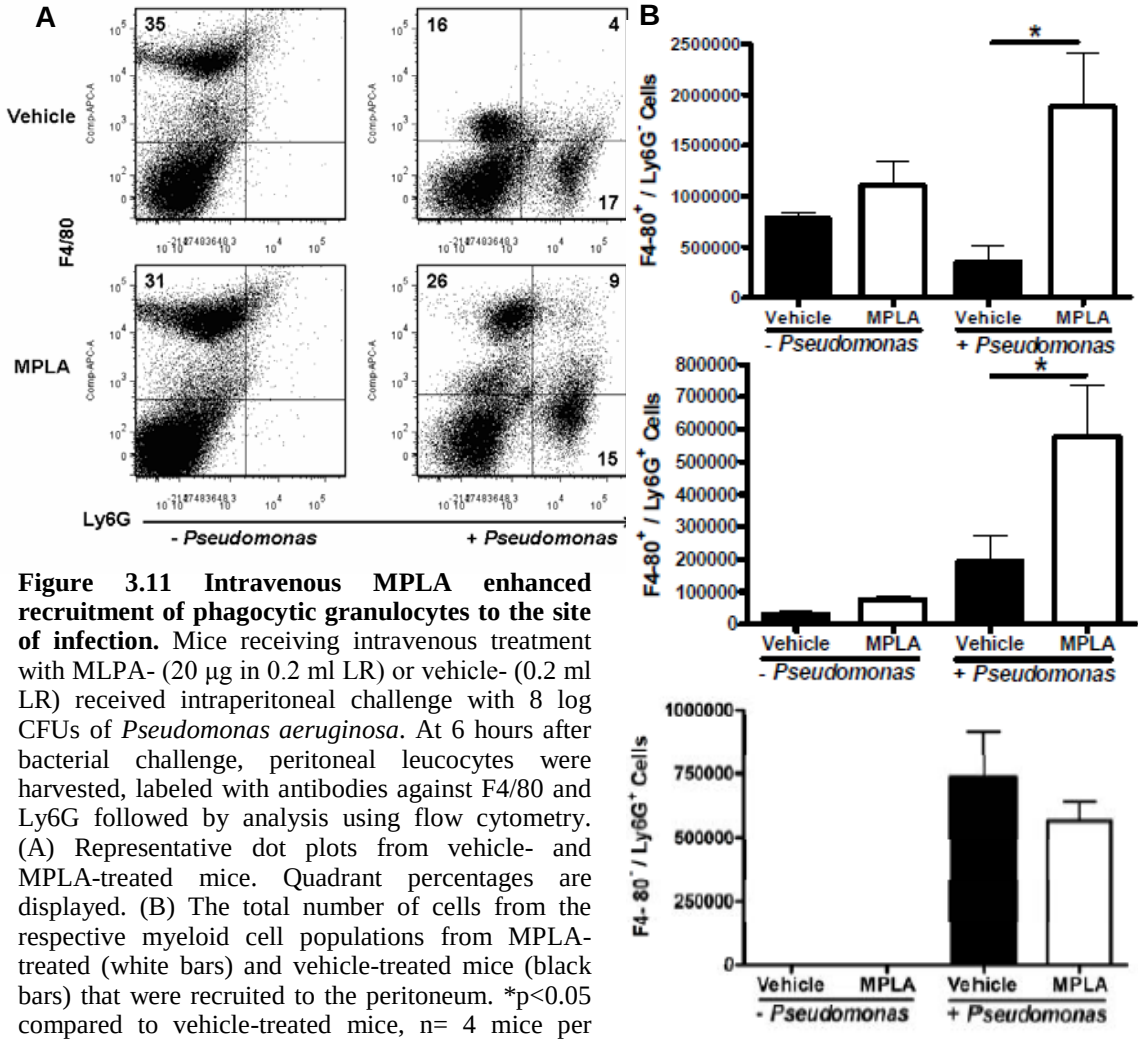


Figure 3.11 Intravenous MPLA enhanced recruitment of phagocytic granulocytes to the site of infection. Mice receiving intravenous treatment with MLPA- (20 μg in 0.2 ml LR) or vehicle- (0.2 ml LR) received intraperitoneal challenge with 8 log CFUs of *Pseudomonas aeruginosa*. At 6 hours after bacterial challenge, peritoneal leucocytes were harvested, labeled with antibodies against F4/80 and Ly6G followed by analysis using flow cytometry. (A) Representative dot plots from vehicle- and MPLA-treated mice. Quadrant percentages are displayed. (B) The total number of cells from the respective myeloid cell populations from MPLA-treated (white bars) and vehicle-treated mice (black bars) that were recruited to the peritoneum. *$p<0.05$ compared to vehicle-treated mice, n= 4 mice per group.

myeloid cell population consisted of F4/80^{+}Ly6G^{-} resident macrophages. After intravenous priming with MPLA or vehicle followed by intra-peritoneal challenge with *Pseudomonas aeruginosa*, MPLA-primed mice had a larger percentage and total number of intra-peritoneal macrophages (F4/80^{+}Ly6G^{-}) and undifferentiated myeloid cells (F4/80^{+}Ly6G^{+}) compared to vehicle-treated mice (Figure 3.11B). There was not a significant increase in neutrophils in MPLA-treated mice after infection as compared to vehicle-treated controls.

MPLA priming increased the expression of myeloperoxidase by peritoneal macrophages, but not neutrophils and immature myeloid cells

Myeloperoxidase (MPO) is an important antimicrobial enzyme produced in neutrophils and macrophages responsible for the generation of reactive oxygen and

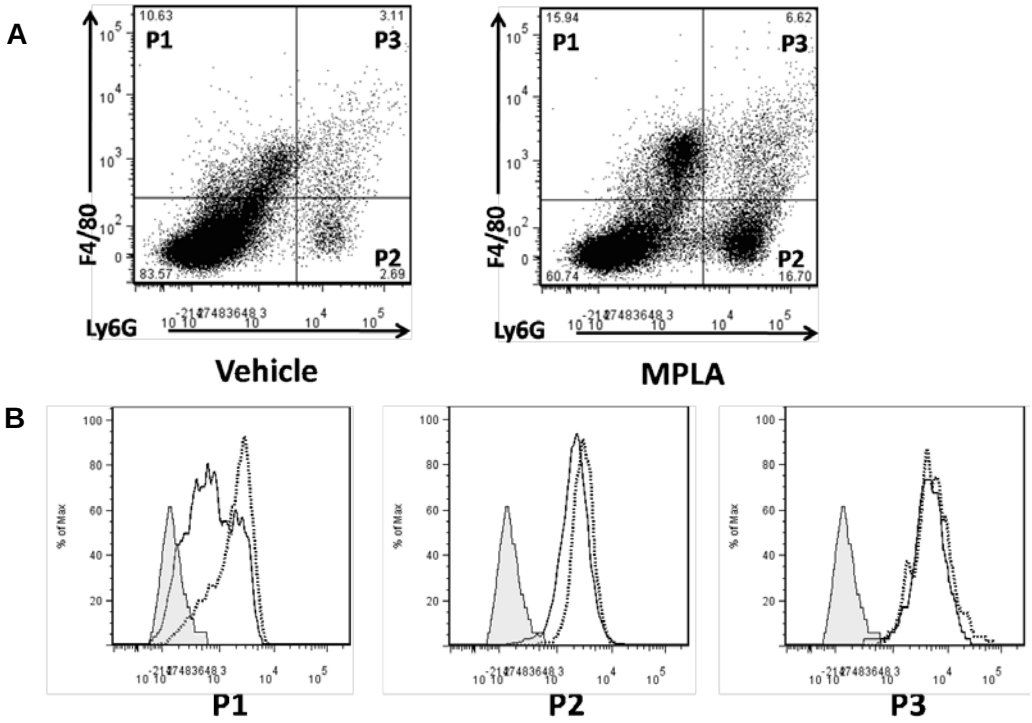


Figure 3.12 MPO expression after infection. Mice were primed i.p with MPLA or vehicle and then infected i.p. with 8 log CFUs *P. aeruginosa*. (A) 1 hour after infection peritoneal cells were recovered and assayed for F4/80, Ly6G, and MPO expression. (B) Histogram demonstrates intracellular MPO staining from the 3 populations in panel A. Isotype control: shaded curve, Vehicle treated mice: solid line, Dotted line: MPLA treated mice. Data representative of 3 experiments.

nitrogen compounds capable of killing pathogens.[260] MPO expression levels decline as monocytes mature, and excessive levels of MPO are associated with tissue destruction in hyper-inflammatory states. To assess the effect of MPLA on this important anti-microbial mechanism, mice received intraperitoneal priming with MPLA or vehicle and were then infected with 10^8 CFU of Pseudomonas. At 1 hour after infection, peritoneal cells were harvested and stained for MPO and myeloid cell surface marker expression. MPLA increased the expression of MPO in F4/80+, Ly6G- monocytes after infection (Figure 3.12). MPLA pretreatment did not increase the expression of MPO in Ly6G+, F4/80- neutrophils, or Ly6G+, F4/80+ immature myeloid cells. The total number of MPO+ cells was increased in MPLA primed mice after infection and was related to the general leukocytosis commonly seen at infections sites in primed mice (data not shown).

MPLA treatment did not impact splenic leukocyte apoptosis

It has been repeatedly demonstrated that sepsis induces apoptosis in splenocytes, and that this increased apoptosis correlates with increased mortality in various murine models of sepsis.[261, 262] To examine the effect on MPLA priming on splenocyte apoptosis, mice pretreated with MPLA or vehicle underwent CLP. At 16 hours post-CLP, splenocytes were harvested and stained for Annexin V and 7AAD. The percentage of apoptotic splenocytes (Annexin V+, 7AAD-) was not statistically different between MPLA primed and unprimed mice 16 hours after CLP (Figure 3.13). Annexin V expression was actually slightly higher in the MPLA primed mice, but this was not statistically significant. Dead / necrotic splenocytes were identified by Annexin V+, 7AAD+ double positive staining, once again there was no statistical difference in this population between MPLA primed and unprimed mice after CLP. There was also no significant difference in apoptosis or necrosis staining of peritoneal leukocytes after CLP between MPLA primed and unprimed mice (data not shown). It appears that the

protective immune-modulated phenotype elicited by MPLA is not mediated through decreased apoptosis within lymphoid organs such as the spleen.

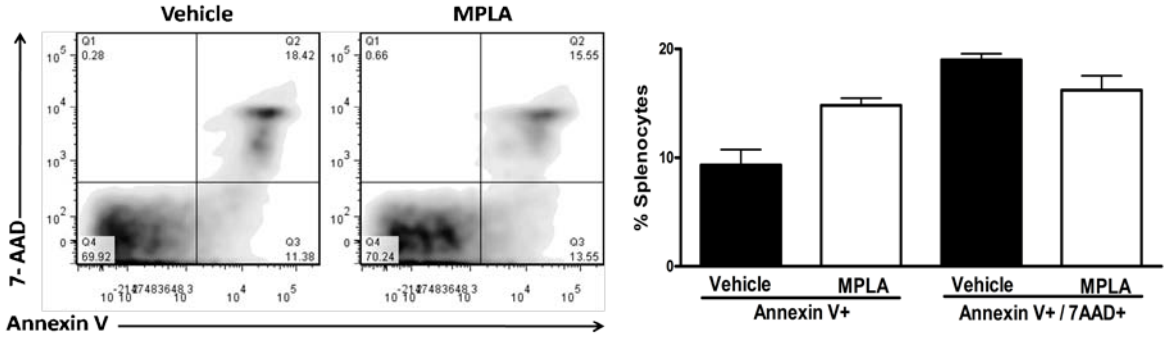


Figure 3.13 MPLA priming did not affect CLP induced splenic apoptosis. Splenocytes recovered from MPLA primed and unprimed mice 16 hours after CLP were stained for Annexin V & 7AAD then analyzed by flow cytometry. There was no statistical difference in apoptosis (Annexin V+) or necrosis (Annexin V+, 7AAD+) between primed and unprimed mouse splenocytes.

Neutrophil depletion eliminated the protective effect of MPLA

As shown in figures 3.7-3.9, MPLA priming causes a significant influx of $Ly6G^+$ neutrophils and immature myeloid cells, into the peritoneum and this population is increased further after infection. To determine the importance of these neutrophil populations in the phenomenon of MPLA immuno-modulation, mice received treatment with Ly6G specific antibody (clone 1A8, 100 µg IP)) after priming with saline or MPLA. To verify that the neutrophil population was depleted mice were infected with 10^8 CFUs of *Pseudomonas aeruginosa* 24 hours after Ly6G antibody administration; peritoneal cells were collected within 3 hours after infection and stained for myeloid cell markers. Treatment with anti-Ly6G antibody eliminated the Ly6G+, $F4/80^-$ neutrophil population (<1% of recovered peritoneal cells) as well as the $Ly6G^{+,}$ $F4/80^+$ immature myeloid population, which was also reduced to <1% of recovered cells (Figure 3.14A).

In further studies, MPLA- and vehicle-primed mice were treated with anti-Ly6G and underwent CLP. At 16 hours after CLP, temperature and bacterial counts were measured. Depletion of neutrophils with anti-Ly6G eliminated the preservation in core

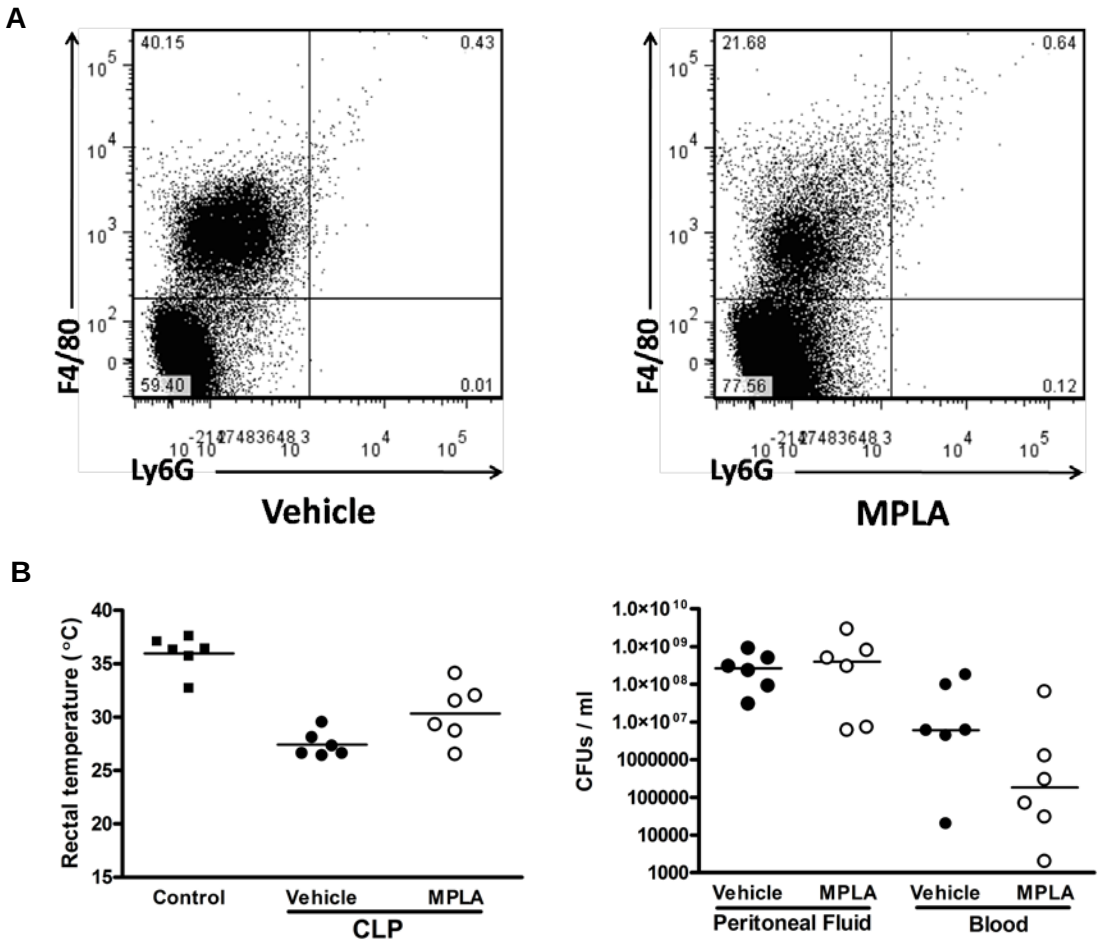


Figure 3.14 Neutrophil depletion with anti-Ly6G eliminated the protective effect of MPLA in a CLP model of sepsis. Mice were primed i.p. with MPLA or vehicle alone, followed by neutrophil depletion with i.p. administration of anti-Ly6G antibody (clone 1A8). (A) 24 hours after neutrophil depletion mice were infected i.p. with 8 log CFUs of *P. aeruginosa*. 3 hours after infection peritoneal cells were recovered and stained for Ly6G and F4/80 surface marker expression to assess the effectiveness of neutrophil depletion. Antibody treatment reduced the Ly6G single positive and Ly6G+, F4/80+ double positive population to <1% of recovered leukocytes. (B) MPLA and vehicle primed mice were treated with anti-Ly6G antibody 24 hours before undergoing CLP. 16 Hours after CLP there was no significant difference between the core temperature in MPLA and vehicle primed mice. (C) Neutrophil depletion with anti-Ly6G also eliminated the reduced bacterial burden associated with MPLA priming after CLP.

temperature normally seen in MPLA-primed mice after CLP (Figure 3.14B). Research from our laboratory and others has shown that core temperature correlates with survival in mice subjected to CLP; a decline in core temperature is a negative predictor of survival after insult. Neutrophil depletion also eliminated the reduced bacterial burden in MPLA-

primed mice after CLP (Figure 3.14C). Neutrophil-depleted, MPLA-primed mice still had higher core temperature and lower bacterial burdens both within their peritoneum and in systemic circulation; however these differences were below the level of statistical significance. It appears that Ly6G+ myeloid cells are necessary for MPLA protection from CLP induced sepsis.

Monocyte depletion attenuated the protective effect of MPLA

Monocytes are also recruited in large numbers by MPLA administration and further expanded by peritoneal infection or CLP (as depicted in figures 3.7-3.9). To determine if monocytes / macrophages were required for the protective effect elicited by MPLA, this population was depleted by treatment with clodronate liposomes. Two days before the first dose of MPLA, mice were injected intraperitoneally with 200 μl of clodronate liposomes or vehicle liposomes. To ensure maximal depletion of monocytic cells, a second dose of liposomes was administered 24 hours after the final priming dose of MPLA or vehicle. The day after the final dose of liposome administration, mice underwent intraperitoneal challenge with 10^8 CFU of *Pseudomonas aeruginosa*. Peritoneal cells were collected 3 hours after infection and stained for myeloid surface marker expression to determine the efficacy of clodronate liposome-mediated monocytes depletion. In vehicle primed mice, $F4/80^+$ monocytes were reduced to roughly 2% of peritoneal leukocytes after clodronate liposome treatment, and $F4/80^+$, $Ly6G^+$ immature myeloid cells declined to <1% of leukocytes (Figure 3.15A). In MPLA primed mice, $F4/80^+$ monocytes were reduced to around 6% of peritoneal leukocytes, and $F4/80^+$, $Ly6G^+$ immature myeloid cells accounted for <1% of cells (Figure 3.15A). In further studies, MPLA- and vehicle-primed mice treated with liposomes underwent CLP. At 16 hours after CLP, temperature and bacterial burden was assessed. Monocyte depletion abrogated the preservation in core temperature normally associated with MPLA pre-

treatment (Figure 3.15B). Interestingly, MPLA-induced enhancement of bacterial clearance was still preserved in mice treated with clodronate liposomes (Figure 3.15C). Treatment with control liposomes did not change the improvement in rectal temperature and bacterial clearance conferred by MPLA.

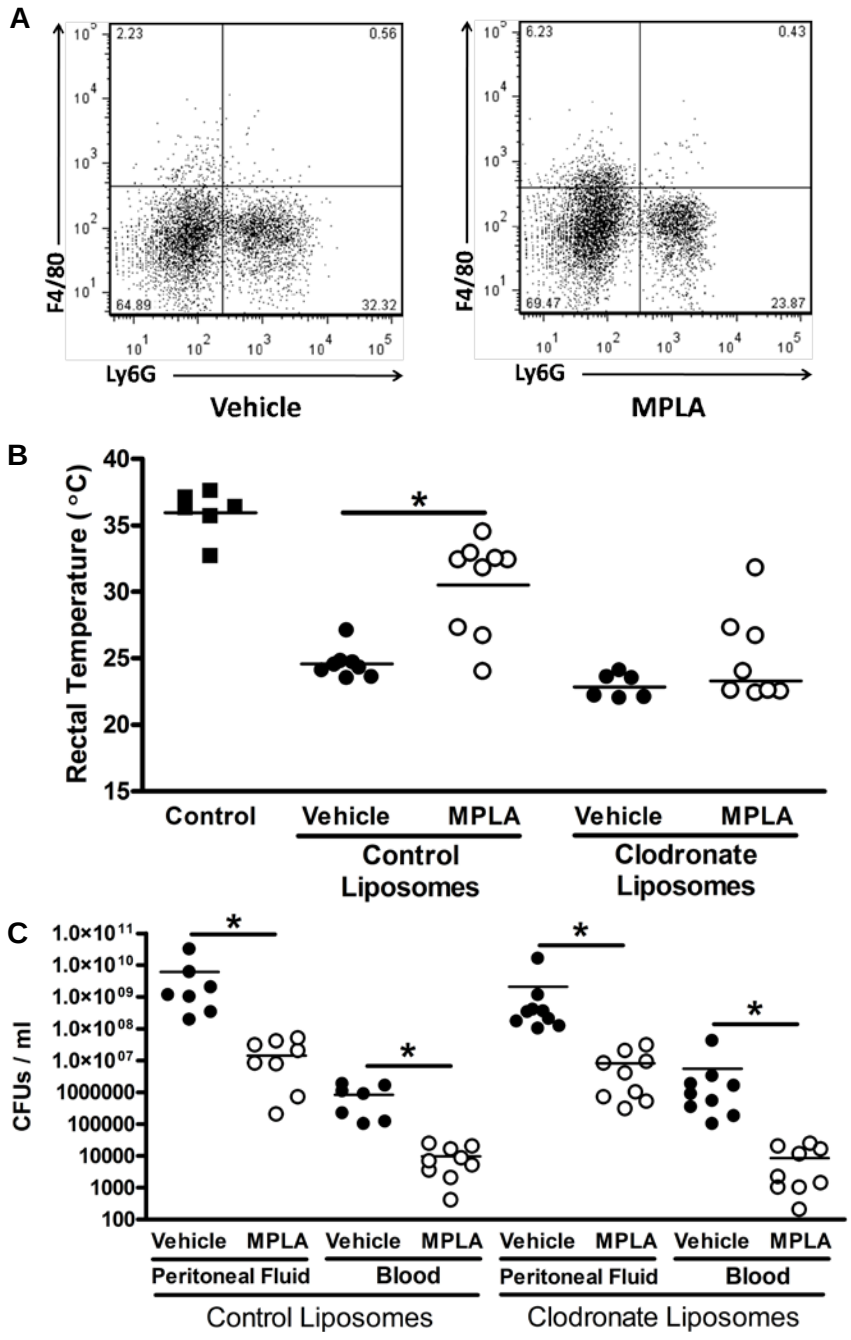


Figure 3.15 Clodronate depletion of Monocyte / Macrophages. (A) 24 hours after monocyte depletion mice were infected i.p. with 8 log CFUs of *P. aeruginosa*. 3 hours after infection peritoneal cells were recovered and stained for Ly6G and F4/80 surface marker expression to assess monocyte depletion. Clodronate treatment reduced the F4/80 single positive and F4/80+, Ly6G+ double positive populations from the peritoneum. (B) Control liposomes did not impact MPLA induced resistance to hypothermia after CLP, whereas Clodronate liposomes eliminated the effect of MPLA on core temperature. (C) Clodronate liposome mediated monocyte depletion did not eliminate the enhanced bacterial clearance associated with MPLA. Control liposomes did not impact clearance as well. *=P<0.05, n=8 mice per group.

These results indicated that monocytes may exert a regulatory role in MPLA-mediated immune-modulation, but were not required for the enhanced bacterial clearance after MPLA treatment. In addition the absence of the immature, double positive myeloid population did not adversely affect bacterial clearance in MPLA-primed mice.

MPLA attenuated the decline in peripheral blood leukocytes after infection

Intravenous MPLA treatment had a significant effect on peripheral blood leukocyte counts (Figure 3.16). Treatment with MPLA, in the absence of infection, did not significantly alter total blood leukocyte counts compared to vehicle-treated mice but the numbers of blood monocytes were significantly higher in MPLA-treated mice. At 4 hours after infection, total leukocyte numbers decreased significantly in vehicle-treated

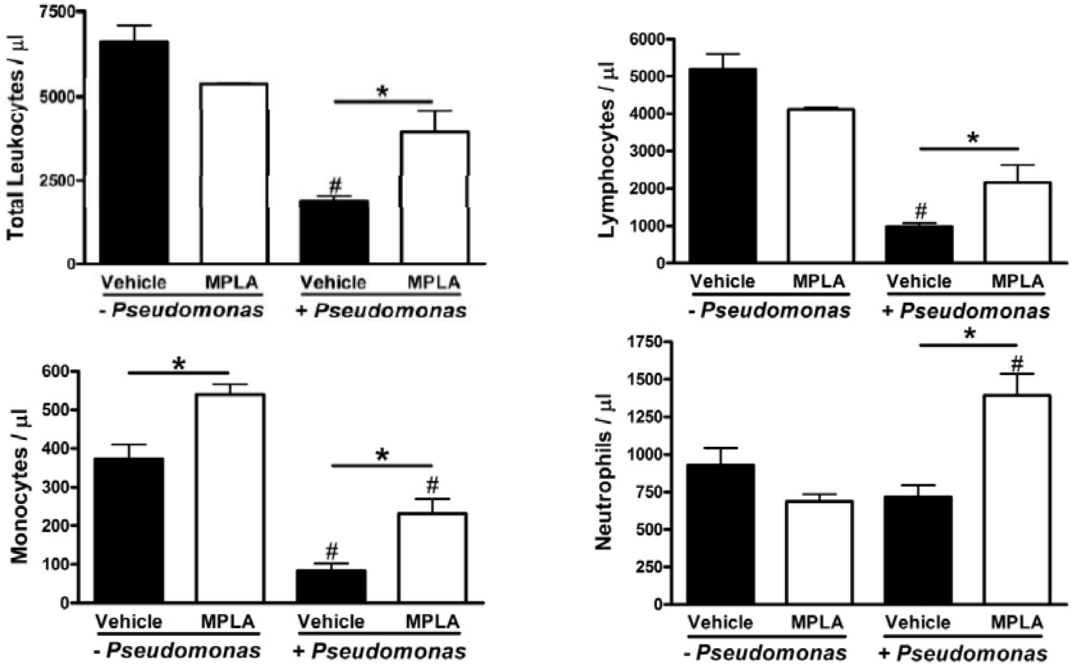


Figure 3.16 MPLA attenuated the decline in peripheral blood leukocytes after infection. Mice receiving intravenous treatment with MLPA- (20 µg in 0.2 ml LR) or vehicle- (0.2 ml LR) received intraperitoneal challenge with 8 log CFUs of *Pseudomonas aeruginosa* or vehicle alone. At 4 hours after bacterial challenge heparinized whole blood was collected and the cellular profile was analyzed using the Hemavet 950FS. * = statistical difference between MPLA & vehicle treated mice with or without infection ($P<0.05$), # = Significant difference from un-infected controls within treatment groups ($P<0.05$), n= 3 mice for un-infected groups, n= 6 mice for infected groups.

mice but this decline was significantly attenuated in mice receiving MPLA treatment (Figure 3.16). The fall in leukocyte numbers seen in vehicle-treated mice was primarily due to a decrease in the numbers of lymphocytes and monocytes. Blood neutrophil numbers were not changed in vehicle-treated and infected mice but were increased after infection in MPLA-treated mice (Figure 3.16).

MPLA attenuated the post infection decline in complement activity

The complement activity of plasma recovered from MPLA- and vehicle-treated mice was assessed prior to and after *Pseudomonas* challenge. Basal complement activity levels were not statistically different between MPLA- and vehicle-treated mice (Figure 3.17). After challenge with *Pseudomonas*, the plasma hemolytic activity of control mice was 15% compared to 30% in MPLA-treated mice, which was a significant difference (Figure 3.17).

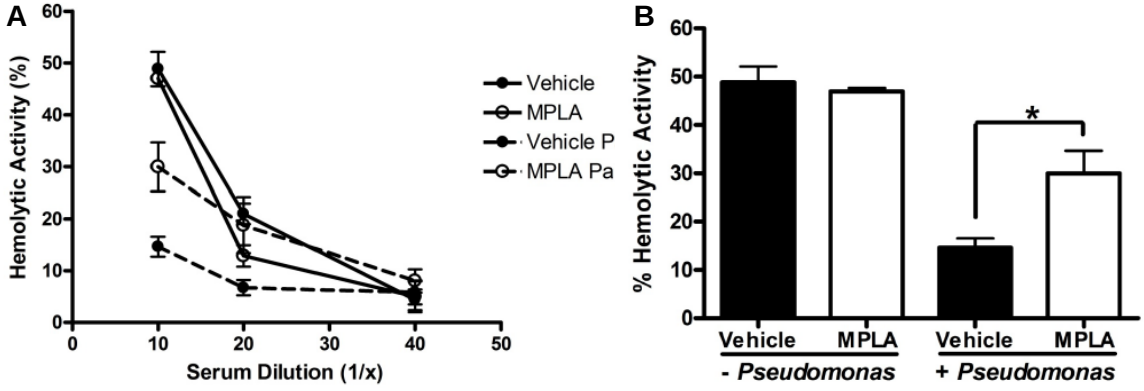


Figure 3.17 MPLA attenuated the post infection decline in complement activity. The hemolytic activity of plasma recovered from MPLA- and vehicle-treated mice was assessed in non-infected mice and at 6 hours after intraperitoneal challenge with 1 x 10^8 CFU of *Pseudomonas aeruginosa*. (A) Hemolytic activity of serially diluted plasma from vehicle- and MPLA-treated mice before and after *Pseudomonas aeruginosa* infection. (B) Total hemolytic activity in plasma from vehicle- and MPLA-treated mice before and after *Pseudomonas aeruginosa* infection *$p<0.05$, n= 5 mice per group.

MPLA treatment post-CLP enhanced bacterial clearance but did not prevent infection associated hypothermia

While prophylactic MPLA treatment has been demonstrated to be of benefit in murine models of sepsis, its efficacy when administered in a therapeutic regimen has not been elucidated. If the immunomodulatory effect of MPLA is dependent on chromatin remodeling and reprogramming of TLR4 signaling pathways, post-insult administration may prove ineffective. To assess this, MPLA or vehicle was administered 1 hour after CLP. At 16 hours post CLP, core temperature, IL-6, and bacterial clearance were measured. Administration of MPLA after CLP did not prevent the decline in core temperature which occurs normally in control or vehicle-primed mice (Figure 3.18A). However, when a less severe model of CLP was employed with only 0.4 cm of cecum ligated instead of 0.7 cm, there was a slight attenuation of hypothermia with MPLA treatment. However the difference was not statistically significant. Nevertheless, bacterial clearance in the peritoneal cavity and blood was significantly enhanced in mice treated with MPLA after severe CLP (Figure 3.18B). When using the less severe model of CLP, there was a significant decrease in bacterial burden in the blood of MPLA-treated mice but the bacterial burden in the peritoneal cavity was notsignificantly lower. Plasma IL-6 concentrations were reduced by MPLA post-treatment and this reduction was enhanced in the less severe model of CLP. However, this reduction was not statistically significant. Higher doses of MPLA (100 μg bolus instead of 20 μg) did not improve the therapeutic effect of MPLA in the more severe (0.7 cm) CLP model (data not shown). These results indicate the MPLA may confer protection when administered after a septic insult, but is less effective than when given prophylactically. The induction of enhanced bacterial clearance is rapidly elicited with MPLA, however the reduction in

cytokine production and preservation of core temperature is not fully achieved when MPLA is administered 1 hour after CLP.

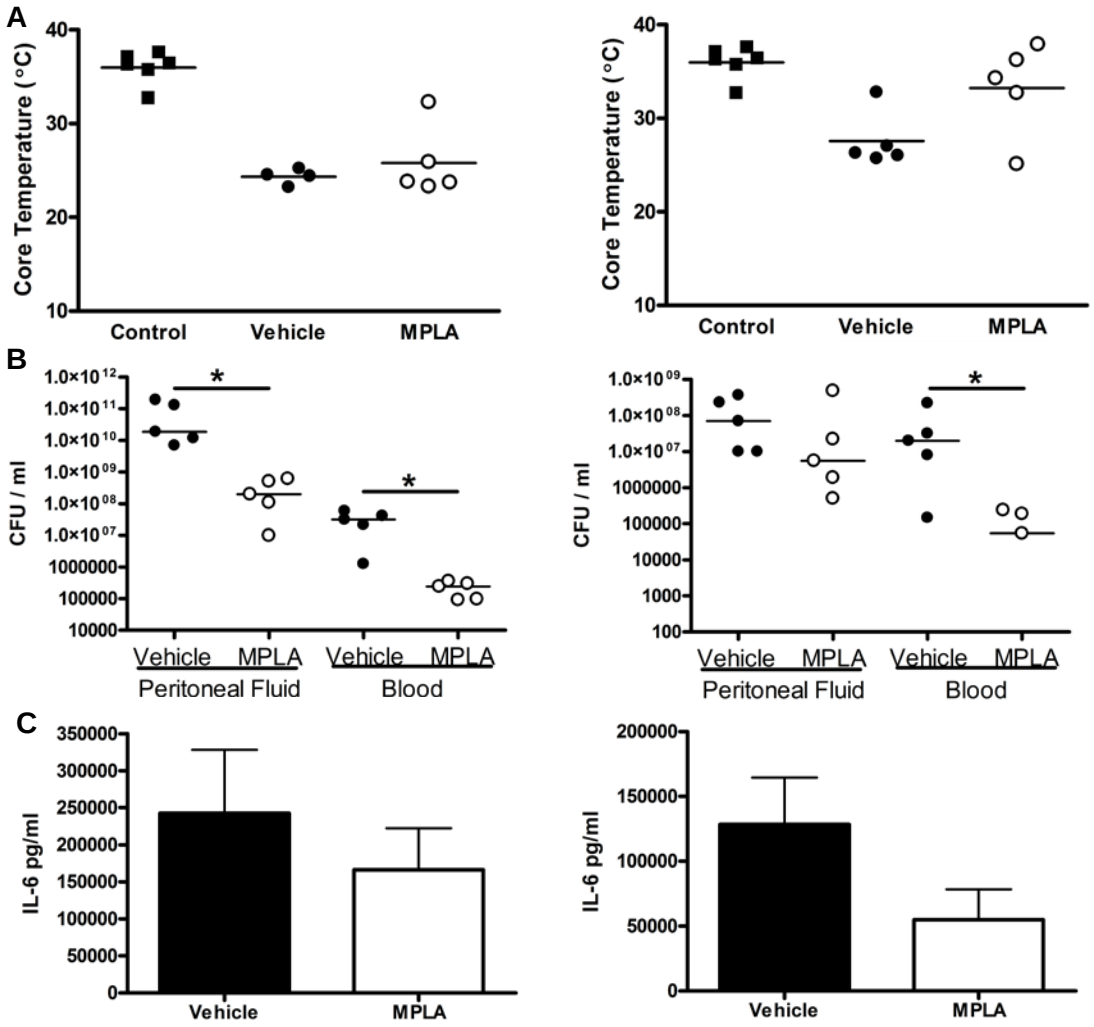


Figure 3.18 MPLA treatment post-CLP enhanced bacterial clearance but did not prevent infection associated hypothermia. Mice underwent CLP with .7cm of cecum ligated (left column), or the less severe .4cm cecum ligation (right column). 1 hour post CLP mice were injected i.p. with 20µg MPLA or vehicle alone. (A) 16 hours post-CLP MPLA and vehicle treated mice experienced equivalent declines in core temperature, with non-statistical improvement in the less sever model after MPLA treatment.(B) Bacterial burden both within the peritoneum and in peripheral blood was decreased in mice treated with MPLA post-CLP compared to vehicle treated mice. This effect was statistically significant in blood but not peritoneal fluid in the less severe model. IL-6 production was attenuated in MPLA primed mice in both CLP models however this was not statistically significant. *$p<0.05$, n=5 mice per group.

Discussion

The results of this study show that mice treated with the TLR4 agonist MPLA show increased resistance to clinically relevant models of systemic infection. MPLA pretreatment improved survival, prevented hypothermia, attenuated pro-inflammatory cytokine production and enhanced clearance of bacteria in the CLP model of sepsis and in mice with *Pseudomonas aeruginosa* burn wound infection. The improved resistance to infection occurred independently of the route of MPLA administration, since both intra-peritoneal and intravenous routes of administration were protective. The beneficial effects of MPLA, as utilized in this study, required prophylactic administration. However, a prophylactic strategy has potential utility in clinical scenarios in which patients are at increased risk of developing infectious complications. Among these are patients that have suffered major trauma, large burns, or have undergone high-risk surgery. Alternatively, patients scheduled for high risk surgery could receive MPLA treatment prior to surgery to potentially decrease surgery-induced inflammation, improve resistance to infection and potentially decrease the incidence of surgical wound infections.

Patients with severe cutaneous burns are highly susceptible to burn wound and systemic infections. In the present study, treatment of burned mice with MPLA markedly decreased local spread and systemic dissemination of *Pseudomonas aeruginosa* in a clinically relevant model of wound infection. Patients undergoing major intra-abdominal surgery are also at high risk of infection, especially bacterial peritonitis. Prophylactic treatment with MPLA markedly improved resistance to peritonitis caused by cecal ligation and puncture. This approach has potential clinical relevance since immuno-modulators such as MPLA could be given pre-operatively to decrease the incidence or severity of post-operative infections, as noted above.

Previous studies from our laboratory and others have demonstrated that treatment with LPS is capable of conferring resistance to infection by mechanisms that have not been fully elucidated.[172, 174, 175] However, the systemic administration of LPS in humans is not an attractive option due to inflammation-associated toxicity and a narrow therapeutic index. MPLA provides a more attractive approach because of decreased toxicity and side effects. MPLA is produced by hydrolysis of native lipid A resulting in removal of a single phosphate group and varying degrees of deacylation.[254, 263] These structural alterations decrease the toxicity of MPLA to less than 1% of that observed with LPS or diphosphoryl lipid A.[217] As shown in this report, MPLA retains potent immunomodulatory properties and improves the host response to infection by increasing bacterial clearance while attenuating the systemic inflammatory response.

MPLA improved bacterial clearance, at least in part, by promoting the recruitment of phagocytic myeloid cells to sites of infection. Intraperitoneal injection of MPLA, in the absence of infection, facilitated the recruitment of neutrophils to the peritoneal cavity. After infection, MPLA-treated mice showed higher numbers of phagocytic macrophages, neutrophils and undifferentiated myeloid cells at the primary site of infection compared to vehicle treated mice. In mice receiving intravenous MPLA treatment, macrophages, neutrophils, and undifferentiated myeloid cells were the dominant leukocyte populations present at the primary sites of infection. The numbers of macrophages and undifferentiated myeloid cells were significantly higher in MPLA-treated mice compared to vehicle-treated controls after intravenous treatment. Irrespective of the route of administration, MPLA treatment increased the numbers of myeloid cells that were recruited in response to live infection, although the phenotypic characteristics of the recruited leukocytes differed somewhat.

Leukocytes recruited to the infected peritoneal cavity of MPLA-treated mice showed effective phagocytic function. In general, the overall numbers of phagocytic myeloid cells at sites of infection were increased in mice receiving MPLA treatment. However, phagocytic function, on a per cell basis, was not altered. There was no significant difference between MPLA and vehicle treated mice in apoptosis / necrosis levels of splenic or peritoneal leukocytes after infection; indicating that the increased number of cells seen in primed animals appears not to result from enhanced cellular survival. Myeloid cells expressing Ly6G were the predominant cell type mediating bacterial phagocytosis in MPLA-treated mice and depletion of $Ly6G^+$ cells ablated the ability of MPLA to enhance bacterial clearance. The depletion of monocyte/macrophages did not significantly alter MPLA-induced clearance mechanisms. The $Ly6G^+$ myeloid cell populations were composed of true neutrophils and other myeloid cells that co-express the macrophage/monocyte marker F4/80 and the neutrophil marker Ly6G. Both true neutrophils and the double positive myeloid cell population showed high levels of phagocytic activity in mice treated with MPLA. Interestingly, the macrophage/monocyte depletion protocol resulted in depletion of the double positive myeloid cell population but did not markedly alter the improved bacterial clearance in MPLA-treated mice. Because neutrophil numbers remained intact, it is possible that the recruited neutrophils were able to bear the burden of bacterial clearance in the absence of the double positive population. Nevertheless, it is clear that $Ly6G^+$ cells are the primary mediators of the improved bacterial clearance seen in MPLA-treated mice.

The large influx of myeloid cells into sites of infection was not associated with increased pro-inflammatory cytokine production. Paradoxically, the production of most cytokines was markedly attenuated in MPLA-treated mice. Treatment with MPLA, like other endotoxin derivatives, has been shown to attenuate subsequent cytokine production

normally triggered by inflammatory insults such as infection or LPS exposure.[264] In the present study, infection-induced production of most cytokines was markedly attenuated in MPLA-treated mice. Several reports have shown that exposure of leukocytes to endotoxin analogs causes intrinsic alterations in intracellular signaling mechanisms that result in decreased production of pro-inflammatory gene products [265-267]. The primary alterations that have been described such as decreased MAP kinase phosphorylation and alterations in NF-κB translocation are primarily associated with the TLR4-associated MyD88 signaling pathway. In the present study, MPLA-treated mice showed attenuated production of cytokines that are regulated primarily through the MyD88 signaling pathway [268]. Interestingly, secretion of hemopoietic factors and chemokines that are considered to be dependent on Trif-mediated signaling, such as G-CSF, MCP-1, and RANTES, were relatively unaffected by *Pseudomonas aeruginosa* challenge in MPLA-treated mice [202, 203, 226]. These observations suggest that MPLA treatment causes targeted alterations in infection-induced cytokine secretion in which production of MyD88-dependent secretory products is preferentially attenuated, while the Trif-dependent signaling remains intact. Further studies are needed to fully define these alterations.

Another innate immune mechanism of bacterial clearance is the complement system. Complement activity has been shown to decline during sepsis and this reduction is an indicator of poor prognosis [236, 269]. MPLA treatment alone did not change basal complement activity. However, MPLA-treated mice had a significantly smaller decline in complement function after infection as compared to vehicle-treated controls. Interestingly, MPLA-treated mice also showed a significantly decreased fall in peripheral leukocytes after infectious challenge. These observations were associated with lower concentrations of systemic cytokines. The mechanism of MPLA-induced preservation of

complement activity and peripheral leukocyte counts is unclear, but it may result from reduced systemic spread of infection and/or an attenuated production of pro-inflammatory cytokines.

The ability of MPLA to confer an effect when administered after CLP is encouraging for future development of MPLA, and other compounds, as immuno-modulatory therapies for sepsis. MPLA enhanced bacterial clearance but failed to prevent a drop in core temperature. Unfortunately, hypothermia correlates closely with morbidity in the murine model of CLP induced sepsis. The hypothermic response also correlates with the production of inflammatory cytokines, indicating that MPLA treatment post insult may enhance bacterial clearance independent of its ability to reduce inflammation. Further investigation is required to define this differential effect in the post-insult MPLA treatment model. With the significant health burden of sepsis and relatively high mortality rate these results warrant further research.

In conclusion, these studies show that treatment with the TLR4 agonist MPLA causes improved resistance to systemic infection by *P. aeruginosa* that is characterized by attenuation of systemic pro-inflammatory cytokine production and improved clearance of bacteria. The improvement in bacterial clearance is mediated, in part, by enhanced recruitment of phagocytic myeloid cells. The ability of MPLA to improve survival, reduce inflammation, and enhance bacterial clearance makes it an attractive agent for potential application in patients that are at high risk of developing infectious complications.

Chapter 4: MPLA Immune-Modulation is Associated With Recruitment and Expansion of a $Gr1^+CD11b^+$ Myeloid Population Similar to Myeloid-Derived Suppressor Cells (MDSCs)

Introduction

There is increasing evidence that some subsets of myeloid cells appear to play an important role in regulating the immune system. A subset of myeloid cells, referred to as myeloid-derived suppressor cells (MDSCs), has been shown to exert significant immune-regulatory control in both disease and health. MDSCs were first described in the cancer literature as a means of suppressing the immunogenicity and clearance of tumors and cancerous cells.[270] MDSCs are a heterogeneous population composed of immature myeloid cells of both monocytic and granulocyte morphologies.[271] In mice, MDSCs are classically identified as $Gr1^+CD11b^+$ double positive, and have been associated with high expression levels of Arginase 1.[272] Up to 40% of bone marrow cells may express the surface markers Gr1 and CD11b concurrently. However, in the periphery the double positive population declines to 1-4% of leukocytes. Another identifier of MDSCs is a characteristic ring shaped nuclei. However, given the heterogeneity of this population, this cannot be used as a definitive marker.[273]

Both the monocyte and granulocyte MDSC subsets are capable of suppressing T-cell activation, which is believed to be a major mechanism of MDSC mediated immune-modulation.[274] An immature $Gr1^+CD11b^+$ myeloid population has been shown to expand during CLP-induced sepsis, and may, in part, mediate the immuno-dysregulation associated with sepsis and CARS.[275] MDSCs are expanded by exposure to LPS and IFNγ alone.[276] However, MDSCs may play a protective role in regulating inflammation, and may offer a novel treatment modality in immune mediated diseases.

MDSC recruitment and expansion has been shown to protect against allograft rejection.[277, 278] MDSCs have been shown to be protective in a murine model of inflammatory bowel disease.[279]

As depicted in chapter 3, MPLA priming recruits a large immature myeloid population exhibiting a mixed phenotype typified by co-expression of the monocyte cell marker F4/80 and the neutrophil marker Ly6G (see figure 3.7). This recruitment correlated with an attenuated pro-inflammatory cytokine response to infection (see figure 3.6), as well as with enhanced survival and decreased bacterial burden. The immature myeloid population recruited or expanded by MPLA may consist, at least in part, of MDSCs that play a regulatory role in preventing a hyper-inflammatory state from developing in murine models of sepsis.

Results

Peritoneal $Gr1^+CD11b^+$ myeloid cells expanded in response to MPLA and infection

MPLA priming caused the $Gr1^+CD11b^+$ cell population to increase in numbers within the peritoneal cavity from <1% of recovered leukocytes to around 10% of peritoneal leukocytes (figure 4.1). Intraperitoneal infection with *Pseudomonas* elicited the expansion of peritoneal $Gr1^+CD11b^+$ leukocytes in control mice. However, the expansion was not statistically significant compared to un-infected controls. After infection, MPLA primed mice experienced an even larger increase in peritoneal $Gr1^+CD11b^+$ myeloid cells, expanding to over 20% of recovered leukocytes. This was a heterogeneous population as can been seen in the flow cytometry dot plots, where the double positive gated cells in the MPLA treated groups appear as a poorly delineated doublet (Figure 4.1A). Upon further investigation it was determined that the

$Gr1^+CD11b^+$ population from MPLA primed mice were partially $F4/80^+Gr1^+$ double positive and $Gr1^+F4/80^-$.

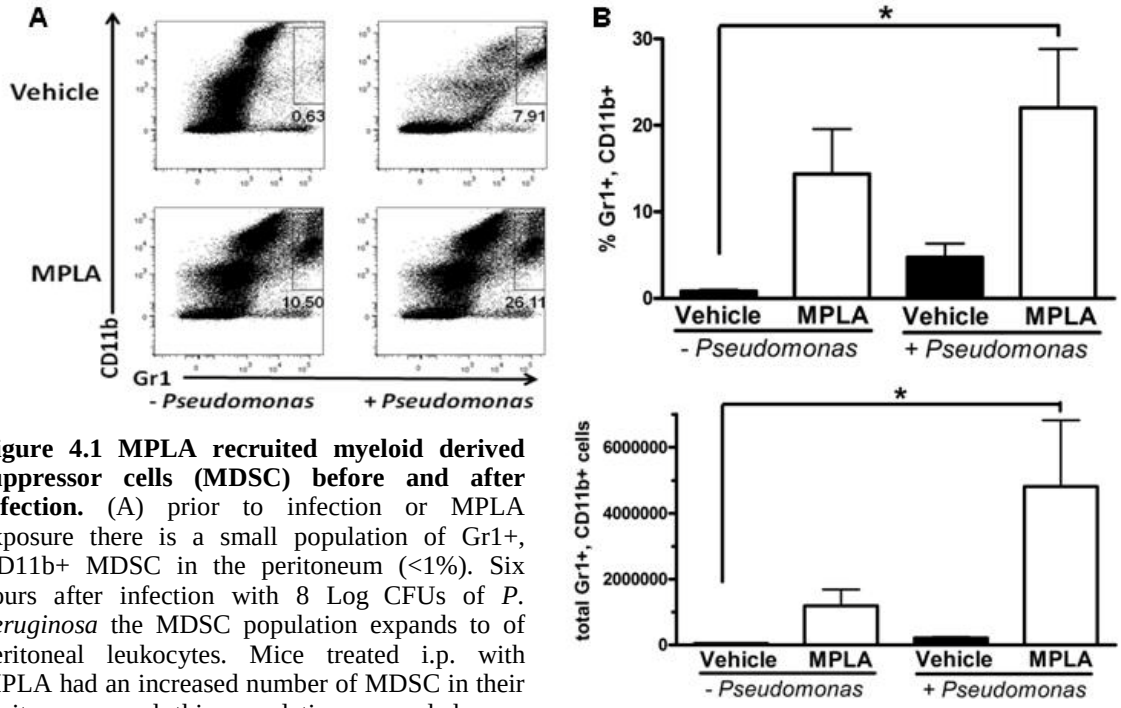


Figure 4.1 MPLA recruited myeloid derived suppressor cells (MDSC) before and after infection. (A) prior to infection or MPLA exposure there is a small population of Gr1+, CD11b+ MDSC in the peritoneum (<1%). Six hours after infection with 8 Log CFUs of *P. aeruginosa* the MDSC population expands to of peritoneal leukocytes. Mice treated i.p. with MPLA had an increased number of MDSC in their peritoneum, and this population expanded even further after infection. (B) MPLA increased the percentage of Gr1+, CD11b+ cells recovered from there peritoneum before and after infection. Dot-plot representative of multiple trials, Bar graph: n=3, * $P<0.05$.

MPLA expanded the $Gr1^+CD11b^+$ population in the bone marrow

Because $Gr1^+CD11b^+$ cells account for a significant segment of the bone marrow leukocyte population, the effect of MPLA treatment on the primary production of MDSCs was assessed. Femur bone marrow cells were assayed by flow cytometry for Gr1 and CD11b expression from MPLA and vehicle treated mice before and after infection. Bone marrow from vehicle-treated mice without infection had a significant $Gr1^+CD11b^+$ population, accounting for roughly 40% of cells (Figure 4.2). After infection, this population decreased to half its original size, to around 20% of bone

marrow cells. This correlates with the large influx of $Gr1^+CD11b^+$ cells to the peritoneum after infection and may represent an exodus of immature myeloid / MDSCs from the bone marrow in response to bacterial peritonitis. MPLA significantly increased the percentage of $Gr1^+CD11b^+$ cells within the bone marrow prior to infection (Figure 4.2). After infection MPLA treated mice experienced a similar decline in bone marrow

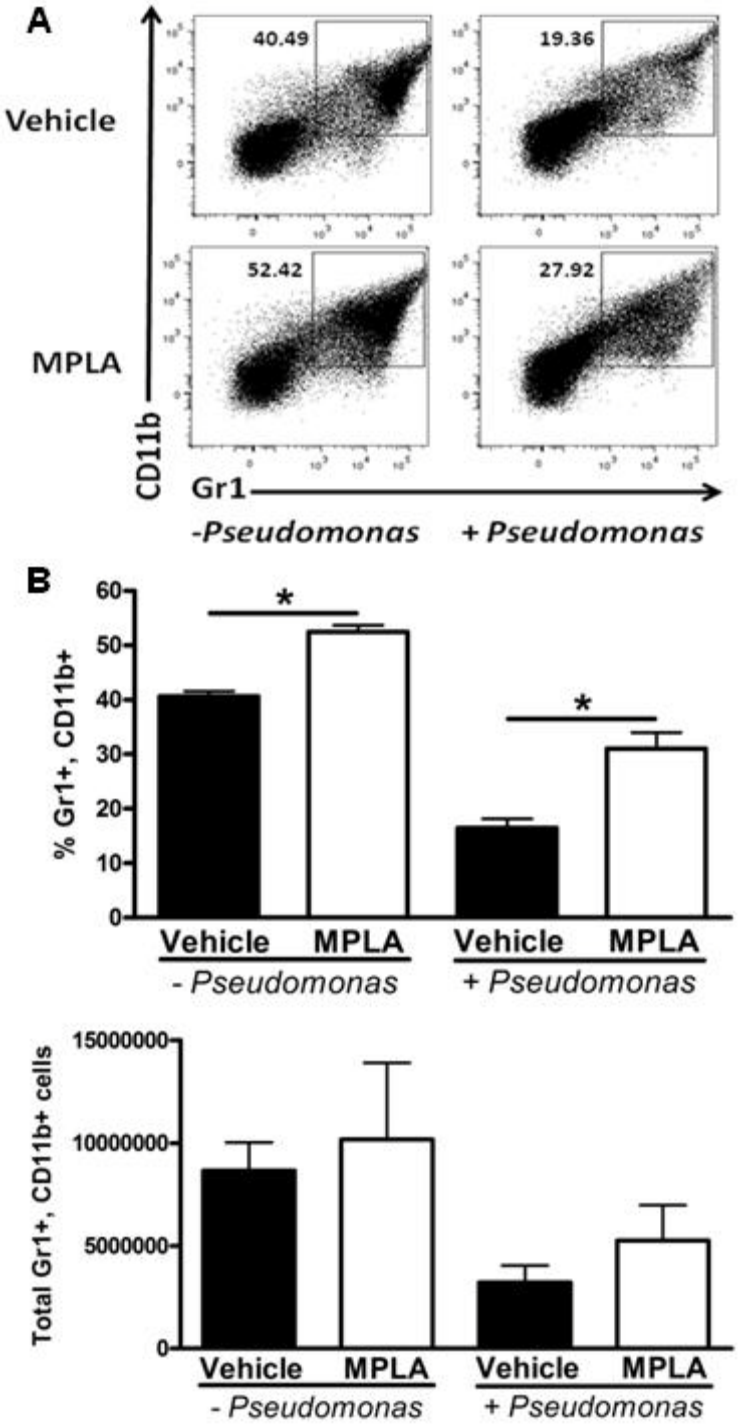


Figure 4.2 MPLA increased the production of Gr1+, CD11b+ myeloid cells in the bone marrow. (A) a large percentage of bone marrow leukocytes recovered from control mice were Gr1+, CD11b+ myeloid cells (40%). This percentage decreased 6 hours after infection (19%). MPLA treated mice had a larger percentage of double positive bone marrow leukocytes as compared to controls. This percentage decreased after infection, but not as much as control infected mice. (B) MPLA increased bone marrow production of Gr1+, CD11b+ myeloid cells and maintained their production after infection as compared to controls. Dot plot representative of multiple trials, Dot-plot representative of multiple trials, Bar graph: n=3, * $P<0.05$.

$Gr1^+CD11b^+$ cells. However the percentage of $Gr1^+CD11b^+$ cells in bone marrow remained significantly higher than in vehicle treated mice after infection.

MDSC analysis using multi-spectral imaging flow cytometry (MSIFS)

To address the concerns in the reliability of our flow cytometry results depicting myeloid cells of mixed marker expression, multi-spectral imaging flow cytometry was utilized. Peritoneal cells from MPLA- and vehicle-primed mice were collected before and after infection, stained normally for surface marker expression, then fixed and shipped to the Amnis Corporation for analysis. At Amnis, cells were stained with propidium Iodide (PI) to allow assessment of nuclear morphology and analyzed using their ImageStream flow cytometer. This method allowed for the discrimination of cellular aggregates from single cell events by comparing the X and Y ratio of cells, with round single cells having a ratio of 1 and cellular debris and aggregates having a ratio greater than 1 (Figure 4.3A). Single cell events were then assayed for focus fidelity. Only cells in sharp focus were utilized for further analysis to ensure adequate marker and morphology assessment (Figure 4.3B). Even after the data was interrogated in this fashion over 8% of peritoneal cells were definitively positive for both Gr1 and CD11b surface marker expression (Figure 4.3C). Multi-spectral imaging flow analysis was also able to identify and quantify the cells with ring shaped nuclei, another marker for myeloid MDSCs. Leukocyte nuclei were identified by PI staining; two “masks” were then created, one that identified positive PI staining intracellular regions, and a second mask that identified dark non-stained regions within the first mask. The combination of these two masks allowed for the positive discrimination of ring shaped nuclei (Figure 4.3D).

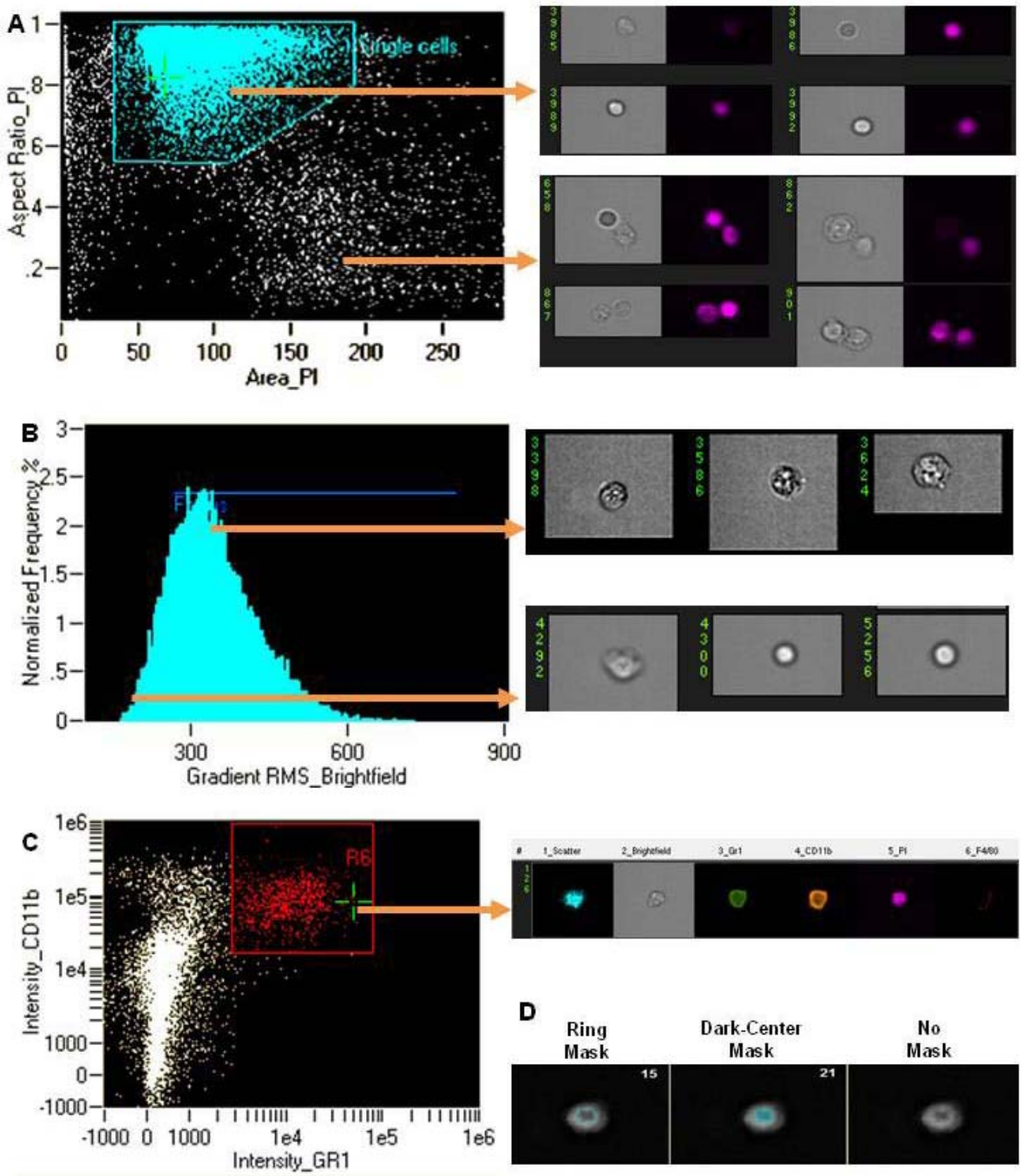


Figure 4.3 Multispectral imaging flow analysis. (A) ImageStream was able to discriminate cell aggregates from individual cells, (B) and analyze only the images in crisp focus. (C) Using this high level of discrimination a robust $Gr1^{+}CD11b^{+}$ double positive population was still identified in MPLA primed animals. (D) Multispectral analysis could also discern ring shaped nuclei.

The ImageStream analysis demonstrated that in mice treated with vehicle alone, $Gr1^+CD11b^+$ cells account for less than 1% of peritoneal leukocytes, after infection this population increased to less than 2%. MPLA treatment alone increased the peritoneal $Gr1^+CD11b^+$ population to almost 9%, and this expanded after infection to nearly 20% of peritoneal leukocytes. While the largest total number of cells with ring shaped nuclei were recovered from MPLA primed mice after infection, the percentage of $Gr1^+CD11b^+$ cells with this nuclear morphology was not significantly different between the treatment groups. This indicates that while there may be a correlation, nuclear morphology may be an inadequate identification criterion for MDSCs, probably resulting from MDSC population heterogeneity.

Myeloid cells recruited by MPLA exhibit both mature and immature morphologies

To Further identify the myeloid cell types recruited by MPLA, peritoneal leukocytes from MPLA-primed and vehicle-primed mice were separated by positive selection of the surface marker Ly6G (a subset marker of Gr1) using a miltenyi Biotec MACs column. Both the positive and negative selection populations were fixed onto slides by centrifugation and stained with Rapi-Diff hematological / cytological staining solution according to the manufactures instructions. Cells were qualitatively assessed by standard bright-field microscopy. Ly6G- cells from vehicle-primed mice had morphologies consistent with resident monocytes and lymphocytes, as did the Ly6G- cells from MPLA primed mice (Figure 4.4). The monocytic cells in MPLA primed mice tended to be slightly larger and more vacuolated. The Ly6G+ subset from vehicle-primed mice exhibited similar morphologies as the Ly6G- population except for a greater number of cells with granulocytic morphology. The Ly6G+ subset of cells from MPLA-primed mice has a large number of cells with polymorphonuclear (PMN) appearance, as well as

many with ring shaped nuclei. This is consistent with previous reports describing MDSCs as a heterogeneous myeloid population with an enriched number of cells containing ring shaped nuclei. Comparison images from peritoneal leukocytes recovered post-infection are omitted due to the large amount of debris and increased cell friability in samples from unprimed mice preventing adequate interpretability.

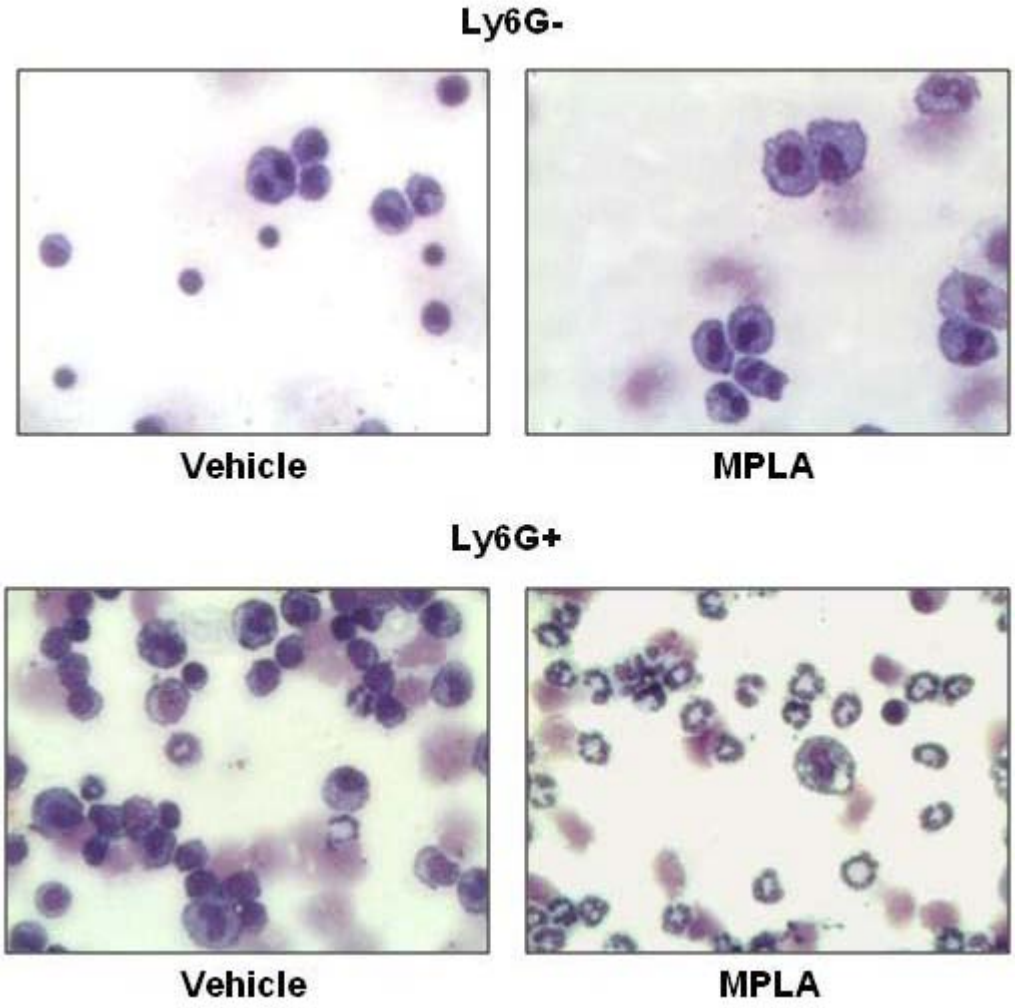


Figure 4.4 MPLA priming altered morphological composition of peritoneal myeloid cells. Ly6G- cells from MPLA primed and un-primed mice exhibited monocytic and lymphocytic morphologies, with only subtle differences in MPLA primed monocytes. Ly6G+ cells from MPLA primed mice were more neutrophil like with many cells exhibiting ring shaped nuclei. Representative bright-field views at 40X.

MDSCs were phagocytic and their expansion in response to MPLA was not age dependent

Since age greatly impacts immune function and the ability to mount an effective immune response, the generation of MPLA-elicited MDSCs was assessed in aged mice. This is of significant interest because such a large proportion of bone marrow cells are $Gr1^+CD11b^+$ cells (see figure 4.2) and bone-marrow derived leukocytes are known to decline with age.[280] Aged mice (14-16 month old) were primed with MPLA or treated with vehicle alone as previously described. Mice were then infected intraperitoneally with 10^9 CFUs FITC labeled, heat-killed *P. aeruginosa*. Peritoneal cells recovered at 3 hours after infection were assayed for Gr1, CD11b expression and uptake of bacteria by flow cytometry. Less than 1% of peritoneal cells recovered from vehicle treated and uninfected mice were $Gr1^+CD11b^+$ cells (figure 4.5). After infection this population

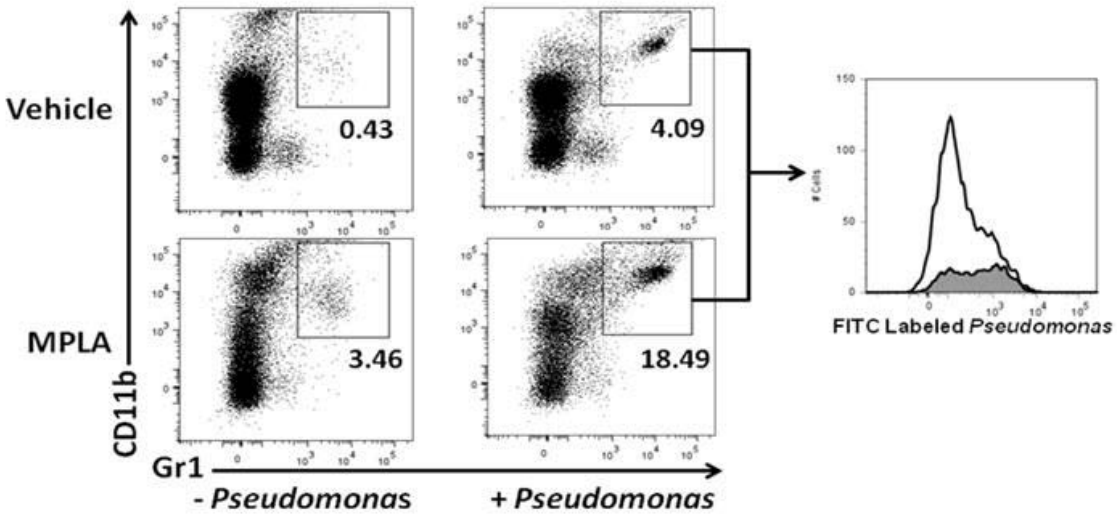


Figure 4.5 MPLA mediated $Gr1^+CD11b^+$ recruitment and phagocytosis in aged mice. Aged mice treated with MPLA had a greater percentage of Gr1+, CD11b+ double positive myeloid cells (3.46%) within the peritoneum compared to controls (0.43%). The double positive population increased with infection in untreated animals to ~4% of peritoneal leukocytes. This population increased to ~18% of peritoneal leukocytes in MPLA treated infected mice. The Gr1+, CD11b+ cells from infected controls and MPLA treated infected mice expressed similar levels of phagocytosis as measured by uptake of FITC labeled heat-killed *P. aeruginosa*.

expanded to around 4% of peritoneal cells, less than in young adult mice, as reported previously. In MPLA-primed mice, $Gr1^{+}CD11b^{+}$ cells accounted for less than 4% of peritoneal leukocytes, much less than in young adult mice that were primed with MPLA. After infection the $Gr1^{+}$, $CD11b^{+}$ double positive population expanded to over 18% of peritoneal cells in MPLA-primed mice, almost the same proportion as seen in young MPLA-primed mice after infection. While the FITC uptake levels were comparable between MPLA-primed and vehicle primed mice, there was a larger $Gr1^{+}$ $CD11b^{+}$ double positive phagocytic population in MPLA treated mice.

MPLA expanded myeloid cells were capable of suppressing T-cell proliferation ex-vivo

The hallmark activity associated with MDSCs is their ability to suppress T-cell proliferation and activation. To assay the suppressive ability of the myeloid cells elicited by MPLA, peritoneal myeloid cells were co-cultured with OT II T-cells and antigen specific T cell proliferation was assessed. Peritoneal leukocytes were harvested after MPLA priming and separated based upon Ly6G surface marker expression. Both the positive and negative fractions were then incubated with mitomycin C and co-cultured at various ratios with CD4 T cells isolated from OT-II mice, that are responsive to ovalbumin. These co-cultures were incubated for up to 4 days in the presence of OVA323-356 peptide, after which time T cell proliferation was assessed using a modified MTT assay. CD4 T cells cultured without myeloid cells proliferated in response to ovalbumin during the 4 day incubation period. However, this replication was reduced by nearly half when T cells were co-cultured with $Ly6G^{+}$ cells from MPLA-primed mice at a 1:1 ratio (Figure 4.6). Conversely co-culture with the $Ly6G^{-}$ fraction at a 1:1 ratio caused T cell proliferation to nearly double. When T cells were cultured with either $Ly6G^{+}$ or $Ly6G^{-}$ cells from MPLA-treated mice at a ratio greater than 1 (T cell): 2 (MPLA elicited

peritoneal cell) proliferation was reduced. This may have resulted from medium depletion or pollution by the high cell count per well during the 4 day incubation. It appears that MPLA-primed $Ly6G^+$ cells are capable of suppressing T-cell proliferation. The $Gr1^+$, $CD11b^+$ MDSCs would fall within this $Ly6G^+$ population.

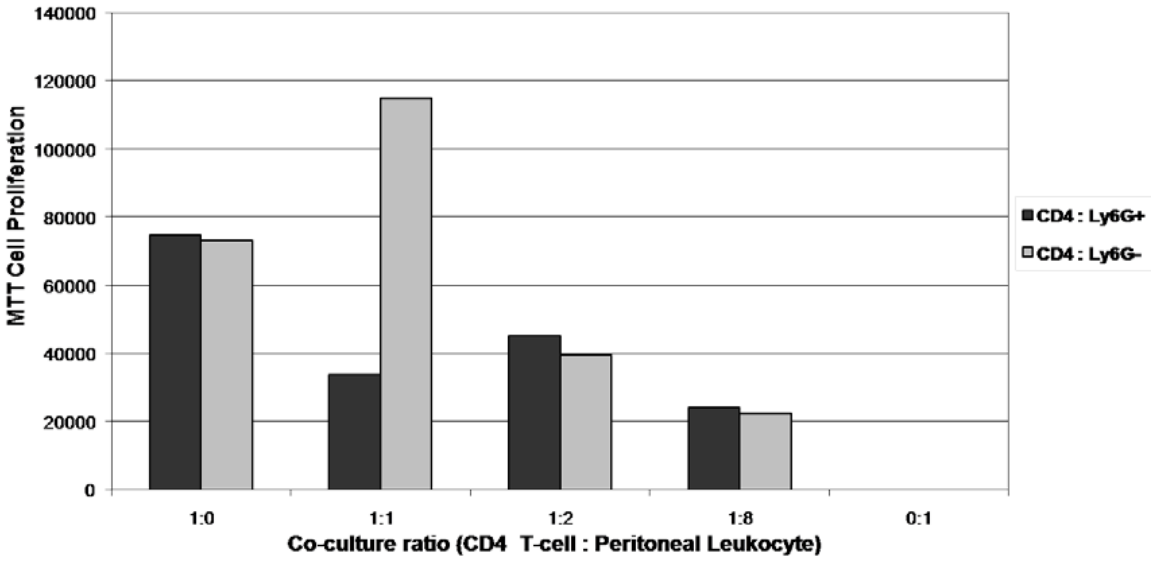


Figure 4.6 MPLA elicited Ly6G+ leukocytes suppressed T-cell proliferation ex-vivo. At a ratio of 1:1 Ly6G+ cells from MPLA primed mice suppressed T-cell proliferation, however Ly6G- cell enhanced T-cell proliferation. At higher concentrations both Ly6G+ and – fractions were suppressive.

Discussion

MDSCs have been demonstrated to possess significant regulatory capacity in a variety of models, both with beneficial and deleterious effects. Studies presented in Chapter 3 of this dissertation show that MPLA priming is associated with a significant increase in an immature myeloid cell population that expresses surface markers traditionally associated with monocytes and macrophages (F4/80), concurrent with the granulocyte marker Ly6G. When this population has been back gated to forward and side scatter plots during flow cytometry analysis, they fall within a broad region, indicating that this is a mixed myeloid population of varying activation and maturation

states. These finding are consistent with descriptions of MDSCs outlined in the introduction of this chapter. MDSCs may play a role in the immuno-modulated state elicited by MPLA, and the findings described in this chapter support that hypothesis.

MPLA priming increased the number of cells within the peritoneum bearing the most common markers used in identifying MDSCs, Gr1 and CD11b. While neither is specific to a given cell type, their co-expression within the peritoneum is uncommon in vehicle-primed animals. Infection further expanded this population in MPLA- and vehicle-primed mice. The increase in $Gr1^{+}CD11b^{+}$ cells in vehicle-primed mice after infection supports the findings by Delano and colleagues that this population can expand in models of sepsis. However, their expansion is even more pronounced in MPLA- primed mice after infection, that are more resistant to sepsis than vehicle-primed and infected mice. If $Gr1^{+}CD11b^{+}$ MDSCs were purely deleterious, one would expect their increased recruitment in MPLA-primed mice to correlate with increased immuno-suppression and increased morbidity and mortality. Thus the presence of this population may play an important regulatory role in the native response to infection. However, in vehicle-primed mice this recruitment may be inadequate to curtail the massive inflammatory response elicited by a major infection or insult.

The reliability of flow cytometry results can be a major concern when dealing with atypical populations of mixed composition. The incorporation of multispectral imaging flow cytometry analysis lends greater reliability to the results of this study and the multi-staining results described in chapter 3. It has long been known that cell aggregates are often confused with large cells or double positive staining; this becomes even more problematic after infection when cells become larger, more adhesive, and cell-cell associations are common.[281] The discrimination afforded by MSIFS allows for definite identification of single cell events, and even morphological

characterization. This allows confident assertion that MPLA increased the $GR1^{+}CD11b^{+}$ double positive myeloid population and causes even further expansion after infection.

Due to the highly heterogeneous nature of the immature myeloid population recruited by MPLA and infection, standard flow cytometric analysis is at times inadequate for fully characterizing specific cell populations. For example, MDSCs have been shown to exhibit both monocytic and granulocytic morphologies, leading to un-interpretable results when back gating double-positive populations to forward and side scatter plots. Instead of falling with the normal regions associated with monocytes or PMNs, immature myeloid cells spread over a broad swath of the forward and side scatter bi-plots. Another concern is that leukocyte populations which stain positive for multiple surface markers and lack definitive forward scatter / side scatter patterns may be the result of cellular aggregates or debris. This is especially true with large vacuolated monocytes-like cells.

The ability of $Ly6G^{+}$ cells from MPLA primed mice to suppress T-cell proliferation further supports the assertion that MPLA priming increases the recruitment of MDSCs. However this should be tempered until further investigation is possible. The $Gr1^{+}CD11b^{+}$ expression levels within this population were not assayed since the labeling with Ly6G for separation would theoretically interfere with Gr1 binding because both of these antibodies recognize a shared epitope. In addition, the suppressive capabilities of $Ly6G^{+}$ cells from vehicle-primed mice is unknown and difficult to assess because there is such a small $Ly6G^{+}$ population in the peritoneum of vehicle-primed mice. Agents used to elicit the $Ly6G^{+}$ cell recruitment in the absence of MPLA priming could inadvertently affect the activation state of these cells. It would be desirable to deplete the MDSC population to definitively assess its role in MPLA mediated immuno-modulation. Unfortunately, because of the heterogeneous nature of this cell population and the shared

surface markers with other cell types an effective and specific depletion method has yet to be developed.

Chapter 5. The Role of TLR4 Signaling in Immuno-Modulation

Introduction

As illustrated in chapter 1, the signaling cascade and regulatory mechanisms implicated in tolerance induction with LPS are numerous and complex. The mechanistic understanding becomes even more uncertain with the development of alternative TLR4 agonist molecules such as MPLA. Conflicting information exists in the literature about the most basic aspects of signaling pathways involved in MPLA immuno-modulation. TLR4 has long been held as the receptor for LPS and MPLA. However, some reports in the literature have provided results indicating the TLR4 may not be necessary for all the effects attributed to LPS. A recent report by has shown that the activation of some inflammatory gene by LPS can occur in TLR4-deficient dendritic cells.[282] Some species of bacteria have been shown to produce heterogeneous LPS macromolecules capable of activating both TLR2 and TLR4.[283] As described earlier, MPLA has been shown to activate NF-kB through TLR2 under some conditions.[224] The priming effect of MPLA has even been shown to occur in the absence of TLR4 and CD14.[225] While concerns about agonist purity have been raised regarding the results of some of these studies they do cast doubt on the dogma that LPS and its derivatives mediate their immuno-modulatory effect through TLR4 alone. To address this concern we have studied the effects of immuno-modulation in TLR4-deficient mice.

MPLA has been proposed to signal though the MyD88-independent, or TRIF-dependent arm of the TLR4 signaling complex.[203] Because there is significant cross talk between these two cascades definitive discrimination as to which arm is activated by assessing downstream mediator activity or production the of associated pathway products

is insufficient. In the studies outlined in chapter 3 it was demonstrated that while MPLA decreased the production of many pro-inflammatory cytokines, select agents associated with the TRIF pathway (such as G-CSF, RANTES, and MCP-1) were still produced in high levels after infection or CLP (see table 3.1). These results indicate that there might be preservation of the TRIF pathway in re-stimulated, MPLA-primed animals. This point towards selective inhibition of the MyD88 pathway in MPLA primed mice. To further clarify these findings the immuno-modulatory effect of MPLA has been assessed in Trif-deficient mice.

Results

TLR4 was required for MPLA immuno-modulation.

To assess the role of TLR4 in MPLA-mediated immuno-modulation C3H/HeJ mice, that are deficient in TLR4, were primed with MPLA or vehicle as previously described before undergoing CLP. C3H/HeOUJ mice were used as controls in these experiments because this is the background strain for C3H/HeJ mice. MPLA priming did not protect C3H/HeJ mice from developing hypothermia 16 hours after CLP (Figure 5.1A), MPLA was protective in C3H/HeOUJ mice. MPLA pre-treatment also did not increase bacterial clearance in the peritoneum, or prevent the spread to peripheral blood in TLR4-deficient mice (Figure 5.1B), once again C3H/HeOUJ mice with proficient TLR4 were protected. Similar results were seen in C57BL/ScNJ mice that are also deficient in TLR4 (data not shown). Based upon these studies TLR4 is required for MPLA-mediated protection from CLP induced sepsis.

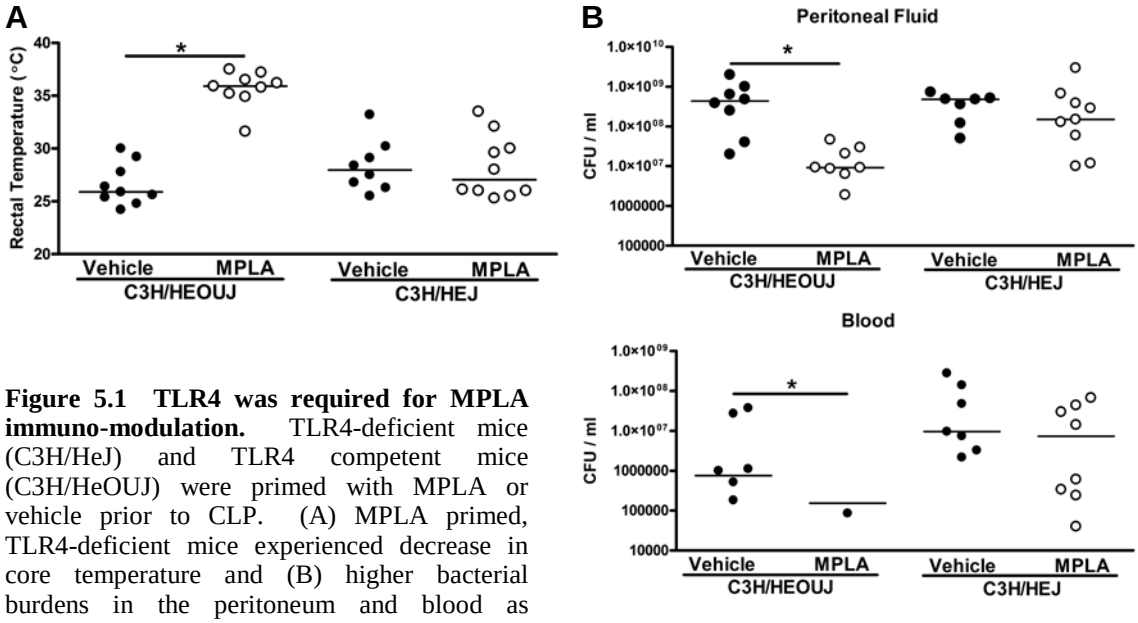


Figure 5.1 TLR4 was required for MPLA immuno-modulation. TLR4-deficient mice (C3H/HeJ) and TLR4 competent mice (C3H/HeOUJ) were primed with MPLA or vehicle prior to CLP. (A) MPLA primed, TLR4-deficient mice experienced decrease in core temperature and (B) higher bacterial burdens in the peritoneum and blood as unprimed mice. *p<0.05, n=7-10 mice per group.

Ticam1 (TRIF) is not necessary for MPLA immuno-modulation.

C57BL/6J-Ticam1^{Lps2} / J mice, which are deficient in TRIF, were treated with MPLA or vehicle alone prior to CLP. Deficiency in TRIF did not abolish the preservation in core temperature after CLP associated with MPLA immuno-modulation (Figure 5.2A). In addition, MPLA pretreatment of TRIF-deficient mice significantly reduced the bacterial burden in peripheral blood compared to un-primed Trif-deficient mice; but did not affect the bacterial burden within the peritoneum (Figure 5.2B). These results indicate that TRIF may not be required for MPLA mediated immuno-modulation to confer protection in the murine CLP sepsis model. However the enhanced bacterial clearance normally elicited by MPLA pre-treatment was attenuated to a certain degree in

Trif-deficient mice. Preliminary experiments in MyD88 deficient mice have generated conflicting results, further experiments are required.

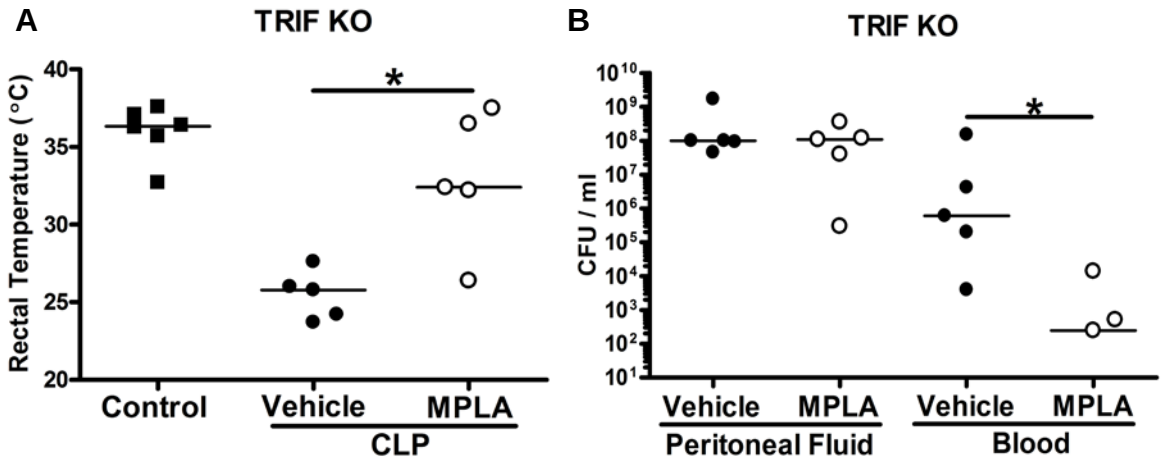


Figure 5.2 Trif-deficient mice were partially responsive to MPLA immuno-modulation. Trif-deficient mice were primed with MPLA or vehicle alone then underwent CLP. (A) MPLA-primed Trif-deficient mice had significantly higher core temperatures compared to unprimed Trif-deficient mice after CLP. (B) MPLA-primed and unprimed Trif-deficient mice had equivalent peritoneal bacterial burdens post CLP, however the significantly fewer bacteria were recovered from peripheral blood in MPLA primed Trif-deficient mice compared to un-primed Trif-deficient mice. *$p<0.05$, n=5 mice per group.

Cytokine / chemokine production in TLR4- and Trif-deficient mice after MPLA and CLP

A major effector of innate immune system activation is the production of cytokines and chemokines. As shown earlier, MPLA treatment of wild type mice prevents the massive increase of inflammatory cytokines after infection, which has been linked with much of the dysfunction in sepsis. However the preserved production of some factors such as G-CSF, MCP-1, and RANTES indicate that immuno-modulation does not elicit a state of complete anergy, but a refined response to insult. To clarify the signaling pathways responsible for this alteration in cytokine production in immuno-modulated animals, cytokines were assessed in TLR4- and Trif-deficient mice primed with MPLA or vehicle alone after CLP.

Plasma levels of IL-1β were equal in MPLA- primed and vehicle-primed TLR4-deficient mice after CLP; however their control strain C3H/HeOUJ also exhibited unaltered IL1-1β production after CLP in MPLA-primed mice (Figure 5.3). TRIF-deficient mice were tolerized by MPLA as seen be reduced production of IL-1β after CLP. Production of IL-6 was also unaltered by MPLA priming in TLR4-deficient mice after CLP, but Il-6 production was attenuated in C3H/HeOUJ mice treated with MPLA. TRIF-deficient mice primed with MPLA produced significantly less IL-6 after CLP when compared to vehicle-primed Trif-deficient mice. The pro-inflammatory cytokine TNF-α was surprisingly low in all C3H strain mice after CLP, irrespective of MPLA pre-treatment. Trif-deficient mice primed with MPLA had significantly lower levels of plasma TNF-α after CLP as compared to un-primed mice. IFN-γ production was un-affected by MPLA priming in all C3h strain mice (both TLR4-deficient and proficient), as well as Trif-deficient mice after CLP. Plasma levels of IL-12 (p70) were unaltered by MPLA priming in TLR4-deficient mice after CLP, but were reduced in C3H/HeOUJ mice by MPLA priming.

MPLA-primed Trif-deficient mice exhibited reduced levels of IL-12 (p70) in circulation as compared to unprimed controls, but this was below the level of statistical significance. IL-10 production was unaltered by MPLA priming in TLR4-deficient mice; however vehicle-primed TLR4-deficient mice produced significantly lower levels compared to their control strain after CLP, indicating TLR4 may be required for normal activation of IL-10 production. MPLA pre-treatment reduced the production of IL-10 after CLP in Trif-deficient mice as well as in wild-type C3H/HeOUJ mice, however this was below the level of statistical significance. G-CSF production was equivalent in MPLA primed and unprimed mice regardless of genetic background or signaling proficiency. A similar response was also seen regarding the production of GM-CSF.

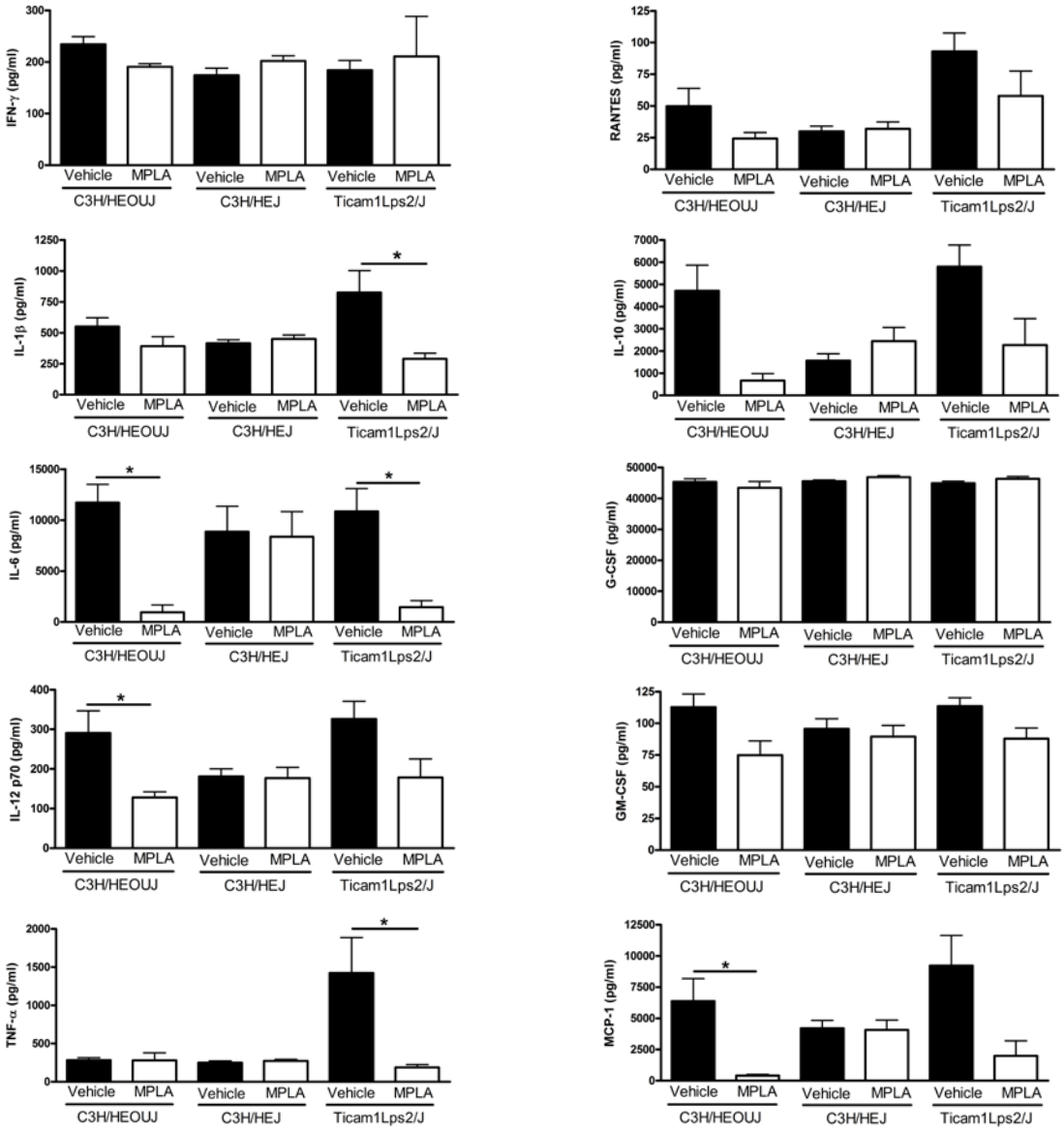


Figure 5.3 Cytokine / chemokine production in TLR4- and Trif-deficient mice in response to MPLA priming and CLP. TLR4-deficinet (C3H/HEJ) and Trif-deficient (Ticam1Lps2/J) mice were treated with MPLA or vehicle prior to CLP. 16 hours post CLP blood was collected and plasma analyzed for Cytokine / Chemokine production by Bio-Plex. *$p<0.05$, n=5=7 mice per group.

MCP-1 production in response to CLP was un-altered in TLR4-deficient mice by MPLA pre-treatment, but was significantly lower in the C3H/HeOUJ control strain after MPLA.

Primed Trif-deficient mice produced less MCP-1 after CLP compared to un-primed mice, however this was not statistically significant.

Plasma levels of RANTES were low in TLR4-deficient mice after CLP with or without MPLA pre-treatment. The control strain C3H/HeOUJ had higher levels in un-primed mice after CLP, but this difference was not statistically significant. Trif-deficient mice produced less RANTES in response to CLP after MPLA pre-treatment however this difference was also not statistically significant. These results indicate that TLR4 is needed for MPLA priming to prevent the production of cytokines such as IL-6, but also TLR4 deficiency alone attenuates the production of IL-12, and IL-10. TRIF deficiency did not alter the production of IL-1, IL-6, IL-10, and TNF or prevent MPLA mediated attenuation in their production. MPLA attenuation of MCP-1 production was more significant in Trif-deficient mice compared to wild-type C57BL/6J mice.

TRIF-dependent factors (IFN-β and CXCL10) were not down regulated by MPLA priming

To further investigate whether the TRIF signaling pathway activation is preserved in MPLA primed mice, CXCL10 and IFN-β (two soluble immune mediators produced in response to TRIF signaling) were measured by ELISA in plasma from MPLA-primed and vehicle-primed wild-type C57BL/6J mice before and after CLP.[284, 285] In MPLA primed and unprimed mice, very little of each factor was detectable in plasma prior to insult (Figure 5.4). 16 hours after CLP Production of both IFN-β and CXCL10 was increased without any statistical difference between MPLA primed and vehicle primed mice (Figure 5.4). The total amount of IFN-β detected was very small, possibly reflecting a more auto- or paracrine-effect.[286]

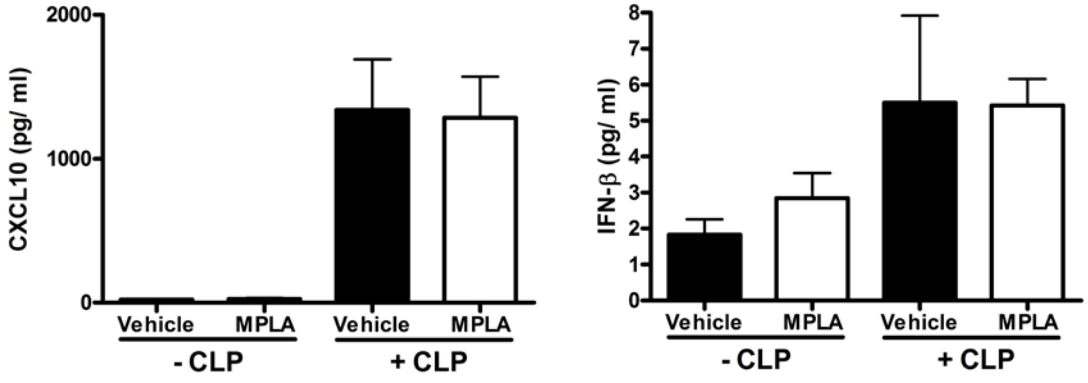


Figure 5.4 TRIF-dependent factors were not suppressed by MPLA priming before CLP. Wild-type mice were treated with MPLA or vehicle prior to CLP. 16 hours post CLP plasma levels of CXCL10 and IFN-β were measured by ELISA. Prior to CLP there were very low expression levels of both factors. After CLP CXCL10 and IFN-β levels increased to equivalent levels in both MPLA primed and unprimed animals. Total IFN-β levels remained relatively low level after CLP in both groups. n=5 mice per group

Differential induction of CXCL10 and IL-6 production by MPLA in TRIF and MyD88 bone marrow-derived macrophages.

Bone marrow cells from wild-type (C57BL/6J), Trif-deficient (C57BL/6J-Ticam1^{Lps2}/J), and MyD88-deficient mice (C57BL/6J-*Myd88*^{poc}/Mmcd) were cultured over night, after which time non-adherent cells were removed. The resultant adherent bone marrow-derived monocytes were incubated overnight in medium containing MPLA (1 μg/ml), or medium alone. Culture supernatant was assayed for CXCL10 and IL-6 by ELISA. MPLA induced a small increase in IL-6 production (less than 50 pg/ml) in wild-type mice; however this response was attenuated in Trif-deficient cells (Figure 5.5A). MyD88-deficient cells produced the least IL-6 in response to MPLA pre-treatment and background levels were undetectable. CXCL10 production was significantly increased by exposure to MPLA in wild type cells, reaching an average concentration of > 800 pg/ml (Figure 5.5B). The induction of CXCL10 was attenuated in MyD88 deficient cells, and reduced even further in Trif-deficient cells. These results support the current

hypothesis that MPLA signals primarily through the TRIF-dependent pathway, but also may activate the MyD88-dependent pathway, or results in substantial cross-communication between the two arms of TLR4 mediated signaling.

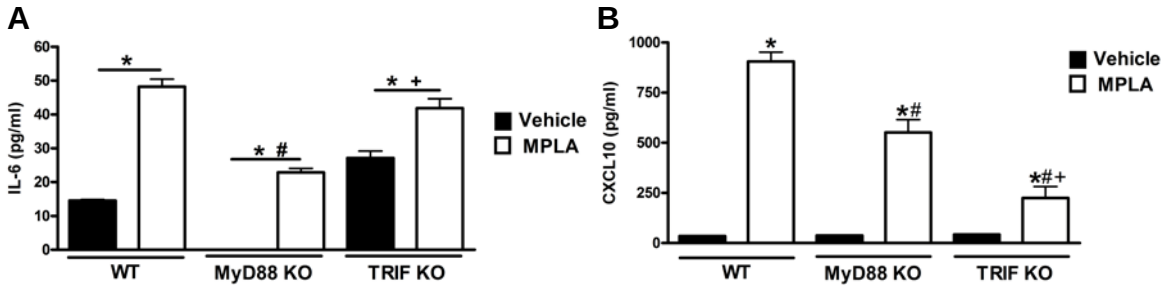


Figure 5.5 Differential induction of CXCL10 and IL-6 production by MPLA in TRIF- and MyD88-deficient bone marrow-derived macrophages. Adherent bone marrow derived macrophages were cultured with or without MPLA overnight. The level of IL-6 and CXCL10 was measured in culture media by ELISA. (A) MPLA exposure modestly increased the production of IL-6 in wild-type (WT), this effect was reduced in Trif-deficient and further reduced in MyD88-deficient cells. (B) CXCL10 production was markedly increased by exposure to MPLA and this effect was attenuated in MyD88 deficient cells, and further reduced in Trif-deficient cells. *$p<0.05$ compared to untreated controls within genotype, # $p<0.05$ compared to MPLA treated WT cells, + $p<0.05$ compared to MPLA treated MyD88 deficient cells, n= 5 samples per group.

Discussion

Immuno-modulation conferred by MPLA is capable of reducing inflammation, enhancing bacterial clearance, and conferring a survival benefit when administered prophylactically in experimental models of sepsis. The exact signaling events required to induce immuno modulation with MPLA has yet to be fully clarified. TLR4 is believed to be the primary receptor for MPLA. However, some reports demonstrate tolerance induction in TLR4 and CD14 deficient animals. In the results of this study show that MPLA-induced immuno-modulation is abolished in TLR4-deficient mice. Mata-Haro and colleagues demonstrated that MPLA signals through TLR4, predominantly via the MyD88- independent (TRIF-dependent) pathway.[226] This finding is supported by the

ability of MPLA to induce high levels of the Trif-dependent chemokine CXCL10 but relatively low production of IL-6, which is primarily MyD88-dependent. Furthermore, MPLA priming appears to selectively decrease MyD88-dependent signaling and leave the Trif-dependent signaling pathway intact. This is supported by our observation that sepsis-induced production of MyD88-dependent cytokines is largely down-regulated in MPLA-primed mice whereas production of cytokines and chemokines that are produced primarily through Trif-dependent signaling remain intact. However, MPLA priming still conferred some benefit to Trif-deficient mice, indicating that immuno-modulation is not solely dependent upon the TRIF signaling pathway. Inflammatory cytokine production was still attenuated in Trif-deficient mice, and even some factors described as TRIF dependent factors such as G-CSF and MCP-1 were still produced post CLP in Trif-deficient mice. Further confounding the ability to discern signaling events involved in tolerance induction is the fact that some cytokine responses appear to be strain specific; with C3H/HeOUJ mice producing certain cytokines at much lower levels compared to previous results with C57BL/6J mice.

Chapter 6: Summary and Conclusions

The illustration presented at the opening of this text is entitled “Morbetto ”; it was originally created by Raphael and depicts a small plague (morbetto) which befell the inhabitants of Crete before the founding of Rome. Infectious disease has been a major factor in sculpting human history throughout our evolutionary journey. It has determined the fate of civilizations and has been the selective pressure shaping our current genetic make-up. Despite this long-standing relationship, we have been ill-equipped to treat major infections until the middle of last century. The inroads made by the development of modern antibiotics have been rapidly eroded by their over-prescription and misguided use, fueling the evolution of multi-drug resistant pathogens. Despite uninterrupted research in the field of critical care, few advances have been made in the treatment of severe infections or sepsis. While activated protein C has been shown to be of benefit in cases of sepsis and septic shock, this advance in treatment addresses a symptom of the immune dysregulation, not the aberrant immune response itself.

Immuno-modulation is an attractive therapeutic approach for treating sepsis since much of the pathology is the result of immunological dysregulation. Numerous microbial products have immunomodulatory properties. Among them, LPS induces an inflammatory response that renders it too toxic to safely use in humans. However MPLA has already received regulatory approval for use as a vaccine adjuvant, a testament to its safety and immunogenicity. Herein, I have demonstrated that MPLA is capable of eliciting an immuno-modulated state in mice, conferring protection against a variety of septic insults, both acute and prolonged. This immuno-modulation is distinct from the immuno-paralysis associated with major infection or trauma in-that important immune functions such as bacterial clearance are enhanced. In contrast to the traditional

interpretation of immuno-paralysis, the immuno-modulated state elicited by MPLA is not associated with broad-scale suppression of soluble immune mediators, but the selective inhibition of pro-inflammatory cytokines and the preservation of a number TRIF-associated factors. The ability of MPLA to enhance bacterial clearance appears to be dependent upon a mixed myeloid population, most importantly granulocytic cells. However a rather heterogeneous population of cells are recruited and mediate the immuno-enhanced state conferred by MPLA; possibly even including MDSCs; which may explain, in part, the reduced inflammation seen in primed mice. The myeloid cells recruited in MPLA-treated mice do not seem to exhibit enhanced antimicrobial functions on a per cell basis, rather the sheer increase in numbers appears to be responsible for the enhanced bacterial clearance. Surprisingly, this large aggregation of immune cells at a sight of major infection does not elicit large-scale systemic cytokine production as one would expect, indicating that MPLA must reprogram myeloid cell responsiveness to infection.

The heterogeneity of cells implicated in MPLA immuno-modulation emphasizes the importance of studying such phenomena in vivo. An intact immune system is most likely critical for the complete induction of MPLA-mediated immuno-modulation given the number of cell types recruited, soluble factors elaborated, and organ systems impacted by MPLA priming. Many previous studies of MPLA- and LPS-induced immuno-modulation have been performed *ex vivo* or with immune cell lines, but this is inadequate to replicate the complex interactions which occur during MPLA treatment and infection in vivo.

MPLA priming is capable of eliciting a tolerized state, whether it is administered by the intraperitoneal or intravenous route, indicating that the priming effect is systemic and not localized to the site of administration. This is further supported by the expansion

of immature myeloid cells in the bone marrow of MPLA-primed animals. These findings are important for clinical applicability of immuno-modulating agents, since administration of a therapeutic agent at the site of infection is often impractical, and the greatest need is for novel agents to treat systemic infections. However, any positive benefits seen in murine sepsis models should be tempered by skepticism about applicability to the clinical arena. Anti-TNF therapy proved very promising in pre-clinical trials but failed to exhibit a clear benefit in clinical trials.[287] This can be attributed to a number of factors, including the genetic heterogeneity of the patient population in the clinical trials, timing of therapy initiation, and additional underlying pathologies.

A major confounding factor hindering the translation of pre-clinical discoveries into future therapeutics for sepsis it the distinct immunological differences between mice and humans. Mice, much like cats, are highly resistant to LPS, with the minimum inflammatory dose of LPS being about 250-50,000 times higher in mice than in humans.[205] Surprisingly, rabbit and horse sensitivity to endotoxin is very close to that of humans. The exact mechanism responsible for this variation in sensitivity has not been discovered, but it was recently shown that TLR4 signaling pathway regulation can be mediated by siRNAs, and significant differences exist in the expression of these regulatory siRNAs between humans and mice (data presented at a Keystone innate immunity research conference). The differences in TLR4 signaling and LPS sensitivity should give pause to applying findings in murine innate immunity research to human subjects.

To address the concerns about applicability of MPLA immuno-modulation to clinical sepsis, we conducted a pilot study using a sheep pneumonia model. This afforded for instrumentation and serial monitoring of clinically relevant outcomes over

the course of an infection. In that experiment, MPLA did not confer protection against pneumonia and the pre-treated animal actually died earlier than the control. Roughly 6 hours before its demise, the sheep began to run a fever and exhibited physiological abnormalities associated with septic shock. The sheep's heart rate increased to over 120 BPM, its central venous pressure declined to 2 mmHg, and its Mean Arteriole Pressure declined to 79 mmHg. Concurrently its PaO_2 fell to 78 mmHg, while its respiratory rate increased to over 30 breaths per minute. This animal exhibited signs consistent with high output cardiac failure and pulmonary congestion. Over the course of its infection, the sheep experienced a bi-modal peripheral leukocyte response, initially there was an increase in total WBCs and neutrophils followed by a decline to levels below baseline right before death. After its passing, tissue samples were homogenized and bacterial burden was assessed (by comparison to an un-treated with MPLA prior to pneumonia). Interestingly the primed sheep actually had lower numbers of bacteria at the primary sight of infection in the lungs, as well as the liver. However, bacterial burden in the spleen was higher in the MPLA primed animal (Figure 6.1). These results are reminiscent of what was seen when MPLA was administered post-CLP. While inflammation and the pathological response to infection were not ablated by MPLA, it appears that bacterial clearance may have been improved. This study is lacking in that MPLA was administered as a single dose less than 24 hours before induction of pneumonia, and a significant instillation of bacteria was used to induce infection. This was a supra-physiological dose of bacteria, and does not model the natural occurrence of pulmonary pneumonia, which tends to be insidious and develop over time. The results of this trial are interesting and warrant further investigation.

While the results of the sheep study were admittedly disappointing, they do not abrogate the added understanding of MPLA, TLR4 signaling, and mechanisms involved

in immuno-modulation afforded by the work presented in this dissertation. These final findings merely highlight the complexity and challenges inherent to the field of sepsis; and act as a reminder that critical care researchers should proceed cautiously when attempting to augment the immune response to serious infections.

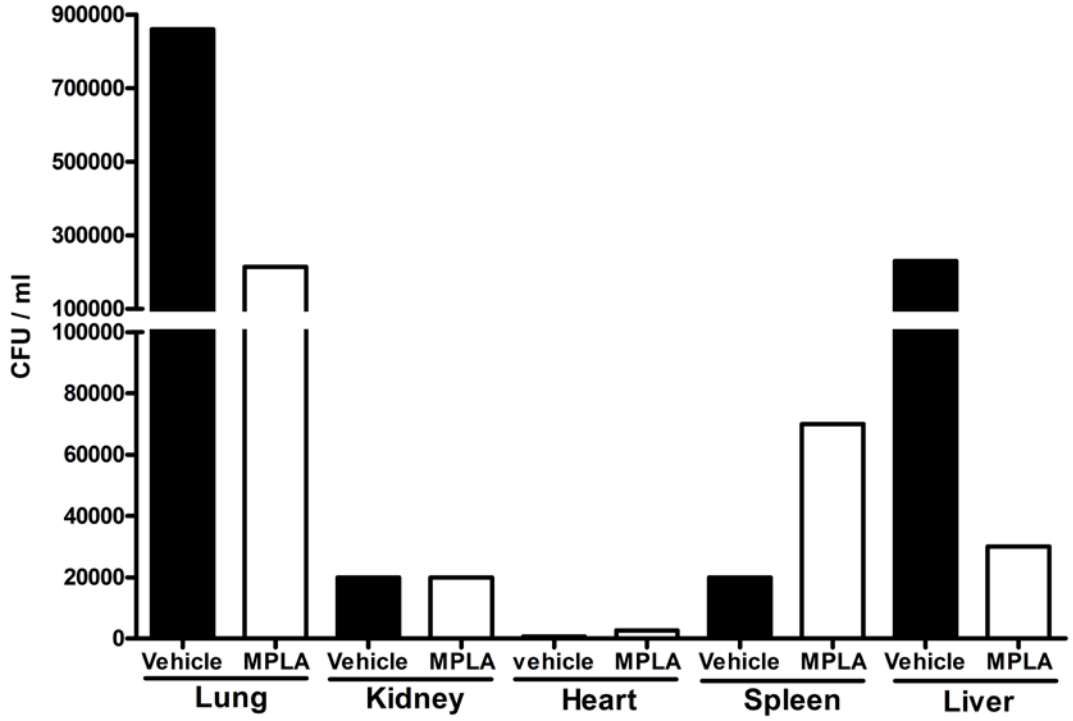


Figure 6.1 Bacterial burden in MPLA-primed and un-primed sheep after pneumonia. Data represents a single animal per group.

Future Directions

Some future directions for this project include assessing the effect of MPLA priming in MyD88-deficient mice, and performing flow cytometric analysis on the leukocyte populations recruited in TLR4-, TRIF-, and MyD88-deficient mice. Leukocyte analysis would also be beneficial in the post-infection model of MPLA priming. It would also be valuable to investigate how long post-challenge can MPLA be administered in a less severe model of CLP and still confer some benefit. Adoptive

transfer of myeloid cells from MPLA-primed mice to naïve mice prior to insult may provide greater understanding of which leukocyte population are necessary for the immuno-modulated state, or even if it is an acellular soluble factor which is responsible for MPLA induced immuno-modulation.

Given the large number of potential intracellular regulator mechanisms which may be responsible for tolerance induction by MPLA, it would be beneficial to perform a broad cDNA array to assess which genes are significantly up and down regulated in MPLA-primed mice. Unfortunately this approach becomes rapidly time and cost prohibitive because of the variety of cell types involved in MPLA immuno-modulation. Also, to fully discern the impact of MPLA at the transcription level it would be necessary to perform such array analysis at multiple time points in order to capture the events responsible for the effect of MPLA. The results from such studies could provide guidance for further investigation utilizing *in-vivo* siRNA to squelch the expression of genes that correlate closely with MPLA immuno-modulation.

The history of gene array utilization in biomedical research has yielded mixed results, often resulting in a signal to noise ratio which precludes definitive interpretation. This is also exacerbated by the high post transcriptional modification, and translation control which regulates quantity and activity of the functional end product. For this reason western blot analysis of select TLR4 signaling components should be conducted to determine if MPLA up or down regulates the production of MyD88, or TRIF cascade proteins. Phospho-protein analysis in addition to total protein determination by western blot would provide important insights into the effect of MPLA on TLR4 signaling cascade reprogramming. Once again this is a challenging strategy given the heterogeneous leukocyte population involved in tolerance induction, numerous potential targets, and various time points required to properly assess changes in signaling

events. TLR4 signaling pathway regulation is also mediated by ubiquitination and splice variant production, which would not necessarily be detectable by standard phospho-protein western blot analysis.

Recent findings have further emphasized the well known importance of the endothelium in response to infection and bacterial clearance. Dr. Cubes and colleagues have shown that mice which express TLR4 exclusively in endothelial cells were able to clear systemic bacterial infections more efficiently than wild type mice.[288] This adds another potential layer of complexity to the phenomenon of immuno-modulation with TLR4 agonists. The impact of immuno-modulation on endothelial function is still unknown. Evans Blue Dye could be used to assess vascular permeability in MPLA primed and control mice prior to, and after infection to assess basic vascular barrier function response to MPLA. Intravital microscopy could also be used to quantify any enhanced leukocyte extravasation conferred by MPLA treatment. This could be coupled with *ex-vivo* co-culture studies utilizing MPLA-primed and un-primed human derived neutrophils in conjunction with primed and unprimed HUVEC cells. This experimental platform could provide insights into the effects of MPLA on binding interactions, migration, and cytokine production mediated by endothelial reprogramming. The importance of endothelial cell TLR4 pathway signaling could be investigated further with bone marrow transfers from WT mice into irradiated TLR4 deficient mice; as well as TLR4 -/- to WT, TRIF -/- to WT, MyD88 -/- to WT, WT to TRIF -/-, and WT to MyD88 -/- mice. This would allow for discrimination between the importance of tissue (particularly endothelial) TLR4 signaling capabilities and leukocyte LTR4 signaling competency in both the native response to sepsis as well as the effect of MPLA.

While MPLA immuno-modulation may not prove to be the definitive answer to treating immune dysregulation in sepsis and sepsis like conditions, to may afford greater

mechanistic understanding of the pathways, cell types, and soluble factors capable of enhancing survival, reducing inflammation, or improving bacterial clearance. The results from the post-insult experiment indicate that the enhanced phagocytic function may be rapidly elicited by MPLA but not the reduction of inflammation. Further investigation may reveal the independent mechanisms responsible for enhancing these two immune functions and allow for the development of even more specific treatments. The immune response is involved in a vast array of conditions and developing greater mechanistic understanding and more targeted treatment options will benefit every field of health care.

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Vita

Christopher-David M. Romero was born in Concord, Massachusetts, on October 2nd, 1979 to Roy and Barbara Romero. At the age of 3 his family moved to the lower Rio Grande Valley where he spent the rest of his youth. After graduating from the Science Academy of South Texas in 1998 he worked for Tropical Management Company, Troy Giles Real Estate, Pinkerton Realty, and Richard Stockton Photography to raise funds for college. In 2000 he enrolled at Texas A&M University-Corpus Christi (TAMU-CC). While at TAMU-CC he became the head work study in the microbiology department, and worked part time as a tutor for microbiology, immunology, pathophysiology, and genetics. From 2002 to 2004 he engaged in an NSF sponsored self-directed research project, and in 2003 was awarded a TAMU-CC undergraduate research grant. During the summer of 2004 he participated in an undergraduate research internship at the National Center for Complementary and Alternative Medicine at the NIH in Bethesda, Maryland. After Graduating Summa Cum Laude from TAMU-CC in December of 2004 he was soon married to Nicole Anne Ekstrom. In 2005 he entered the MD / PhD combined degree program at The University of Texas Medical Branch in Galveston; and in 2007 commenced his graduate research under the mentorship of Dr. Edward R. Sherwood. Since his entry into the combined degree program he has been named both a Blocker and McLaughlin Scholar, and is also the recipient of an NIH F31 Fellowship. He has served as president of the MD / PhD Student Organization and the Sailing Club at UTMB; through which he initiated the Coastal Community Care project.

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