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Altered phagocyte function precedes death in polymicrobial sepsis

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ALTERED PHAGOCYTE FUNCTION PRECEDES DEATH IN
POLYMICROBIAL SEPSIS

by

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DEDICATION

I dedicate this work to my Mom, Dad, and my two older Brothers. Thank you for shaping the person I was, the person I am, and the person I will become.
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ALTERED PHAGOCYTE FUNCTION PRECEDES DEATH IN POLYMICROBIAL SEPSIS

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ABSTRACT

Sepsis is an immunological condition defined by a pathogen inducing the Systemic Inflammatory Response Syndrome (SIRS), which itself is a clinical diagnosis involving temperature, heart rate, respiration, and white blood cell (WBC) count.

Our lab uses Cecal Ligation and Puncture (CLP) to induce polymicrobial sepsis in mice, with a mortality rate of 50 percent. Previous research in our lab has demonstrated that the plasma levels of IL-6 collected six hours after the start of sepsis can be used to predict which mice will live (Live-P) and which mice will die (Die-P) during the acute phase (<5 days post CLP). This predictive tool enables stratification of mice prior to mortality to determine immunological differences between groups. With this approach it was found that both Live-P and Die-P mice have equivalent bacterial burden and phagocyte recruitment within 6 hours of CLP. Yet by 24 hours, Die-P mice have increased bacterial burden while recruiting more phagocytes than Live-P. This suggested a phagocytic impairment.

This study reproduced the aforementioned findings and subsequently determined that Die-P peritoneal phagocytes kill fewer bacteria than Live-P. This bactericidal deficit
was associated with multiple cellular defects. The reduced cellular function included: decreased phagocytosis, decreased phagosomal acidification, and decreased generation of reactive oxygen species (ROS). All of these are integral components of the bacterial killing process. Furthermore, it was found that this deficit was due to cellular suppression and not to cellular exhaustion. The study of phagocytic function was then extended to the bone marrow, a source of phagocytes, and to the peripheral blood. Die-P bone marrow phagocytes showed increased phagocytic activity despite a similar capacity to respond to bacteria as Live-P. Additionally, Die-P bone marrow phagocytes were found to express higher levels CD11b, a marker of activation. Conversely, Die-P peripheral blood phagocytes expressed higher levels of activation markers while exhibiting decreased phagocytic functions.

This study then recapitulated the phagocytic dysfunction of septic cells with naïve healthy cells. A surge in pro and anti-inflammatory mediators is a hallmark of sepsis, with Die-P mice producing a significantly larger surge. Naïve phagocytes were incubated with plasma or peritoneal fluid from Live-P and Die-P mice and it was found that Die-P fluids significantly compromised the phagocytic activity of naïve phagocytes. These studies collectively suggest that mortality from CLP induced sepsis is due to failure to kill bacteria and that differential production of inflammatory mediators contributes to the differences in phagocytic function.
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LIST OF ABBREVIATIONS

3x-labeled: Triple labeled

A2BR: Adenosine Receptor 2B

Alexa-350 SE: Alexafluor-350 succinimydl ester

AKI: acute kidney injury

APC: recombinant activated protein C

ARDS: acute respiratory distress syndrome

AUC: area under the curve

AST: aspartate transaminase

BUMC: Boston University Medical Center

BM: Bone Marrow

BSA: Bovine Serum Albumin

CASP: colon ascendens stent peritonitis

CD: Cluster of Differentiation

CFU: colony forming units

CLP: cecal ligation and puncture

DAMP: Damage Associated Molecular Patter

DCF: dichlorofluorescein

DCF-SE: dichlorofluorescein succinimydl ester

DEX: Dexamethasone

DHR: DiHydroRhodamine-1,2,3
DIC: disseminated intravascular coagulation
Die-P: predicted to die
DMSO: dimethyl sulfoxide
E.coli: Escherichia coli
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FACS: fluorescence activated cell sorting
FcR: Fc Receptor
FDA: Food and Drug Administration
fMLP: formil-methionyl-leucyl-phenylalanine
G-CSF: granulocyte colony stimulating factor
GFR: glomerular filtration rate
gMFI: geometric mean fluorescent intensity
GM-CSF: granulocyte-macrophage colony stimulating factor
H&E: hematoxylin and eosin
HBSS: Hanks’ Balanced Salt Solution
Hr: Hour
IACUC: Institutional Animal Care and Use Committee
ICAM-1: inter-cellular adhesion molecule 1
ICR: Institute for Cancer Research
ICU: intensive care unit
IFN\(\gamma\): interferon \(\gamma\)
**IgG**: immunoglobulin G

**iIgG**: inhibitory Immunoglobulin G

**IL**: interleukin

**IL-1ra**: IL-1 receptor antagonist

**Indo-1am**: Indo-1 Acetoxymethyl Ester

**ITAM**: Immunoreceptor tyrosine-based activation motif

**ITIM**: Immunoreceptor tyrosine-based inhibitory motif

**KC**: keratinocyte-derived chemokine

**LTB₄**: leukotriene B₄

**LASC**: Laboratory Animal Science Center

**Live-P**: predicted to live

**LPS**: lipopolysaccharide

**Mac**: macrophage

**MFI**: mean fluorescence intensity

**MCP**: monocyte chemotactic protein

**Min**: minute

**Mo**: macrophage

**MODS**: multi-organ dysfunction syndrome

**MPO**: myeloperoxidase

**NADPH**: nicotinamide adenine dinucleotide phosphate

**NE**: neutrophil

**NRF-2**: nuclear response factor 2
NOX: NADPH Oxidase

O-E.c: Opsonized E.coli

OD: optical density

PaCO₂: partial pressure of carbon dioxide

PAMP: pathogen associated molecular patterns

PB: Peripheral Blood

PBS: Phosphate buffered saline

PC: peritoneal cavity

PDL1: Programmed cell death ligand 1

pHrodo-SE: pHrodo succinimydyl ester

PIRO: predisposing factors, insult or infection, response of the host organ dysfunction

PMA: Phorbol myristate acetate

PMN: polymorphonuclear neutrophil

PMT: photo multiplier tube

PRR: pattern-recognition receptors

R-123: rhodamine 1,2,3

RBC: red blood cells

ROC: receiver operator characteristic

ROS: reactive oxygen species

RLU: Relative light units

SA-HRP: streptavidin-conjugated horseradish peroxidase

SEM: standard error of the mean
**SIRS**: systemic inflammatory response syndrome

**TLR**: Toll-like receptor

**TNFα**: tumor necrosis factor α

**TNF-srI and II**: TNF soluble receptor I and II

**TMB**: 3, 3’, 5, 5’-tetramethyl-benzidine

**WBC**: white blood cells
1 CHAPTER ONE: GENERAL INTRODUCTION

Introduction

Sepsis is the leading cause of morbidity and mortality in hospital intensive care units. It is the 10th leading cause of death in the United States [1]. It costs society an estimated 24 billion dollars annually and this cost is increasing [2]. The overall prevalence and cost of sepsis has doubled from 2000-2008 [3, 4] and this rate is projected to increase as the increased use of immunosuppressive therapies and an aging population takes its toll. Since the advent of antibiotics in the middle of the last century, little progress has been made in reducing sepsis mortality [5, 6], which ranges from ~25-60%, depending on severity [7]. If sepsis outcome is considered beyond the standard 28 day window, mortality increases with nearly 80% mortality within 5 years. Sepsis is classically defined as a systemic dysregulation of the immune system. Clinically, sepsis is defined as the co-presentation of infection, or suspicion of one, and Systemic Inflammatory Response Syndrome (SIRS), where SIRS is a diagnosis involving temperature (\(>38.3\, ^\circ\text{C}\) or \(<36\, ^\circ\text{C}\)), heart rate (\(>90\) beats/min), respiration (\(>20\) breaths/min or \(\text{PaCO}_2 <32\, \text{mmHg}\), and white blood cell (WBC) count (\(>12\times10^9/\text{L}\) or \(<4\times10^9/\text{L}\)) [8]. As disease severity progresses, additional classifications are made. Severe sepsis, which compounds sepsis with organ dysfunction, and septic shock, which is sepsis in addition to hypotension despite adequate fluid resuscitation are more severe forms of sepsis. However, these classifications do little to direct the course of treatment.
Sepsis Pathophysiology

Sepsis is thought to originate from trauma or infection that results in an aberrant inflammatory response. Early work on this concept was performed with the endotoxin lipopolysaccharide (LPS), a cell wall component of gram negative bacteria [9]. LPS, a Pathogen Associated Molecular Pattern (PAMP) binds to TLR-4, a Pattern Recognition Receptor (PRR) that is expressed on mammalian cells and most notably on macrophages. TLR-4 ligation results in the secretion of pro-inflammatory cytokines and chemokines that initiate an inflammatory response and recruit additional immune cells such as neutrophils (PMNs) to eradicate the infection [10, 11]. However, too much PAMP/PRR signaling may be detrimental to the host.

It was thought that the early stages of sepsis were the result of a powerful pro-inflammatory response that led to systemic activation of the immune system and resulted in SIRS. During SIRS, hyper-activation of immune cells is thought to lead to increased organ damage. Neutrophil derived inflammatory mediators can damage the vascular endothelium, resulting in necrosis and apoptosis [12] and subsequent hemodynamic changes leading to tissue hypoxia [13, 14]. This is thought to lead to multiple organ failure in sepsis [15, 16] The SIRS phase is then followed by the Compensatory Anti-inflammatory Response Syndrome (CARS) [17] that results in global immunosuppression and increased susceptibility to secondary infection [18, 19]. However, recent evidence suggests that in sepsis, SIRS and CARS occur simultaneously
[20, 21] and the magnitude of these concomitant responses predicts mortality [22, 23]. Although the overlap of SIRS and CARS fits within feed-forward immunological feedback loop models, it is likely that the traumatic or infectious insult is of sufficient magnitude so as to overwhelm the immunological control mechanisms responsible for a coordinated response. A prototypical pro-inflammatory immune response is such that an inflammatory trigger results in activation of pro-inflammatory signaling that propagates in a self-amplifying feed-forward manner (Fig 1.1A). This self-amplifying process would enable the quick mobilization of immunity. Once the pro-inflammatory drive crosses a signaling threshold [24], a negative feedback loop is triggered to prevent the dangers of unrestrained inflammation [25]. In the context of sepsis, the initial insult may be sufficiently large to trigger both responses simultaneously (Fig 1.1B), thus acting to blunt the immune response before it can eradicate the infection. This model may in part explain why immune-suppression develops in sepsis.
Figure 1-1 Immune Deregulation in Sepsis. A- Prototypical immune response. B- Uncoordinated immune response in sepsis.

Sepsis Treatment

Current treatment for sepsis includes antibiotic therapy, infectious source control, fluid resuscitation, and other procedures aimed at maintaining hemodynamic stability [26]. Although this approach has reduced 28-day mortality, it is not known for certain how this is achieved [27]. For every hour passed between the onset of hypotension to antibiotic administration, there was a 7.6% increase in in-hospital mortality [28], highlighting the
importance of bacterial clearance. Despite this regimen, complications frequently develop such as acute respiratory failure, acute renal failure, shock, coagulopathy, and multiple organ failure [8, 29]. The idea that mortality derives from excessive inflammation led to therapies such as Anti-Tumor Necrosis Factor (TNF), and anti-Interleukin-1 (IL-1). While these therapies showed promise in lab animals, they ultimately failed to improve outcome in humans [8].

Similarly, trials with recombinant activated protein C (APC), an inhibitor of coagulation; eritoran, a Toll-like Receptor 4 (TLR-4) blocker, and talactoferrin, an immune stimulant, either failed or were stopped due to worsened outcome [30]. It is likely that heterogeneity exists within and across the different classifications of sepsis and this complicates treatment. SIRS, sepsis, severe sepsis, and septic shock do not factor in variables such as the pathogen, comorbidities, or patient demographics. To address this, consideration of predisposing factors, the infection, the response, and organ failure (PIRO) of the host have been used to stratify patients by likelihood of mortality [31]. Further complicating sepsis therapy is that we still do not know exactly why patients die from sepsis, thus highlighting the need for more research into the mediators of mortality in sepsis.

Animal Models of Sepsis

An appropriate model is at the heart of studying sepsis. A TNF blockade improved survival in a murine model of LPS-induced endotoxemia sepsis, however, the same study
demonstrated that a blockade of TNF in a CLP model did not improve survival [32]. This stresses the importance of selecting a sepsis model with clinical relevance.

There are three general categories of sepsis models: Endotoxin administration (i.e. LPS), exogenous bacterial administration, or host-barrier disruption models such as CLP which results in necrotizing tissue and the release of intestinal flora into the peritoneum. Each model has its advantages and disadvantages. Endotoxemia can be tightly controlled by the amount of toxin given, however it results in a rapid rise and decline in cytokine levels which differs from the sustained and more moderate levels seen in human sepsis. Exogenous bacteria tend not to colonize the host and thus resemble an endotoxemia model to some extent. The choice of bacteria will also affect the host response, as will the site of administration. CLP introduces layers of complexity such as multiple bacterial species, animal age, and animal strain variability [33, 34]. However, CLP is considered the gold standard for sepsis studies and for good reason; it shares many characteristics with human sepsis such as hemodynamic alterations and changes in body temperature [35, 36], improved response to antibiotic therapy [37], a massive parallel pro and anti-inflammatory cytokine storm [38], and a clinical correlate in perforated and/or necrotizing bowels. Furthermore, the severity of CLP may be altered to investigate phenomenon associated with a particular severity of disease[36].

Our lab is interested in what mediates mortality in CLP induced sepsis, with care taken to be as clinically relevant as possible. Outbred mice are used, as they have been shown to
more closely model the heterogeneity prevalent in humans [39] and their course of disease [40]. Fluid resuscitation, broad spectrum antibiotics, and pain control are also provided in a manner similar to the clinic [41].

To determine what mediates mortality in CLP induced sepsis, it would be helpful to know in advance which animals will survive. Using an LD$_{50}$ model of CLP, IL-6 was found to predict mortality with great sensitivity and specificity during the acute phase of sepsis [42]. This predictive tool allows more defined studies to delineate what goes wrong in sepsis, as well as providing a means for more targeted interventions and assessments of efficacy. Indeed, recent work in mice has shown that dexamethasone treatment fails to improve overall survival from CLP-induced sepsis, however, by stratifying mice into Live-predicted (Live-P) versus Die-predicted (Die-P) groups, a marked improvement in survival was found for mice that would have otherwise succumbed [43]. Stratification has also yielded interesting clues for the mechanisms of mortality. Six hours Post-CLP, the peritoneal cavity of both Live-P and Die-P mice contain similar numbers of neutrophils and macrophages (phagocytes), and bacterial CFUs.

Yet by 24 hours post-CLP, Die-P mice have significantly more peritoneal phagocytes and CFUs [44]. These data suggests that there is a defect in the phagocytes of Die-P mice.
**Phagocytes and Sepsis**

The phagocytes, primarily neutrophils (PMN) and macrophages/monocytes (Macs) are at the front lines of host defense and are essential for protecting the host from extracellular infection. The principal means by which they protect against microbial infection is via phagocytosis, a process in which they engulf and destroy the infectious agent [45]. Phagocytes possess several pathways by which they can recognize and ingest infectious agents. Antibodies or complement can opsonize microbes, thereby allowing phagocytosis via the Fc-Receptor (FcR) or complement receptor (CR) pathways, respectively. Similarly, phagocytes possess scavenger receptors (SR) that are capable of directly binding microbes in an opsonin independent manner. Although PMNs and Macs phagocytize to kill pathogens, they each have their own signature bactericidal processes. At the heart of PMN bactericidal processes is the NADPH Oxidase (NOX) complex and various granules. PMN granules are classified as Azurophilic, Specific, Tertiary, or Secretory. Azurophilic granules are filled with lysosomal proteases and are considered the bactericidal compartment mobilized by phagocytosis. Specific granules contain additional antimicrobial contents, tertiary granules contain enzymes for extravasation of PMN into tissues, and secretory granules contain multiple types of receptors (i.e. CD11b) which are brought to the surface upon activation [46]. NOX is a transmembrane protein complex which catalyzes the reduction of oxygen to superoxide anions, the primary Reactive Oxygen Species (ROS) which gives rise to various other ROS [47]. NOX is located throughout the plasma membrane of PMNs which positions it to pump ROS into the extracellular environment, as well as
intracellularly as when the plasma membrane invaginates around a phagocytic target.
During membrane invagination, granules fuse with the budding phagosomes. Once phagosome formation is complete, NOX is activated and generates a large intraphagosomal ROS burst [48]. As superoxide dismutates into peroxide, it encounters myeloperoxidase which catalyzes the formation of bleach (HOCl) which is thought to kill the bacteria [49]. Additionally, the large ROS influx is thought to setup an ionic gradient that results in $K^+$ influx which releases granule proteases from their inactivating matrix so that they may digest the bacteria [50].

In addition to their phagocytic activity, PMNs are also known to produce Neutrophil Extracellular Traps (NETs). NETs can be composed of either nuclear or mitochondrial DNA [51, 52]. Their function is to help sequester microbes in a manner similar to that of a spider’s web. Bacteria bound by NETs cannot disseminate and represent easy targets for elimination.

Monocytes and macrophages have a less pronounced ROS burst than PMNs [53, 54] and ROS may not be essential for macrophage bacterial killing as NOX deficient macrophages were equally capable of killing bacteria as their wild type counterparts [55]. Furthermore, whereas PMNs have prepackaged granule proteases to deliver to the phagosome, macrophages rely on an endosomal-like maturation process that results in decreasing phagosomal pH and eventual fusion with lysosomes to destroy their cargo [56]. Although differences exist in the extent to which neutrophils and macrophages use
ROS for microbial killing, the absence of NOX activity, such as in Chronic Granulomatous Disease (CGD), underscores the importance of ROS in fighting infection [47]. However, it must be kept mind that ROS is not just a byproduct of an intense inflammatory reaction; it is also a specific signaling molecule responsible for various actions such as chemotaxis [57], gene transcription [58], granule release from inactivating matrix [59], and endothelial cell function [60].

A frequently used measure of bactericidal potential comes from measuring the magnitude of the ROS burst from phagocytes. Recently it was shown that neutrophils from CLP mice displayed a decreased ROS burst 3-days post CLP, but this impairment returned to normal by day 7 post-CLP. The group also showed that Day-3 mice were much more susceptible to pneumonia than day 7 mice [61]. However, other groups have shown NOX-derived ROS to contribute to disease pathogenesis and mortality in sepsis [62]. Similarly the process of phagocytosis has been shown to be impaired in sepsis due to decreased SR expression [63], decreased FcγRIII (CD16) expression on PMN released from the bone marrow [64], or even due to PMN mediated suppression of inflammatory monocytes via PMN derived IL-10 [65].

The aforementioned studies indicate that modulation of phagocytosis and ROS generation occur in sepsis, but these studies compare sham operated animals to CLP. While this approach is sufficient for examining the host response to sepsis, we are interested in the differences between survivors and non-survivors of sepsis. Our group previously found
that six hours post-CLP, the peritoneal cavity of both Live-P and Die-P mice contain similar numbers of neutrophils and macrophages (phagocytes), and bacterial CFUs. Yet by 24 hours post-CLP, Die-P mice have significantly more peritoneal phagocytes and CFUs [44]. These results form the basis for this study and our hypothesis that *Mice Predicted to Die during the Acute Phase of Sepsis have Cellular Alterations that Impair Bacterial Killing.*
CHAPTER TWO: PERITONEAL PHAGOCYTE FUNCTION IN THE CLP MODEL OF SEPSIS

Introduction

Sepsis is an immune mediated immune disorder that costs society approximately $24 billion annually [2], and claims over 200,000 lives per year [66]. Despite being on par with cancer in lives lost, there are few treatment options available. Currently, patients receive an aggressive treatment of fluid resuscitation, vasopressors, and broad spectrum antibiotics [26]. Despite these interventions, post-mortem studies revealed the majority of patients still had an infectious foci present [67], thus suggesting a deficit in bacterial clearance.

Neutrophils and macrophages comprise the phagocytic arm of the immune system and are largely responsible for eradicating a bacterial infection. Following infection, tissue macrophages engage the pathogen and secrete distress factors to recruit neutrophils and inflammatory monocytes. Neutrophils help to neutralize the infection by secreting Neutrophil Extracellular Traps (NETs) [68], and/or by phagocytizing microbes and exposing them to Reactive Oxygen Species (ROS) and cationic proteases [69]. Similarly, macrophages and inflammatory monocytes phagocytize microbes and process them in a manner similar to endosomal cargo, ultimately fusing with lysosomes and digesting the bacteria via pH sensitive proteases [56]. As the first responders in an immune response, they are central to the initiation of sepsis.
Cecal Ligation and Puncture (CLP) induced peritonitis in mice produces a rampant inflammatory response that is thought to become dysregulated, leading to immune paralysis, bacterial overgrowth, and death [70]. Studies have shown that altering leukocyte recruitment [71], or enhancing leukocyte function result in decreased bacterial burden [72], increased organ perfusion [73, 74], and ultimately increased survival. However, the majority of these studies compare sham-operated animals to CLP-operated. This approach is well-suited to define the host response to sepsis, but it may be less appropriate for delineating the mechanisms of sepsis mortality.

Previous studies have shown that within 6hrs of CLP, circulating biomarkers can be used to accurately predict mortality during the acute phase of sepsis [75]. This powerful capability enables stratified interventions to assess efficacy [76, 77] and it provides windows of time in which to observe the detrimental divergences between survivors and non-survivors. This approach revealed that similar peritoneal bacterial CFUs and phagocyte recruitment occurs 6hr post-CLP. Yet by 24hr, non-survivors recruited significantly more phagocytes and yet they also showed significantly increased bacterial burden [22].

We hypothesized that non-survivor phagocytes were impaired in their bacterial killing capacity.
Methods

Animals

Female ICR (CD-1) mice (Harlan-Sprague Dawley, Inc., South Easton, MA) between 24-28 grams were used for all studies. Mice were received and allowed to acclimate to our housing room for at least 96 hours prior to surgery. Mice were kept in a temperature and humidity controlled room with a diurnal cycle of 12 hours light, 12 hours dark. Mice were given food and water ad libitum for the entire duration of the experiment. The experiments were approved by the Boston University Animal Care and Use Committee.

Sepsis Model

Cecal ligation and puncture was performed as first described[29] with minor modifications[41]. Mice were first anesthetized in an induction chamber with 5% isofluorane, followed by 3% for maintenance during surgery. A midline incision was performed through the skin and then the linea alba. The cecum was exteriorized and ligated below the illeocecal valve with 4-0 silk. Approximately two-thirds the length of the cecum from the distal tip was ligated, then double punctured longitudinally with a 16 gauge needle. A small amount of cecal contents was squeezed out to ensure patency, then the cecum was returned to its original position. The abdomen was closed with 4-0 silk sutures, and the skin closed with wound glue (Nexaband- Veterinary Products Laboratories, Phoenix, Az). Mice were resuscitated with 1mL warm saline (37°) with buprenorphine (0.05mg/kg) for pain management (1 injection every 12hours for the first two days post-CLP). Antibiotic treatment with 25mg/kg Imipenem was administered 2
hours post-CLP, and then once every 12 hours for the first 5 days. Mice sacrificed at 6 hours received one dose of drugs, while mice sacrificed at 24 hours received 3 doses.

**Sampling**

Blood sampling was performed at 6 and 24 hours to generate IL-6 Receiver Operator Characteristic curves. For all other experiments, sampling was performed once at 6 or 24 hours, according to the planned experiments. 20uL of blood was collected by facial vein puncture and diluted 1/10 in PBS containing 3.38mM EDTA tripotassium salt, and immediately placed on ice. Blood was centrifuged for 5 min at 1000 X g at 4°C, and the plasma was collected and frozen at -20°C until subsequent analysis for IL-6. In the mice that were sacrificed, blood was collected from the retro-orbital venous plexus under anesthesia (87 µg/g Ketamine and 13 µg/g Xylazine in normal saline), followed by euthanasia via cervical dislocation. The peritoneal cavity was then lavaged with 1mL warm Hanks’ Balanced Salt Solution (HBSS, Mediatech Inc., Herndon, VA) containing 10U/mL Heparin, followed by 20mL cold HBSS with 0.5mM EDTA and strained through a 70uM strainer. 100uL of the 1st mL was used for bacterial cultures. Then the two lavage samples were centrifuged separately and the supernatant of the first wash was saved for future use. The cell pellets were then resuspended in 2mL wash buffer (PBS + 0.5% Bovine Serum Albumin) and 100uL retained for total cell counts and cytospins. The remaining cell volume was underlaid with 2mL 30% sucrose (w/v) in PBS and then centrifuged for 8 minutes at 450 X g to remove extracellular bacteria [78]. The supernatant containing bacteria was aspirated and the cell pellet was resuspended in 3mL
wash buffer. 1mL of 100% isotonic Percoll in PBS was added to cell volume to generate a 25% Percoll solution. This was underlaid with 1mL Histopaque 1.119 and then centrifuged for 25 min at 500 X g. The viable cells were located at the Percoll Histopaque interface. Light debris was aspirated from the 25% Percoll layer, dead cells were removed from the pellet by pipette, and the remaining viable cells were washed once, followed by resuspension in HBSS containing Ca\(^{2+}\) and Mn\(^{2+}\) + 1% BSA. Total cell counts were performed with a Beckman-Coulter particle counter model ZF (Coulter electronics Inc., Hialeah, Fl).

Bone Marrow cells were flushed from the two hind tibias with cold HBSS + heparin using a 25 gauge needle. Cells were centrifuged, the pellet lysed with 1mL 0.2% hypotonic saline for 40 seconds, then restored to isotonicity with 1.6% hypertonic saline. Cells were washed once and then counted.

**Bacterial Cultures**

100\( \mu \)L of the \( 1^{st} \) mL of lavage fluid was serially diluted with HBSS 1:10 to 1:10 and 50\( \mu \)L of each dilution were cultured on 5% sheep blood agar plates (Fisher Scientific). Plates were incubated for 24 hours at 37°C in anaerobic or aerobic conditions and then the number of Colony Forming Units (CFU) was counted.

**ELISA**
An aliquot of 1/10 diluted plasma collected at 6 and/or 24 hours was diluted 1/5 in dilution buffer for a final dilution of 1/50, and then analyzed for IL-6 concentrations by ELISA as previously described [79].

**Bacterial Killing**

Streptomycin resistant Escherichia coli (*E. coli* strain HB101) were grown to log-phase in Tryptic Soy Broth containing streptomycin. Bacteria were opsonized with normal ICR mouse plasma for 20 minutes at 37°C at which point they were directly added to peritoneal cells (4*10^6/ml) at a 5:1 microbe: cell ratio. Cells/E.coli were centrifuged at 50*g* for 5 minutes to facilitate contact. Cells/E. coli were then incubated for 1 hour at 37°C. The reaction was stopped by lysis with H_2O pH 11 [80]. Serial dilutions were plated on Tryptic Soy Agar plates infused with streptomycin and grown overnight at 37°C in aerobic conditions. The % bacteria killed was calculated by dividing CFUs of sample + bacteria by CFUs of bacteria alone.

**pHrodo and ROS Measurement**

Heat killed pHrodo-red E.coli (Life Technologies, Carlsbad, CA) were opsonized with E.coli specific antibodies. Bacteria were incubated with peritoneal cells (4*10^6/ml) at a 20:1 ratio for 30 minutes at 37°C. pHrodo fluorescence was calculated as gMFI of cells incubated at 37°C minus the gMFI of cells incubated on ice.

ROS was measured by the conversion of non-fluorescent Dihydrorhodamine 1,2,3 (DHR-123) to fluorescent R-123. Cells were loaded with 2uM DHR-123 and stimulated with
opsonized heat-killed *E.coli* or 100nM Phorbol-12 Myristate-12 Acetate (PMA). PMA stimulation was limited to 20 minutes. The magnitude of the ROS burst was calculated by the % increase of R123 fluorescence (gMFI) of stimulated cells over unstimulated cells. Reactions were stopped by placing cells on ice, followed by extracellular marker staining for flow cytometry.

**3X-Labeled Bacteria Preparation**

Labeling was performed as previously published[81]. Briefly 2*10^{10}/mL of heat-killed (80°C 1hr) HB101 E.coli (same strain used for bacterial killing assay) were sequentially labeled with 50uM DCF-SE, 50uM pHrodo-SE, and 159uM Alexafluor-350-SE (Life Technologies). Labeling was performed in PBS pH 9.0, degassed and purged with N₂ to minimize auto oxidation of DCF. Bacteria were washed 3X with PBS pH 8.0, and resuspended in PBS pH 7.4 (N₂) and stored in glass vials flushed with N₂. Bacteria (~1.5*10^{10}/mL) were opsonized with 1/10⁸ volume of normal mouse plasma (heparinized) and anti-*E.coli* antibodies (50ug/mL) (Life Technologies).

**3X-Labeled Bacteria Phagocytosis**

Peritoneal cells were loaded into polypropylene cryovial tubes at 4*10⁶/mL. While on ice, ~150 bacteria/cell were added to cells (10uL of OD₆₀₀ = 0.105). DNase was added (50ug/mL) to prevent aggregation of cells. Tubes were transferred to a water bath at 37°C, placed on heated stir plate, and agitated at lowest speed for 30 minutes with 7x2mm micro stir bars. For controls, samples were also incubated on ice for 30 minutes.
Following incubation, all cells were kept on ice until data acquisition with a flow cytometer. No wash steps were performed.

**Flow Cytometry**

*All data was acquired on an LSRII (BD Biosciences).*

*pHrodo/DHR-123*- Cellular FcReceptors were blocked with Fc Block (Bd Biosciences). The following antibodies were used: CD11b (clone M170), CD19 (1d3), CD3e (145-2C11), Ly6G (1A8), Gr-1 (RB6-8C5), F480 (Ci-A13), Ly6C (HK1.4). Only Sytox Blue negative events (Live Cells) were used for gating. Doublet discrimination was performed by Fsc-A vs Fsc-H. BD compensation beads (BD biosciences) were used for compensation. Additionally, Sytox Blue and R-123 were used to stain cells and compared to unstained cells for compensation against those parameters.

*3x Labeled Bacteria*- DCF, pHrodo, and Alexafluor-350 fluorescence was collected by (wavelength/band pass) 530/15, 590/10, and 450/50 filters. It was important to use a custom 590/10 filter for pHrodo because Trypan Blue auto fluoresces when bound to protein and its emission begins at ~615nm. The stock filter supplied by the core facility was a 610/20bp.

Initial PMT settings were chosen such that the MFI of pHrodo and Alexa-350 had a 1:1 ratio. DCF PMT was set between 0-100 (DCF has little fluorescence prior to oxidation). Once the bacterial settings were in place, a positive control (healthy cells + 3x bacteria)
was acquired to ensure all events were on scale. PMTs were adjusted as needed, and then “locked in” with calibration particles. Prior to sample acquisition, Spherotec rainbow 7-peak calibration particles were run to ensure that the PMT settings used were appropriate (<±5% of the first experiment’s MFIs). 3x bacteria were also collected for quality control purposes. Equal volumes of beads, bacteria, and diluent (PBS) were used, and events were acquired at the same flow rate from day to day.

Ice controls were acquired first. ~20,000 events were collected to measure attachment. Then, Trypan Blue was added to tube (0.25% Final concentration), incubated on ice for 1 minute, and then ~30,000 gated events were collected. Cells that were allowed to phagocytize were only acquired with TB present.

Data was analyzed with Flowjo (Treestar Inc.). Non-debris cells were gated on by Fsc/Ssc. Doublets were removed by fsc-h vs. fsc-w, with care taken to not remove activated cells from analysis. Phagocytosis + gates were constructed for each sample based off its own ice control (no phagocytosis occurs.) The MFI of quenched ice control cells was subtracted from each channel of the 37°C sample, to account for the minimal contribution of autofluorescence (minimal because the PMTs were set low enough to get positive single bacterial events to read near zero.) Derived parameters were constructed within Flowjo so that the DCF or pHrodo fluorescence for each event was divided by the fluorescence of Alexa-350 for that event. The ROS index was then calculated by dividing
the DCF/Alexa ratio of the cell by the DCF/Alexa ratio of the bacteria. Similar calculations were performed for the Acidification Index (pHrodo).

**Calcium Signaling**

*Indo-1 loading*: Cells were loaded with Indo-1am as previously detailed [82]. Briefly, 5-10*10^6 cells/mL were incubated with 5uM Indo-1am in Krebs-Ringer Phosphate (KRP) for 10 minutes at 37°C, followed by 10 minutes at room temperature. Cells were washed and resuspended in complete KRP + 1.5mM MgSO_4 + 0.9mM CaCl_2.

Of note, all polypropylene conical vials were washed 3X with 3X Distilled water to remove traces of chemical plasticizers which will prematurely activate PMNs.

Flow Cytometry: A custom device that allows for thermostating, stirring, and stimulus injection was borrowed from the Elizabeth R Simon’s lab and modified to work with the LSRII flow cytometry platform. Cells were loaded into the device at 2*10^6/mL, warmed for 2-3 minutes at 37°C, followed by acquisition on the LSRII. Once a steady baseline of Indo-1 fluorescence was reached (10-20s), stimulus (~150 bacteria/cell) was injected into the sample. Acquisition proceeded for 120s minimum.

Indo-1 fluorescence was collected with 405/20 (violet) and 500/30 (blue) bandpass filters.

**Luminol Chemiluminescence**

Peritoneal cells were added to white-opaque microplate wells in duplicate at a concentration of 4*10^6/mL. Luminol was present at 20uM. Cells were placed in a temperature controlled fluorescent plate reader (Tecan Infinite m1000) and warmed to
37°C for 5 minutes. Stimulus was then added (100nM PMA, 20:1 bacteria:cell) and chemiluminescence was measured every ~10s (0.5s integration time) followed by mechanical shaking.

**Statistical Analysis**

Statistics were performed using Prism 5 software (Graphpad Software, San Diego, CA). For comparison between 2 groups, an unpaired Student t test was used.

**Results**

*Characterization of CLP-Induced Sepsis: A Similar Beginning, a Dissimilar Fate.*

To study the mechanisms of mortality, our lab uses a CLP model that produces approximately 50% mortality within the first five days of sepsis (Acute phase) (Figure 2-1). Monitoring plasma IL-6 levels of survivors/non-survivors shows that mice that succumb to sepsis have significantly elevated IL-6 at 24hr post CLP as well as at 6hr post-CLP (data not shown). Receiver Operator Characteristic curves were used to generate IL-6 discrimination values to stratify mice as predicted to live (Live-P) or predicted to die (Die-P), as described previously [75]. With this approach, mice were sacrificed at 6hr and 24hr post-CLP and posthumously stratified.

As reported previously by this lab, Die-P mice have similar numbers of bacteria in their peritoneum as compared Live-P mice at 6hr Post-CLP (Figure 2-1 D). Similarly, there is no difference in overall cell numbers or cell phenotype between Live-P and Die-P groups.
(Figure 2-1C) [22]. This demonstrates that our CLP model is consistent, but more importantly, that both Live-P and Die-P groups were subjected to a similar initial insult with a similar cellular response. However, by 24hr post-CLP Die-P mice have increased bacteria and increased phagocyte recruitment to the peritoneum. The divergence from similar phagocyte recruitment and bacterial burden at 6hr post-CLP, to increased phagocytes and bacterial numbers at 24hr post-CLP suggests a defect in phagocyte function in Die-P mice.
Figure 2-1 Phagocyte Dysfunction Precedes Death from CLP-induced Sepsis. (a) CLP was performed to induce ~50% mortality in ICR CD1- outbred mice. (b) Plasma was collected while animals were monitored for survival. ROC curve analysis was used to generate IL-6 values predictive for mortality. (c-d) Peritoneal bacterial loads and cellular recruitment between Live-predicted/Die-predicted mice were compared following euthanasia at 6 or 24 hours post-CLP. *=p<0.05, ***= p<0.0001
Die-P Phagocytes Show Impaired Bactericidal Activity

When examined for bacterial killing using live *E.coli* (HB101), Die-P mice showed a marked reduction in bacteria killed compared to Live-P mice within 6hr post-CLP and this persists through 24hr (Figure 2-2). These data strongly suggest that the early onset of reduced bacterial killing precipitates subsequent mortality.

![Bacteria Killing Assay](image)

*Figure 2-2 Die-P Mice Exhibit Impaired Bacterial Killing of E.coli at 6 and 24hr post-CLP.* Data from at least 3 independent experiments. N=7-13/group at 6hr, 7-8/group at 24hr, where N represents an individual mouse. ***= p<0.0001

To determine why Die-P peritoneal phagocytes kill fewer bacteria than Live-P, the bactericidal mechanisms were further examined. ROS generation is integral to the microbial killing process, as evidenced by those with Chronic Granulomatous Disease (CGD), a condition where patients are prone to recurrent bacterial infections due to a mutation that renders the ROS producing NADPH Oxidase (NOX) complex inoperable.
To assess NOX activity, peritoneal cells were stimulated with opsonized bacteria or PMA, and then analyzed by flow cytometry.

Neutrophils were defined as (CD11b⁺, LY6G Hi) and monocytes/macrophages were defined as CD11b⁺, LY6G lo/neg (Figure 2-3) [83, 84]. Though the analyzed macrophage population expressed the classical macrophage marker F4\80, its variable level of expression in Die-P (Figure 2-3, G) mice made its use unsuitable for gating purposes. This fits with recent work published by Misharin et al where it was shown that eosinophils express F4\80, and, inflammatory conditions induced by thioglycollate result in decreased F4\80 expression by macrophages [85]. This is likely due in part to newly recruited monocytes/macrophages that express little F4\80 [86].
Figure 2-3 Flow Cytometry Gating Strategy for Peritoneal Phagocytes. A-C: Removal of debris, doublets, and dead cells. D-F: Neutrophil & Monocyte/Macrophages (Macs) identified as (Cd11b+, Cd3ε/Cd19-) LY6G-Hi or LY6G-Low, respectively. G: Fluorescence Minus One (FMO) staining of LY6G-low cells shows variable F480
staining on LY6G-low cells (Die-P, Red; Live-P, Blue; Naïve Resident Macs, Orange; FMO controls shaded).

Within 6hr post-CLP, both Die-P neutrophils and Die-P macrophages produce significantly less ROS than their Live-P counterparts in response to Opsonized E.coli (O-E.coli) (Figure 2-4 A-C), and this deficiency persists through 24hr post-CLP. The use of O-Ec as a stimulus is physiologically relevant; it measures how a cell would likely respond in-vivo when encountering cecal bacteria. This process leads to NOX activation subsequent to cell surface receptor ligation and signaling. However, if there is decreased receptor expression, desensitization, or some other means of blunted receptor signaling, NOX may fail to activate despite being fully capable to do so. To determine if the differences in NOX mediated ROS were due to impaired PRR signaling, or decreased capacity of intracellular NOX to activate and generate ROS, PMA was used to stimulate the cells. PMA is a non-specific activator of NOX, which bypasses the requirement of PRR signaling for NOX activation. Following PMA stimulation, Die-P phagocytes were shown to be capable of generating ROS, albeit significantly less so than Live-P at both time points (Figure 2-4 D,E).
Figure 2-4: Die-P Peritoneal Phagocytes have Decreased ROS Burst. A: Representative histograms for Rhodamine 1,2,3 fluorescence (ROS) of cells incubated
with (red histogram) or without stimulus (blue) at 37°C for 30min. B-C: Physiological ROS Burst via opsonized bacteria stimulus. D-E: Cellular Capacity for ROS Burst via PMA stimulus. N=4-7 per group from 3 independent experiments, where N represents an individual mouse.

Interestingly, Die-P phagocytes produce less ROS in response to O-E.coli stimulation than they do if left unstimulated, relatively. It is important to note that “unstimulated control” cells are not naïve cells; they were harvested from the peritoneums of mice undergoing extreme peritonitis. These cells are already stimulated, but even so, there are significantly different responses at 6hr post-CLP, a time at which bacterial loads (i.e. pre-assay stimulation) are similar. A caveat to end-point measurements for ROS lies in that our analysis, and many others’, employ normalization to an unstimulated control that is producing basal ROS. Since these cells were procured from inflamed tissue, it may be that Die-P phagocytes generate less ROS than Live-P because the Die-P cells are already generating maximal ROS (i.e. hyperstimulated), making any observable increase difficult to detect. To determine if Die-P cells are hyperstimulated, a chemiluminescence based kinetic assay was used to measure ROS. Here too, Die-P mice are significantly impaired to generate ROS in response to both phagocytosis and PMA (Figure 2-5 B, D-E).

Furthermore, the basal ROS data suggests that Die-P cells are not hyperstimulated, but may instead be exhausted or suppressed (Figure 2-5 A,C).
**Figure 2-5 Die-P Peritoneal Cells are not Hyperstimulated.** Representative ROS Kinetics for unstimulated (A), O-E.coli (B) and PMA (C) stimulated cells for Live-P and Die-P mice, as measured by Luminol based chemiluminescence (RLU). Area Under the Curve was calculated for samples E. coli (D) and PMA (E) burst. (N=3 mice / group). Samples were assayed in duplicate wells (D). *=p<0.05, **=p<0.01, ***=p<0.001

Diminished ROS generation alone does not explain decreased bacterial killing, as macrophages with defective NOX and inducible nitric oxide synthase (iNOS) enzymes
can still phagocytize and kill bacteria as well as their NOX/iNOS competent counterparts [55]. This is because, following internalization, the phagosome acidifies and enables pH-sensitive antimicrobial products to destroy the phagosome’s microbial cargo. Thus, impaired ROS generation alone may not account for the impaired bacterial killing observed in Die-P mice. In light of this, phagocytosis was examined by pHrodo labeled E.coli. pHrodo is a fluorophore with low fluorescence at neutral pH but whose fluorescence intensity increases with decreasing pH.

Following incubation with pHrodo:E.coli, Die-P neutrophils and macrophages show significantly less pHrodo fluorescence (Figure 2-6) at both time points, suggesting decreased phagocytosis overall. However, pHrodo is pH sensitive and phagosomal pH conditions vary, potentially confounding pHrodo data interpretation. As such, the term “pHrodocytize” is used in lieu of phagocytosis to reflect this.

Taken together, these data indicate that the failure of Die-P cells to generate bactericidal conditions (ROS) or internalize bacteria (pHrodo) result in decreased bacterial killing in-vivo and ex-vivo, leading to death.
Die-P Phagocytes “pHrodocytize” Less. Die-P peritoneal neutrophils phagocytize less pHrodo-E.coli at 6 and 24hr post-CLP. Macrophages phagocytize less at 24hr post-CLP. N=4-7 per group from 3 independent experiments where N represents an individual mouse. *=p<0.05, ***=p<0.001

Die-P Phagocytes are Immunosuppressed

The preceding data suggest that Die-P phagocytes are impaired as compared to Live-P, but the data does not indicate how or why they are impaired. It may be that Die-P cells are exhausted, meaning that the majority of their cells are refractory to stimulus and any activity that is observed comes from only a subset of cells responding. Alternatively, a similar percentage of cells between groups may respond to stimulus, but Die-P cells are weaker in their response, meaning that they are actively suppressed in their function. To determine this, a saturating dose of labeled bacteria was used to stimulate the peritoneal phagocytes. It was important to use a saturating dose of bacteria in order to ligate all available receptors because, despite equal binding of ligand by all cells, only a subset of cells will actually respond (i.e. phagocytize) [82]. A saturating dose induces the
maximum proportion of cells to respond. In conjunction with Trypan blue, cells that have
internalized bacteria can be discriminated from those that have not.

Using a saturating dose, similar proportions of cells phagocytize the bacteria at both time
points (Figure 2-7 B,D). However, Die-P cells phagocytize significantly less bacteria
overall than Live-P at both time points (C,E). The term phagocytic strength is used to
reflect the amount of internalized bacteria, as calculated by the gMFI of phagocytosis+
events after quenching of extracellular bacterial fluorescence by trypan blue. These data
suggest that Die-P peritoneal cells are suppressed in their function, but they are not
exhausted since a similar percentage of cells do phagocytize, albeit significantly less than
seen in Live-P.
Figure 2-7 Die-P Phagocytes Are Suppressed in Phagocytosis. Gating for Phagocytosis\textsuperscript{+} events: Ice control left, 37°C incubation right. Trypan blue was used to quench extracellular fluorescence (A). 6hr post-CLP, the percentage of Peritoneal cells from Live-P and Die-P mice that phagocytize bacteria (B), and overall amount of bacteria internalized (C). 24hr post-CLP (D,E) Data collected from 3-4 independent experiments. N=4/group for 6hr, N=5-7/group for 24hr, where N represents an individual mouse. *p<0.05, ***p<0.0001
Decreased phagocytosis could be due to decreased attachment of the bacteria to the cell surface. However, this does not appear to be the case as both groups’ exhibit similar levels of bacterial fluorescence when incubated on ice and without addition of TB (Figure 2-8).

**Figure 2-8 Attachment of Bacteria to Cell Surface.** No significant difference exists in binding of bacteria to the phagocyte cell surface 6hrs post-CLP. N=4/group from 3 independent experiments, where N = an individual mouse

In the same set of experiments, intraphagosomal processes were examined to determine the extent of the suppression and see whether once inside, bacteria were still exposed to the harsh microbicidal environment. This was accomplished using a recently developed technique in which *E.coli* were simultaneously labeled with Dichlorofluorescein (ROS sensor), pHrodo, and Alexafluor-350 (ROS/pH insensitive). Since TB quenching made extracellular staining for Mac/PMNs impractical, cellular responses were subdivided into High ROS and Low ROS cells. This was done by dividing each event’s DCF
fluorescence by its Alexafluor-350 fluorescence. In this manner, DCF fluorescence can be attributed to the amount of oxidation, and not to differences in the amount of labeled bacteria within the cell. A similar approach was used to determine phagosomal acidification, but with pHrodo normalized to Alexafluor-350. It was important to subdivide the cell populations so that small differences in a subset of cells would not potentially be masked by no differences in the majority of the cells. Furthermore, high ROS cells are loosely considered PMN and Low ROS cells are considered macrophages. This is based on the propensity of PMN to produce increased ROS [53], while macrophages have more phagosomal acidification [54], and the macrophage’s increased capacity for phagocytosis owing to its ability to synthesize new granule proteins [87] (Figure 2-9).

To determine if the decreased phagocytic strength of Die-P cells was due to decreased phagocytosis by either macrophages or neutrophils, the peritoneal phagocytic strength data (Figure 2-7 C,E) was re-examined by first stratifying the cell populations based on High or Low phagosomal ROS production (Figure 2-9, top panel). This approach shows that both Die-P neutrophils and macrophages are suppressed in their ability to phagocytize.
Figure 2-9 Gating for Putative Neutrophils and Macrophages based on Phagocytic Activity. Above-Contour Plot for Phagocytosis+ events, showing normalized ROS and pH fluorescence with High ROS as neutrophils and Low ROS cells as macrophages. Below- The Phagocytic strength, or overall amount of internalized bacteria, for High/Low ROS cells was calculated via its Alexa-350 fluorescence. *p<0.05,**p<0.01 ***p<0.001 N=4 at 6hr and 5-7 at 24hr from 3 independent experiments, where N = an individual mouse

As observed with phagocytosis, Die-P peritoneal phagocytes show decreased phagosomal maturation as compared to Live-P (Figure 2-10). Although significant differences in phagocytosis exist by 6hr, there are not significant differences in phagosomal ROS or pH in PMNs. However, there is significantly less phagosomal acidification in Die-P macrophages within 6hr (Figure 2-10 C), thus suggesting that multiple bactericidal
processes are suppressed soon after sepsis begins. By 24hrs post-CLP, the suppression of phagosomal maturation is more evident. Die-P PMNs produce significantly less phagosomal ROS, in addition to decreased phagosomal acidification (Fig 2-11 A-B). Similarly, Die-P macrophages continue to show significantly less phagosomal acidification when compared to Live-P macrophages (Fig 2-11 C). Taken together, these data strongly suggest that Die-P phagocytes are suppressed in their ability to internalize bacteria, and to create the harsh conditions necessary to kill internalized bacteria.

**Figure 2-10 Die-P Peritoneal Phagocytes are Partly Suppressed in Phagosomal Maturation, 6hr Post-CLP.** A, PMN phagosomal ROS was calculated by normalizing DCF (ROS sensitive) fluorescence to Alexa-350 fluorescence (ROS/pH insensitive). B, PMN phagosome acidification was calculated by normalizing pHrodo fluorescence to Alexa-350. C, Macrophage phagosome acidification. *p<0.05 N=4 from 3 independent experiments, where N = an individual mouse
Fig 2-11 Die-P Peritoneal Phagocytes are Suppressed in Phagosomal Maturation, 24hr post-CLP. A, PMN phagosomal ROS was calculated by normalizing DCF (ROS sensitive) fluorescence to Alexa-350 fluorescence (ROS/pH insensitive). B, PMN phagosome acidification was calculated by normalizing pHrodo fluorescence to Alexa-350. C, Macrophage phagosome acidification. *p<0.05, **p<0.01 ***p<0.001 N=5-7 from 3 independent experiments, where N = an individual mouse.

Calcium Signaling

Decreased phagocytosis and phagosomal function despite similar attachment of bacteria to the cell suggest that the signal to phagocytize is not propagated. Intracellular calcium is a key second messenger whose release from internal stores or extracellular influx drives a range of cellular processes. Decreased phagocytosis and phagosomal function despite similar attachment of bacteria to the cell suggest that the signal to phagocytize is not propagated [88, 89].

To determine if impaired Ca^{2+} signaling contributes to impaired phagocyte function in Die-P mice, cells were assayed for their ability to generate a calcium flux. Cells were loaded with the ratiometric probe, Indo-1 and subsequently stimulated with a saturating
dose of bacteria. Confirming earlier reports [90], a dose of 100-200 bacteria per cell was found to generate the maximum change in the Indo-1 ratio (Figure 2-12).

Figure 2-12 Determination of Saturating Dose of Bacteria. A, B- Typical Indo-1 fluorescence shifts for Naïve PMN stimulated with opsonized E. coli. C, A derived parameter of Indo-1 (violet) / Indo-1 (blue) was constructed to visualize calcium flux. D, Kinetic plots of the Indo-1 ratio for several doses of bacteria show dose/response saturation. Arrows indicate time of stimulation.

These preliminary studies were performed in naïve PMN isolated from bone marrow. However, when peritoneal cells from septic mice were used, a reliable calcium flux could
not be observed. This could be due to the cells being pre-activated and in a continuous state of activation, as suggested by the large spread in Indo-1 fluorescence prior to stimulation (Figure 2-13 A, B). This renders the calculation of baseline to peak indo-1 fluorescence shifts impractical. Furthermore, when viewing kinetic plots of some samples, a calcium flux appeared (Figure 2-13 C), as determined by the increase in the Indo-1 ratio. This occurred in the absence of commensurate increases and decreases in the Indo-1 violet and Indo-1 blue fluorescence, respectively (Figure 2-13 D, E). This suggests that other unknown factors influenced the Indo-1 ratio calculation such that it was not a suitable measure for the binding and releasing of Ca\(^{2+}\).

Figure 2-13 Calcium Signaling is not Suitable for Cells from Infectious Foci. Indo-1 plots shows broad range of fluorescence (A,B) before and after stimulation with
fluorescently labeled (TRITC) *E. coli* (C). Kinetic plots of Indo-1 ratio (D), Indo-1 Violet (E) and blue (F) show discordant responses of increasing violet emission without commensurate decrease in blue emission. Arrows indicate time of stimulus. Data representative of several experiments.

To conclude these results, a color coded table was created to illustrate all the differences and similarities between Live-P and Die-P mice at both time points (Table 2-1)

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- **Decrease Relative to Live-P**
- **Increase Relative to Live-P**
- **No significant difference**
- **Not Applicable**
- 

Table 2-1: Results Summary for Die-P mice, as compared to Live-P. Peritoneal cavity ROS results (right most column) obtained from Luminol based chemiluminescence, * indicates results for the putative cell type based since extracellular cell staining could not be performed (see Figure 2-9 Gating for Putative Neutrophils and Macrophages based on Phagocytic Activity. Above-Contour Plot for Phagocytosis+ events, showing normalized ROS and pH fluorescence with High ROS as neutrophils and Low ROS cells
as macrophages. Below- The Phagocytic strength, or overall amount of internalized bacteria, for High/Low ROS cells was calculated via its Alexa-350 fluorescence. *p<0.05,**p<0.01 ***p<0.001 N=4 at 6hr and 5-7 at 24hr from 3 independent experiments, where N = an individual mouse for details.)

Discussion

The most important finding in this study is that despite receiving equivalent inoculums and recruiting similar numbers of phagocytes in response to the inoculum, Die-P peritoneal phagocytes are impaired in their ability to kill bacteria. This provides strong evidence that the CLP model can be standardized, which has been a point of concern within the community [91]. Less obvious but of equal concern to other investigators is that without proper stratification of mice according to their likelihood of survival, considerable variability will permeate their studies. For example, cell function, organ injury, bacteria, and cytokines have been shown to differ significantly between survivors and non-survivors [75, 92]. Stratification would be of considerable benefit in human studies where heterogeneity in individuals and their responses are more pronounced. In support of stratification and our findings of decreased bacterial killing preceding death, Danikas et al showed phagocytic activity as prognostic for outcome in human sepsis [93].

While numerous studies have argued for and against organ injury preceding death from sepsis [92, 94, 95], this study strongly suggests that it is phagocytic impairment early on (within 6hr) that ultimately leads to uncontrolled microbial growth and death. This is despite the use of broad spectrum antibiotics. It may be that failure to contain the initial
infection allows the intense inflammatory reaction to ripple throughout the organism, leading to organ injury. The successful eradication of microbial pathogens requires recruitment of myeloid cells to the site of infection [96, 97]. To that end, other labs have shown that increasing or inhibiting neutrophil recruitment following CLP [98, 99] affects bacterial burden and animal survival. Our lab has shown that prior recruitment of neutrophils also increases survival [22]. However, that study and this one demonstrate that the mice that are predicted to die do not fail to recruit neutrophils or monocytes/macrophages as compared to the mice predicted to live. In fact Die-P mice recruit more cells by 24hr post-CLP. Similar findings were recently reported by another lab that used CLP to produce ~ 60% mortality in control mice [100]. In it they demonstrated that without GM-CSF producing B-cells, CLP resulted in 100% mortality. Most importantly, as it relates to this study, the B-cell GM-CSF^{−/−} mice recruited significantly more neutrophils to the peritoneum, yet still had increased bacterial burden, increased inflammatory mediators, and decreased phagocytosis as observed in our model. Taken all together, this suggests that while phagocyte recruitment is important, their functional status is more important.

Although this study strongly argues for impaired phagocyte function as precipitating death, it does not address the role of lymphocytes, which are fundamental to an immune response. Others have shown that treatments affect lymphocyte apoptosis and function resulting in increased survival [101, 102]. While these studies did not stratify by survival likelihood, it is interesting to speculate that impaired lymphocyte function or
death is driving the phagocyte dysfunction observed in our model. Certainly the report by Rauch et al supports this. Another limitation to this study is that only peritoneal cells were evaluated for performance. It would be interesting to know if Die-P phagocytes become impaired upon their arrival to the peritoneum, or if their impairment exists prior to their arrival. Delano et al showed that CLP results in suppressed ROS burst by monocytic splenocytes and bone marrow neutrophils. It will be important to determine to what extent systemic alterations are implicated in a survivor/non-survivor model of sepsis.

There is considerable evidence that suggests that overstimulation of cells with contradictory stimuli (i.e. pro and anti-apoptosis agents) can result in a terminally non-responsive state, affectionately termed “zombie cells” [103]. Along similar lines, Die-P mice demonstrate a significantly larger surge in pro and anti-inflammatory mediators in their peritoneum’s and plasma [22], potentially creating the conditions for terminally non-responsive cells that render them incapable of defending the host. In light of this, perhaps the most hopeful finding of this study is that Die-P cells are functionally intact and equally capable of responding to bacteria, although they are deficient in both the amount they can phagocytize and how they process their phagosomal cargo. That they are responsive makes them attractive targets for functional modulators, such as antagonists of adenosine receptors or programmed death receptor-1, both of which have been suggested to increase phagocyte function and improve survival from sepsis [77, 104].
CHAPTER THREE: SYSTEMIC ALTERATIONS OF PHAGOCYTE FUNCTION IN THE CLP MODEL OF SEPSIS

Introduction

Mortality from sepsis increases as its pronounced aberrations spread throughout the body, first producing leukopenia, then hypovolemia, Multi-Organ Dysfunction Syndrome (MODS) which may culminate in death. The organs most likely to fail are the lungs, kidneys, liver and pancreas [66, 105-108]. A key component of sepsis therapy is to maintain organ perfusion and blood pressure, with the hope that this provides the host enough time to resolve its disordered state. However, current treatments lack interventions meant to restore immunological function.

Systemic immunosuppression is increasingly seen as a key mediator of sepsis immunopathology [109]. In mice and humans, sepsis results in a profound depletion of splenic lymphocytes and dendritic cells [110-112], as well as their functional suppression [113]. Similarly in mice, it was shown that CLP induced sepsis leads to decreased reactive oxygen species burst (ROS) in splenic and bone marrow granulocytes [114]. Although revealing, these studies did not compare non-survivors to survivors of sepsis.

The previous chapter provided evidence why some mice die from CLP while others do not: Decreased bacterial killing and bactericidal function by peritoneal phagocytes. This does not, however, make the distinction of whether Die-P phagocytes are intrinsically inferior to their Live-P counterparts. It may be that phagocyte immunosuppression is
localized to the peritoneum, following CLP. Before the phagocytes can engage the infection, they must navigate from the bone marrow through the circulation until they reach the peritoneum. We sought to determine if Die-P phagocytes are suppressed in their function prior to their arrival to the peritoneal cavity.

**Methods**

**Animals**

Female ICR (CD-1) mice (Harlan-Sprague Dawley, Inc., South Easton, MA) between 24-28 grams were used for all studies. Mice were received and allowed to acclimate to our housing room for at least 96 hours prior to surgery. Mice were kept in a temperature and humidity controlled room with a diurnal cycle of 12 hours light, 12 hours dark. Mice were given food and water ad libitum for the entire duration of the experiment. The experiments were approved by the Boston University Animal Care and Use Committee.

**Sepsis Model**

Cecal ligation and puncture was performed as first described [29] with minor modifications [41]. Mice were first anesthetized in an induction chamber with 5% isofluorane, followed by 3% for maintenance during surgery. A midline incision was performed through the skin and then the linea alba. The cecum was exteriorized and ligated below the illeocecal valve with 4-0 silk. Approximately two-thirds the length of the cecum from the distal tip was ligated, then double punctured longitudinally with a 16 gauge needle. A small amount of cecal contents was squeezed out to ensure patency, then the cecum was returned to its original position. The abdomen was closed with 4-0 silk
sutures, and the skin closed with wound glue (Nexaband- Veterinary Products Laboratories, Phoenix, Az). Mice were resuscitated with 1mL warm saline (37°C) with buprenorphine (0.05mg/kg) for pain management (1 injection every 12 hours for the first two days post-CLP). Antibiotic treatment with 25mg/kg Imipenem was administered 2 hours post-CLP, and then once every 12 hours for the first 5 days. Mice sacrificed at 6 hours received one dose of drugs, while mice sacrificed at 24 hours received 3 doses.

**Sampling**

Blood sampling was performed at 6 or 24 hours, depending on when the animal was sacrificed. 20uL of blood was collected during exsanguination via the retro-orbital venous plexus and diluted 1/10 in PBS containing 3.38mM EDTA tripotassium salt, and immediately placed on ice. The remainder of the exsanguination volume was collected into 100uL Heparin (1000U/mL). Blood diluted 1/10 with PBS/EDTA was centrifuged for 5 min at 1000 X g at 4°C, and the plasma was collected and frozen at -20°C until subsequent analysis for IL-6 to determine if the mice were predicted to live or die.

Bone Marrow cells were flushed from the two hind tibias with cold HBSS + heparin using a 25 gauge needle. Cells were centrifuged, the pellet lysed with 1mL 0.2% hypotonic saline for 40 seconds, then restored to isotonicity with 1.6% hypertonic saline. Cells were washed once and resuspended in HBSS containing Ca$^{2+}$ and Mg$^{2+}$ + 1% BSA. Total cell counts were performed with a Beckman-Coulter particle counter model ZF (Coulter electronics Inc., Hialeah, Fl).
**pHrodo and ROS Measurement**

Heat killed pHrodo-red *E. coli* (Life Technologies, Carlsbad, CA) were opsonized with *E. coli* specific antibodies. Bacteria were incubated with bone marrow cells (4*10^6/ml) at a 20:1 ratio for 30 minutes at 37°C. pHrodo fluorescence was calculated as gMFI of cells incubated at 37°C minus the gMFI of cells incubated on ice.

ROS was measured by the conversion of non-fluorescent Dihydrorhodamine 1,2,3 (DHR-123) to fluorescent R-123. Cells were loaded with 2uM DHR-123 and stimulated with opsonized heat-killed *E. coli* or 100nM Phorbol-12 Myristate-12 Acetate (PMA). PMA stimulation was limited to 20 minutes. The magnitude of the ROS burst was calculated by the % increase of R123 fluorescence (gMFI) of stimulated cells over unstimulated cells. Reactions were stopped by placing cells on ice, followed by extracellular marker staining for flow cytometry.

**3X-Labeled Bacteria Preparation**

Labeling was performed as previously published[81]. Briefly 2*10^{10}/mL of heat-killed (80°C 1hr) HB101 *E. coli* (same strain used for bacterial killing assay) were sequentially labeled with 50uM DCF-SE, 50uM pHrodo-SE, and 159uM Alexafluor-350-SE (Life Technologies). Labeling was performed in PBS pH 9.0, degassed and purged with N\textsubscript{2} to minimize auto oxidation of DCF. Bacteria were washed 3X with PBS pH 8.0, and resuspended in PBS pH7.4 (N\textsubscript{2}) and stored in glass vials flushed with N\textsubscript{2}. Bacteria (~1.5*10^{10}/mL) were opsonized with 1/10th volume of normal mouse plasma (heparinized) and anti-*E. coli* antibodies (50ug/mL) (Life Technologies).
3X-Labeled Bacteria Phagocytosis

Bone marrow cells were loaded into polypropylene cryovial tubes at 4*10⁶/mL (final volume of 0.25mL). While on ice, ~150 bacteria/cell were added to cells (10uL OD₆₀₀ 0.105). DNAse was added (50ug/mL) to prevent aggregation of cells. Tubes were transferred to water bath 37°C, placed on heated stir plate, and agitated at lowest speed for 30 minutes with 7x2mm micro stir bars. For controls, samples were also incubated on ice for 30 minutes. Following incubation, all cells were kept on ice until data acquisition with a flow cytometer. No wash steps were performed.

240uL of Peripheral blood was loaded into tubes and given 10uL bacteria, as above. After incubation with bacteria, blood was lysed with 12 volumes of Ammonium Chloride lysis buffer on ice for 5 minutes and centrifuged at 450 X g for 8 minutes at 4°C. The pellet was washed once and resuspended in 250uL HBSS containing Ca²⁺ and Mg²⁺ + 1% BSA. Cells were strained through 70uM filter prior to flow cytometry.

Flow Cytometry

All data was acquired on an LSRII (Bd Biosciences).

pHrodo/DHR-123- Cellular FcReceptors were blocked with Fc Block (Bd Biosciences). The following antibodies were used: CD11b (clone M170), CD19 (1d3), CD3e (145-2C11), Ly6G (1A8), Gr-1 (RB6-8C5), F480 (Ci-A13), Ly6C (HK1.4). Only Sytox Blue negative events (Live Cells) were used for gating. Doublet discrimination was performed by Fsc-A vs Fsc-H. BD compensation beads (BD biosciences) were used for
compensation. Additionally, Sytox Blue and R-123 were used to stain cells and compared to unstained cells for compensation against those parameters.

3x Labeled Bacteria- DCF, pHrodo, and Alexafluor-350 fluorescence was collected by (wavelength/band pass) 530/15, 590/10, and 450/50 filters. It was important to use a custom 590/10 filter for pHrodo because Trypan Blue auto fluoresces when bound to protein and its emission begins at ~615nm. The stock filter supplied by the core facility was a 610/20bp.

Initial PMT settings were chosen such that the MFI of pHrodo and Alexa-350 had a 1:1 ratio. DCF PMT were set between 0-100 (DCF has little fluorescence prior to oxidation) (Figure 3-1). Once the bacterial settings were in place, a positive control (healthy cells + 3x bacteria) was acquired to ensure all events were on scale. PMTs were adjusted as needed, and then “locked in” with calibration particles. Prior to sample acquisition, Spherotec rainbow 7-peak calibration particles were run to ensure that the PMT settings used were appropriate (<±5% of the first experiment’s MFIs). 3x-labeled bacteria were also collected for quality control purposes. Equal volumes of beads, bacteria, and diluent
(PBS) were used, and events were acquired at the same flow rate from day to day.

Figure 3-1: 3X-Labeled Bacteria Fluorescence: Histograms are shown to depict how PMT gains were set on the flow cytometer to achieve the desired fluorescence of individual bacteria. Median fluorescent intensities are shown below the histograms.

Ice controls were acquired first. ~20,000 events were collected to measure attachment.

Then, Trypan Blue was added to tube (0.25% Final concentration), incubated on ice for 1 minute, and then ~30,000 gated events were collected. Cells that were allowed to phagocytize were only acquired with TB present.

Data was analyzed with Flowjo (Treestar Inc.). Non-debris cells were gated on by Fsc/Ssc. Doublets were removed by fsc-h vs. fsc-w, with care taken to not remove activated cells from analysis. Phagocytosis + gates were constructed for each sample based off its own ice control (no phagocytosis occurs.) The MFI of quenched ice control cells was subtracted from each channel of the 37°C sample, to account for the minimal contribution of autofluorescence (minimal because the PMTs were set low enough to get
positive single bacterial events to read near zero.) Derived parameters were constructed within Flowjo so that the DCF or pHrodo fluorescence for each event was divided by the fluorescence of Alexa-350 for that event. The ROS index was then calculated by dividing the DCF/Alexa ratio of the cell by the DCF/Alexa ratio of the bacteria. Similar calculations were performed for the Acidification Index (pHrodo).

Results

CLP Leads to Alterations in Bone Marrow

The bone marrow (BM) is the site of myelopoeisis and it contains large reserves of neutrophils and monocytes that are released during an infection [115]. Previous studies have documented systemic granulocytopenia post-CLP in a sham/CLP model. The same study also observed decreased NOX ROS generation by BM granulocytes (CD11b+/GR-1+ cells, which encompass PMNs and monocytes). It was suggested that these systemic alterations account, in part, for increased mortality from a secondary infection [114]. To determine if there was a difference in BM “reserves” after CLP that may contribute to Die-P mortality, total BM counts, granulocyte frequency, and granulocyte function was examined.

Unlike the comparison between sham/CLP mice, there were not significant differences between Live-P and Die-P mice when comparing the total number of BM cells retrieved (Figure 3-2 A) or the percentage of BM cells that were granulocytes (Figure 3-2 B), although there was a marked reduction in cellularity between 6 and 24hr. To determine if
BM granulocytes exhibited differences in function, cells were assessed for two bactericidal processes: ROS Burst and “pHrodocytosis”- a term meant to reflect the increase in pHrodo fluorescence that is due to phagocytosis, but with the caveat that differential pH changes within the phagosome could result in different signal intensities without there being a difference in the number of pHrodo: bacteria internalized.

There was no discernible difference in BM granulocyte pHrodocytosis or ROS generation within 6hr post-CLP (Figure 3-2 C). Interestingly, Die-P cells displayed significantly increased bactericidal activity in response to a physiological stimulus (opsonized *E. coli*) by 24hr (Figure 3-2 C,D). However, when assessed for their capacity to generate ROS via PMA, a non-receptor based activator, there was no significant difference in ROS (Figure 3-2 E). This suggests that that while BM granulocytes from Live/Die-P mice have a similar capacity to respond, when challenged with a physiologically relevant stimulus, their responses differ.
Figure 3-2 Die-P Mice Bone Marrow Granulocytes Show Increased Functional Response. A, Total number of cells recovered from two hind tibias. B, Percentage of bone marrow cells that are granulocytes. C, pHrodo fluorescence of BM granulocytes incubated with pHrodo labeled E.coli. D-E, The percent increase in R-123 fluorescence intensity of BM granulocytes incubated with E.coli or PMA over cells incubated without stimulus. 6hr N= 4-5/group, 24hr N=5-7/group from 3-4 independent experiments, where N represents an individual mouse. **=p<0.01

CD11b, a transmembrane protein involved in phagocytosis and adhesion, is widely considered as a marker of granulocyte activation [116, 117]. To determine if Die-P BM cells are more activated and thus more responsive to bacteria, CD11b expression was measured. 24hr post-CLP, Die-P BM granulocytes have significantly increased CD11b expression (Figure 3-3), which may partly explain their increased activity. Collectively these data suggest that exhaustion of BM granulocyte reserves does not occur in Die-P
mice, although hyper-activation of the Die-P BM granulocyte pool may occur between 6 and 24hr post-CLP.

**CD11b Expression Level**

![Bar graph showing CD11b expression levels for Live-P and Die-P BM granulocytes.](image)

Figure 3-3 Die-P BM Granulocytes Express Significantly More CD11b on Their Surface: 24hr post-CLP, there is significantly more CD11b associated fluorescence on Die-P BM granulocytes, as compared to Live-P. *p<0.05. N=3-4/group where N represents an individual mouse.

**Die-P Mice have Reduced Phagocytic Activity in Peripheral Blood**

We next sought to extend our observations in the BM, to the phagocytes circulating in the peripheral blood (PB). However, there were insufficient cells recovered to perform the assays due to the leukopenia and hypovolemia induced by CLP. To circumvent this, the 3x-labeled bacteria discussed in the previous chapter were used to determine PB phagocyte function in whole blood. Ice controls to prohibit phagocytosis and set phagocytosis+ gates (see chapter 2) were not used due to limited blood volume, and because cell washing steps were necessary following RBC lysis. Because lymphocytes do...
not phagocytize and they are prevalent in mouse blood, they were used to set fluorescence thresholds for phagocytosis+ events (Figure 3-4).

![Figure 3-4 Peripheral Blood Gating Strategy](image)

**Figure 3-4 Peripheral Blood Gating Strategy:** Left Panel, red blood cell debris was excluded from analysis. Thick gates show granulocyte and lymphocyte populations. Center panel, phagocytosis positive events were identified by high Alexa-350 fluorescence, relative to non-phagocytizing lymphocytes. Right panel, Normalizing DCF (ROS) and pHrodo (Acidification) fluorescence relative to Alexafluor-350 fluorescence reveals 2 major populations of High and Low phagosomal ROS.

Incubating whole blood with a saturating dose of bacteria revealed that PB Die-P phagocytes phagocytize significantly less bacteria 24hr post-CLP (Figure 3-5), thus suggesting there is some degree of phagocyte suppression that exists beyond the peritoneum.
Figure 3-5 Die-P Peripheral Blood Phagocytes Phagocytize Less Bacteria, 24hr post-CLP: Left Panel, red blood cell debris was excluded from analysis. Thick gates show granulocyte and lymphocyte populations. Right panel, phagocytosis positive events were identified by high Alexa-350 fluorescence, relative to non-phagocytizing lymphocytes. Bottom Panel, Alexa-350 fluorescent intensity of Phagocytosis+ events reflects the overall amount of bacteria internalized (phagocytic strength). **=p<0.01

We next sought to determine if the suppression of phagocytosis extended to the subsequent bactericidal mechanisms that are activated within the phagosome. After normalizing DCF (ROS sensitive) and pHrodo (pH sensitive) fluorescence to Alexafluor-350 (ROS/pH insensitive) fluorescence, two major cell populations were observed
(Figure 3-4, right panel). Gates were constructed to analyze High ROS and Low ROS cells separately so that important phagosomal differences were not obscured. These distinct populations may reflect different cell types. Based on the prevalence of High ROS cells and their high ROS activity, they are likely PMNs. Low ROS cells are likely monocytes as they produce less ROS than PMNs and are less prevalent in the blood of mice.

Looking within the phagosomes, Die-P High ROS cells generate less phagosomal ROS than Live-P, while acidifying their phagosomes more (Figure 3-6 A,B). This may be due to the decreased ROS production because ROS itself alkalinizes the phagosome. Conversely, there is no difference in acidification in Low ROS cells, although their overall phagocytic strength is reduced (Figure 3-6 C,D). Collectively these data suggest that Die-P phagocytes are suppressed in bactericidal activity prior to their arrival to the infection in the peritoneum.
Figure 3-6 Die-P Peripheral Blood Phagocytes are Suppressed in Phagocytic Activity, 24hr post-CLP: A, Phagosomal ROS Index for High ROS cells, calculated by normalizing DCF fluorescence to Alexafluor-350 fluorescence. B-C, Phagosomal Acidification for High or Low ROS cells, calculated by normalizing pHrodo fluorescence to Alexafluor-350 fluorescence. DE, Alexa-350 fluorescent intensity of internalized bacteria for High or Low ROS cells. *=p<0.05 **=p<0.01

Phagocytosis initiates when a phagocyte makes contact with a microbe via Pattern Recognition Receptors. The most studied of these are the complement and Fc-receptors (FcR), which participate in both opsonin dependent and independent phagocytosis [118]. To determine whether differential expression of these receptors could account for the
differences in phagocytosis observed, peripheral blood cells were stained for FcγRII (CD32), FcγRIII (CD16), and CD11b. Somewhat expectantly, Die-P mice had increased CD11b expression on peripheral phagocytes (Figure 3-7 C), as was observed for the BM cells. Surprisingly, Die-P cells displayed increased FcR expression as compared to Live-P (Figure 3-7 A,B). For FcR staining, cells were stained first with an antibody to CD16 and then with antibody that recognized both CD16 and CD32. It is likely that the increased CD16/32 (Figure 3-7 B) staining is due to increased expression of CD32 as CD16 was pre-blocked with the CD16 antibody.
A. FcγRIII expression

B. CD16/32 expression

C. CD11b expression

Live-P  Die-P
**Figure 3-7 Die-P Peripheral CD11b+/Gr-1+ Granulocytes Express More Markers of Activation and Inhibition than Live-P.** A, Fluorescence intensity of the Activating FcRIII. B, Fluorescence intensity of the Inhibitory FcR, CD32. C, Fluorescence intensity of the activation marker, CD11b.

**Discussion**

Our previous study (chapter 2) showed that peritoneal phagocytic suppression precedes mortality from CLP induced sepsis. This study extended those findings to cellular compartments distal to the infection. While many sepsis studies focus on systemic alterations, many of these look at particular organs (i.e. lungs, spleen, liver) examining organ injury, leukocyte recruitment or adaptive system components [119-122], we believe this is the first to compare phagocytic function of cells in route to the infectious foci and document the differences between survivors/non-survivors.

This study examined systemic alterations in the context of broad spectrum antibiotic administration, which prevents detectable bacteremia. This enabled a look at phagocytic function prior to phagocyte: pathogen interaction. One concern that emanated from our previous findings in the peritoneum was that the peritoneal environment became increasingly hostile towards phagocytic function. Possible reasons for this include the increasing cellular debris present in Die-P samples (data not shown), especially by 24 hr, which may hamper bacterial clearance, and perhaps increased bacterial dissemination within the peritoneum. The most important finding from this study is that peripheral blood phagocytes from Die-P mice are suppressed in phagocytic activity prior to their arrival to the peritoneum. Because the mice used in these studies are heterogeneous
outbred mice, it tempting to speculate that the impaired phagocytic function observed in
the peritoneum and the periphery are due to intrinsic cellular variation; some mice were
born with greater phagocytic capacity. While this argument is difficult to refute in its
entirety, genetics is not suspected to have a significant role in our model. This is because
our model of 50 percent mortality has been reproduced in inbred mouse strains such as
BALB\c [75]. Furthermore, while ICR mice are heterogeneous, they are not wild mice
and are inbred by comparison.

It has been previously shown that the degree of receptor cross-linking can influence
cellular responses such as calcium flux, degranulation, ROS, or switching from a pro-
inflammatory to anti-inflammatory phenotype [123-125]. Here we have shown an
increased density of markers for activation (CD11b, CD16). It may be that the increased
density of receptors (and likely others not measured here) results in increased signaling
for a given concentration of stimulus. It has been previously shown that Die-P mice have
increased circulating cytokines, chemokines [22] and mitochondrial DAMPs (MTDs)
(unpublished data). This could result in neutrophil degranulation [126] or an ROS burst
prior to their arrival in the peritoneum. This may in part explain the switch from hyper-
reactive BM phagocytes, to hypo-reactive peripheral phagocytes in Die-P mice, despite
increased complement receptor and FcR expression. In further support of this is the
increased expression of CD32 on Die-P peripheral phagocytes. CD32, unlike other FcRs,
has an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) intracellular domain
rather than an Immunoreceptor Tyrosine-based Activating Motif (ITAM). This results in
suppression of inflammatory signaling, the importance of which is exemplified by the
development of autoimmunity in its absence [127] or the suppression of autoimmunity
via its engagement by Intravenous Immunoglobulin (IVIG) [128]. It will be important to
determine whether the increased expression of FcR expression is correlated with or
causing of the systemic immune suppression following CLP, as they represent attractive
targets for immune modulation.

Further support of the suppressed phenotype of phagocytes in sepsis was recently
provided by a study using adenosine receptor antagonists. Adenosine accumulates in
hypoxic and inflamed tissues where it suppresses the inflammatory activity of phagocytes
[129]. Belikoff et al showed that blocking adenosine receptor 2b of Die-P mice improved
survival, and this benefit was at least in part due to increasing the phagocytic activity of
macrophages [77].

Immunosuppression is increasingly seen as detrimental to the host during sepsis [130].
Although there is a negligible inflammatory signature during the chronic phase of sepsis,
there is a surge in inflammatory mediators just prior to mortality [131] that is similar to
the acute phase surge, albeit of less amplitude. Although too much inflammation is
implicated in a variety of diseases, for sepsis, it may be that we need to release the
brakes.
4 CHAPTER FOUR: THE EFFECTS OF THE SEPTIC INFLAMMATORY MILIEU ON PHAGOCYTIC FUNCTION

Introduction

Sepsis is a systemic disease that is propagated by immune dysregulation that stems from massive trauma and/or infection. In the prototypical regulated immune response, a traumatic or infectious insult triggers a pro-inflammatory response consisting of biochemical mediators that primes the immune system for action. Once initiated, the pro-inflammatory drive is thought to self-amplify in a feed-forward positive feedback loop. When the pro-inflammatory response is of sufficient magnitude, a compensatory anti-inflammatory response (CAR) is triggered that serves to restrain inflammation and prevent excessive bystander damage to the host [24, 25]. However, there is evidence that this system of checks and balances may itself initiate the immune dysregulation prevalent in sepsis.

Unlike the wave-like transition between pro-inflammatory and anti-inflammatory states in classical immune responses, the enormous insult that precedes sepsis produces a simultaneous surge in pro and anti-inflammatory mediators that are of similar magnitude [132]. It is thought that this produces a state of immunosuppression as evidenced by clinical studies documenting viral reactivation [133, 134], opportunistic infections, and global leukocyte suppression [130].
Previously it has been shown that Die-P mice produce a larger surge in pro and anti-inflammatory mediators as compared to Live-P. This increased surge is evident within 6hr post-CLP in the plasma, and by 24hr in the peritoneum [22]. Similarly, Die-P mice show suppressed phagocytic function within 6hr and this suppression extends beyond the peritoneum and into the peripheral blood (chapters 2-3). This study sought to determine if the inflammatory milieu, produced by Die-P mice within the peritoneum or released systemically into the blood, would suppress phagocytic function of naïve cells, relative to Live-P.

**Methods**

**Animals**

Female ICR (CD-1) mice (Harlan-Sprague Dawley, Inc., South Easton, MA) between 24-28 grams were used for all studies. Mice were received and allowed to acclimate to our housing room for at least 96 hours prior to surgery. Mice were kept in a temperature and humidity controlled room with a diurnal cycle of 12 hours light, 12 hours dark. Mice were given food and water ad libitum for the entire duration of the experiment. The experiments were approved by the Boston University Animal Care and Use Committee.

**Sepsis Model**

Cecal ligation and puncture was performed as first described [29] with minor modifications [41]. Mice were first anesthetized in an induction chamber with 5% isofluorane, followed by 3% for maintenance during surgery. A midline incision was performed through the skin and then the linea alba. The cecum was exteriorized and
ligated below the illeocecal valve with 4-0 silk. Approximately two-thirds the length of the cecum from the distal tip was ligated, then double punctured longitudinally with a 16 gauge needle. A small amount of cecal contents was squeezed out to ensure patency, then the cecum was returned to its original position. The abdomen was closed with 4-0 silk sutures, and the skin closed with wound glue (Nexaband- Veterinary Products Laboratories, Phoenix, Az). Mice were resuscitated with 1mL warm saline (37°) with buprenorphine (0.05mg/kg) for pain management (1 injection every 12 hours for the first two days post-CLP). Antibiotic treatment with 25mg/kg Imipenem was administered 2 hours post-CLP, and then once every 12 hours for the first 5 days. Mice sacrificed at 6 hours received one dose of drugs, while mice sacrificed at 24 hours received 3 doses.

**Sampling**

Blood sampling was performed at 6 or 24 hours, depending on when the animal was sacrificed. 20uL of blood was collected during exsanguination via the retro-orbital venous plexus and diluted 1/10 in PBS containing 3.38mM EDTA tripotassium salt, and immediately placed on ice. The remainder of the exsanguination volume was collected into 100uL Heparin (1000U/mL). Blood was centrifuged for 5 min at 1000 X g at 4°C, and the plasma was collected and frozen at -20°C until subsequent analysis for IL-6 to determine if the mice were predicted to live or die. The peritoneal cavity was then lavaged with 1mL warm Hanks’ Balanced Salt Solution (HBSS, Mediatech Inc., Herndon, VA) containing 10U/mL Heparin, followed by centrifugation and storage of the supernatant at -20°C.
Naïve cells: Bone Marrow cells were flushed from the two hind tibias and femurs with cold HBSS + heparin using a 25 gauge needle. Cells were centrifuged, the pellet lysed with 1mL 0.2% hypotonic saline for 40 seconds, then restored to isotonicity with 1.6% hypertonic saline. Cells were layered onto a discontinuous percoll gradient of 40, 50, and 62.5% percoll, and centrifuged at 550 X g for 30min at 4°C. All but the pellet was aspirated, and the cell pellet (containing the naïve PMNs) was washed once in PBS with 0.5% BSA. Resident Peritoneal cells (primarily B1 B-cells and macrophages [85, 86, 135]) were isolated from the same naïve mouse via peritoneal lavage with warm HBSS containing 0.5mM EDTA. The lavage fluid was immediately mixed with cold HBSS w/ Ca$^{2+}$/Mg$^{2+}$ to prevent intracellular depletion of Ca$^{2+}$/Mg$^{2+}$. Cells were washed and then resuspended in HBSS w/ Ca$^{2+}$/Mg$^{2+}$ 1%BSA at high concentration (15*10^6/mL) to minimize subsequent dilution of peritoneal fluid or plasma. Cells were diluted to final concentration of 4*10^6/mL (~70% PMN, 30% resident peritoneal cells.) Cells were then incubated at 37° For 45 minutes, followed by incubation with bacteria for 30 minutes.

**3X-Labeled Bacteria Preparation**

Labeling was performed as previously published[81]. Briefly 2*10^10/mL of heat-killed (80°C 1hr) HB101 E.coli (same strain used for bacterial killing assay) were sequentially labeled with 50uM DCF-SE, 50uM pHrodo-SE, and 159uM Alexafluor-350-SE (Life Technologies). Labeling was performed in PBS pH 9.0, degassed and purged with N$_2$ to minimize auto oxidation of DCF. Bacteria were washed 3X with PBS pH 8.0, and
resuspended in PBS pH7.4 (N₂) and stored in glass vials flushed with N₂. Bacteria (~1.5*10¹⁰/mL) were opsonized with 1/10ʰ volume of normal mouse plasma (heparinized) and anti-E.coli antibodies (50ug/mL) (Life Technologies).

**Phagocytosis Assay**

Cells were diluted to final concentration of 4*10⁶/mL (~70% PMN, 30% resident peritoneal cells.) with peritoneal fluid, plasma, or HBSS w/ Ca²⁺/Mg²⁺ 1%BSA, and placed into a polypropylene cryovial (final volume = 0.25mL). Tubes were transferred to a 37°C water bath, placed on heated stir plate, and agitated at lowest speed for 45minutes with 7x2mm micro stir bars. After the 45minute incubation, a saturating dose of 3x-labeled bacteria were added to each tube. Following incubation, all cells were kept on ice until data acquisition with a flow cytometer. No wash steps were performed.

**Flow Cytometry**

*All data was acquired on an LSRII (Bd Biosciences).*

Doublet discrimination was performed by Fsc-A vs Fsc-H. BD compensation beads (BD biosciences) were used for compensation. Additionally, Sytox Blue and R-123 were used to stain cells and compared to unstained cells for compensation against those parameters. 3x Labeled Bacteria- DCF, pHrodo, and Alexafluor-350 fluorescence was collected by (wavelength/band pass) 530/15, 590/10, and 450/50 filters. It was important to use a custom 590/10 filter for pHrodo because Trypan Blue auto fluoresces when bound to
protein and its emission begins at ~615nm. The stock filter supplied by the core facility was a 610/20bp.

Initial PMT settings were chosen such that the MFI of pHrodo and Alexa-350 had a 1:1 ratio and were ~100-200MFI. DCF PMT were set between 0-100 (DCF has little fluorescence prior to oxidation). Once the bacterial settings were in place, a positive control (healthy cells + 3x bacteria) was acquired to ensure all events were on scale. Trypan Blue was added to tubes (0.25% Final concentration), incubated on ice for 1 minute, and then ~30,000 gated events were collected.

Data was analyzed with Flowjo (Treestar Inc.). Non-debris cells were gated on by Fsc/Ssc. Doublets were removed by fsc-h vs. fsc-w, with care taken to not remove activated cells from analysis. Phagocytosis+ gates were constructed for each sample based off an ice control (no phagocytosis occurs.) The MFI of quenched ice control cells was subtracted from each channel of the 37°C sample, to account for the minimal contribution of autofluorescence (minimal because the PMTs were set low enough to get positive single bacterial events to read near zero.) Derived parameters were constructed within Flowjo so that the DCF or pHrodo fluorescence for each event was divided by the fluorescence of Alexa-350 for that event.

**Statistical Analysis**

Statistics were performed using Prism 5 software (Graphpad Software, San Diego, CA). For comparison between 2 groups, an unpaired Student t test was used.
Results

To recapitulate the early stages of CLP induced sepsis, naïve peritoneal cells were combined with naïve PMNs in a ratio that mimicked the 6hr post-CLP cellular profile of the peritoneum. Cells were first incubated with peritoneal fluid from Live-P or Die-P mice, or HBSS w/ Ca\(^{2+}\)/Mg\(^{2+}\) 1%BSA as a control, in order to program the cells prior to incubation with bacteria. Peritoneal fluid harvested from mice at 24hr post-CLP was used because the difference between the pro and anti-inflammatory surge of Live-P and Die-P mice was not statistically significant at 6hr [22].

Incubation with Die-P peritoneal fluid did not result in a difference in the percentage of cells phagocytizing (Figure 4-1 B), although overall phagocytosis was decreased (C). This mimics the data shown in chapter 2 (Figure 2-7), where the overall percentage of cells phagocytizing was similar, but the amount of bacteria phagocytized (phagocytic strength) was decreased in Die-P mice. Due to the small sample size for control cells (n=1), it is not clear from this data whether Live-P peritoneal fluid alters phagocytic function relative to cells incubated in HBSS alone.
Figure 4-1 Die-P Peritoneal Fluid Decreases Phagocytosis by Naïve PMN. A, Gating strategy to identify phagocytizing cells. Cells were incubated on ice with bacteria (left panel) to prevent phagocytosis and then used to construct gates to exclude non-phagocytizing cells when analyzing cells incubated at 37°C (right panel). B, The percentage of cells phagocytizing bacteria. C, the overall amount of internalized bacteria, determined by the fluorescent intensity of Alexa-350+ events. N=3/group, with each n representing peritoneal fluid from an individual mouse. N=1 for HBSS/BSA control.

Quenching the extracellular fluorescence of attached, but not internalized, bacteria with trypan blue, enables changes in phagosomal maturation to be observed. To determine if the biochemical mediators of CLP-mice affected intracellular processing of bacteria, phagosomal ROS and acidification indices were calculated by normalizing DCF (ROS sensitive) and pHrodo (pH sensitive) fluorescence to Alexafluor-350 (pH/ROS...
insensitive) fluorescence. Similar to what is seen with septic cells (chapter 2), naïve cells incubated with Die-P peritoneal fluid demonstrate decreased phagosomal acidification and ROS generation (Figure 4-2). This suggests that the pro/anti-inflammatory surge within the peritoneal cavity of Die-P mice mediates, in part, the immunosuppression of, and the reduced bacterial killing by, peritoneal phagocytes that was evident in septic mice (chapter 2). Due to the small sample size for control cells (n=1), it is not clear from this data whether Live-P peritoneal fluid alters phagocytic function relative to cells incubated in HBSS alone.

![Figure 4-2: Die-P Peritoneal Fluid Suppresses Phagosomal Maturation of Naïve PMN](image)

Phagocytosis (Alexa-350+) events were analyzed for Phagosomal ROS (A), and Phagosomal Acidification (B) by ratioing DCF or pHrodo fluorescence to Alexafluor-350 fluorescence, respectively. N=3/group, with each N representing peritoneal fluid from an individual mouse. N=1 for HBSS/BSA control.

While the bulk of bacterial killing occurs at the infectious source, phagocytes must first navigate to the infection via the circulation. While in transit, the immune cells are primed by circulating inflammatory mediators that help to prepare the cells for their encounter with pathogen. To determine if the circulating biochemical milieu of Live/Die-P mice
differentially primes cells, naïve cells were incubated in plasma collected from Live/Die-P mice 6hr post-CLP. Incubation with plasma produced startling observations that were visually apparent based on flow cytometry light scatter alone. Forward scatter (Fsc) provides an indication of a cell’s size while Side Scatter (Ssc) indicates granularity or internal complexity. Live-P plasma incubation (Figure 4-3, bottom panels) results in a distinct population of increasing Fsc and Ssc that is entirely absent from cells incubated with Die-P plasma (top panels). The increasing Fsc/Ssc, and the poorer resolution of the Non-Debris cell population (and cytospin, data not shown) suggests that Live-P plasma primes PMNs to eject Neutrophil Extracellular Traps (NETs). NETs are strings of DNA and histones that ensnare microbes and prevent their dissemination, while also possessing bactericidal activity [51].
Figure 4-3: Live-P plasma promote putative NET Formation in Naïve PMN.
Forward Scatter and Side Scatter profiles for cells incubated in Die-P plasma (top row) or Live-P plasma (bottom row), and then incubated with 3x-labeled bacteria. Live-P plasma promotes formation of debris with increasing forward and side scatter.

Somewhat expectantly, Live-P plasma incubation with naive cells results in significantly fewer cells phagocytizing (Figure 4-4 A). However, the cells that do phagocytize internalize more bacteria, as compared to Die-P (Figure 4-4 B). This might be expected because PMNs are the major cell population and if a subset of them have been induced to eject their chromosomes, their phagocytic capacity may be greatly diminished.

Surprisingly, Die-P plasma results in significantly increased phagosomal ROS (C), although phagosomal acidification is not different. Also evident in both peritoneal and
plasma fluid data is the decreased phagocytic activity of Live/Die-P relative to incubation with naïve plasma. Although a larger sample size is needed for the naïve plasma controls, it is not surprising that the surge of pro and anti-inflammatory mediators in Live/Die-P mice results in less phagocytic activity as compared to naïve cells.

Figure 4-4: Live-P/Die-P Plasma Promote Differential Responses to Bacterial Phagocytosis in Naïve Cells. A, the percentage of Alexa-350+ cells relative to an ice control. B, the geometric mean fluorescent intensity of Alexa-350 for phagocytizing cells (Alexa-350+). C, phagosomal ROS production for phagocytizing cells (DCF ÷ Alexa350 fluorescence). D, phagosomal acidification for phagocytizing cells (pHrodo ÷ Alexa350 fluorescence). N=3/group, with each N representing plasma from an individual mouse.
Discussion

One of the hallmarks of sepsis is an inflammatory surge in conjunction with or followed by a state of immunosuppression. It was previously shown that in animals predicted to die from sepsis, there was profound suppression in bactericidal mechanisms by phagocytes residing in the peritoneum or traversing the circulatory system (chapters 2-3). One concern with those studies is the use of outbred heterogeneous mice, as intrinsic variability in phagocytic capacity by individuals may predetermine the mice that will live versus die. The most important finding in this study was the recapitulation of phagocytic suppression, observed in septic mice, with naïve cells incubated with humoral fluids (peritoneum, plasma) and heat-killed bacteria. This suggests that it is humoral elements secreted in response to sepsis that largely determine phagocytic activity, and by extension, survival. While genetics undoubtedly contribute to the clinical scenario, these studies here and previous ones with homogeneous mice [75] help to minimize genetics as a confounding factor in a model of 50% mortality.

While the findings of phagocytic and phagosomal suppression in this study were somewhat of a surprise, as both Live-P and Die-P mice produce large amount of pro and anti-inflammatory mediators, the idea that soluble mediators affect phagosomal maturation is not new. Studies with macrophage phagocytosis of mycobacterium have shown that the pro-inflammatory cytokines IFNγ and IL-6 augment phagolysosomal fusion [136, 137], while the addition of anti-inflammatory IL-10 decreases
phagolysosomal fusion [138]. Similarly, IFNγ increases ROS production by macrophages [139] while IL-10 depresses neutrophil ROS [140]. However, a more relevant study observed that the simultaneous addition of IFNγ and IL-10 to dendritic cells (DC) significantly suppressed TNF and IL-12p40 production more so than if given just IFNγ or IL-10 alone. More importantly, concomitant administration of IFNγ and IL-10 suppressed DC activation of CD4 T-cells [141]. As both pro and anti-inflammatory mediators surge in Live/Die-P mice, it may be that the significantly stronger surge in Die-P mice results in stronger suppression.

As important as the observation that humoral fluids differentially affect phagocytic function, perhaps more important is that 6hr Live-P plasma induces putative NET formation while Die-P and naïve plasma do not. While formal determination of NET presence has not yet been performed, several findings support that these are NETs: first- The shift in light scatter showing decreased resolution of the PMN population along with increased aggregation of debris and/or bacteria; second- the decreased percentage of cells phagocytizing that is presumably due to a portion of the cells that have ejected their chromosomes [142]; and third- The decreased ROS burst from Live-P plasma incubated cells may reflect their consumption of NOX activity used to generate NETS and their release of myeloperoxidase along with the NETS [143].

The putative NET formation by Live-P incubated cells could further explain why despite similar bacterial loads 6hr post-CLP (chapter 2, [22]), Die-P mice fail to contain the
infection. NET formation has been shown to trap bacteria and prevent dissemination during sepsis [144]. The importance of this was recently shown when following CLP, mice that were injected with DNase showed increased mortality within 24hr, increased bacterial dissemination, increased IL-6, and increased neutrophil recruitment [145]. It is tempting to speculate that the failure to produce NETs allows greater dissemination of bacteria within the peritoneum, leading to increased cellular activation and cytokine secretion, thus partially explaining the difference in phagocytic function, inflammatory profiles, and survival despite equivalent inoculums.
Prior to the start of this study, the prevailing wisdom was that sepsis is mediated at first by a large pro-inflammatory storm and patients died of hyperinflammation. Those that survived the pro-inflammatory drive were then caught in a compensatory anti-inflammatory response. Patients that succumbed during this phase were considered immunosuppressed. Numerous therapies aimed at blunting hyperinflammation in hopes of preventing organ failure, failed. There are likely many reasons why these therapies failed, and poor study design may be at the forefront [30]. This is likely due to treating sepsis as a single static disease defined clinically as SIRS plus infection rather than as a dynamic disorder, and therefore a one size fits all approach is unlikely to be efficacious.

As this study concludes, there are still clinical trials moving forward aimed at blunting the immune response and some being recalled due to their increasing mortality [146]. However, there is progress towards a more holistic understanding of sepsis within the research community, with evidence for enhancing immunity rather than blunting it [101, 102, 113, 147, 148], and attempts at stratifying patients most at risk for progressing beyond SIRS into sepsis [149]. This study supports the path forward and may contribute to new approaches to sepsis disease management.

Like other studies, ours shows immune system suppression, but with a substantial novel information. In a recent review, Hotchkiss et al. [109] stated that although a SIRS/CARS surge is present in sepsis, the initial stages are predominated by hyperinflammation.
Previous studies from our lab support this from a cytokine/chemokine standpoint, as the total contribution from pro-inflammatory mediators is somewhat greater than anti-inflammatory mediators [22, 131]. However, this body of work shows that the mice with a greater inflammatory surge are still immunosuppressed. Can they be simultaneously dying from too much inflammation, and yet, not enough inflammation? Osuchowski et al showed that low dose administration of dexamethasone (DEX), an anti-inflammatory corticosteroid, to Die-P mice resulted in increased survival [76]. Importantly, this benefit was only evident when mice were stratified for treatment, as treating all CLP mice with DEX did not decrease mortality. How can restraining inflammation augment the inflammatory response? The answer may reside in the role of PMNs and how they are ill-suited for hyperinflammatory conditions

Although PMNs are often regarded primarily as the first responders to an infection and as the cells frequently associated with host tissue injury, their role as immuno-regulators is gaining more prominence. In cancer, PMNs were shown to suppress CD8-Tcell activation via an IL-10 dependent mechanism [150]. In sterile lung inflammation PMNs suppressed inflammation via ROS production [151]. LPS stimulation of human whole blood results in anti-inflammatory cytokine production by neutrophils [152]. In CLP induced sepsis, PMNs have been shown to be significant producers of IL-10 and decrease mortality by limiting inflammation [153, 154]. However, neutrophils have also been shown to be dispensable during some infections. In their absence, inflammatory monocytes pick up the slack and eradicate the infection [155], and this is likely due to
alleviating PMN mediated suppression of monocytes via IL-10 [71]. How then may the positive contributions of PMNs go awry in CLP-induced sepsis?

Severe trauma such as CLP results in hyperinflammatory conditions due to the widespread dissemination of DAMPs from necrotic cecum, and bacterial PAMPs from the stool. Although clinically relevant, these conditions may not represent the more typical scenarios of localized inflammation that is less severe, and for which neutrophil and immunity likely evolved. In such conditions, neutrophils are attracted by chemokines produced at the site of infection. As PMNs arrive, they engage the pathogen and are induced to quickly release pre-packaged stores of chemokines that call for more PMNs [156] in a feed forward manner similar to that depicted in chapter 1 (Figure 1-1).

Attenuation of inflammation should increase as more PMNs arrive, and this is likely due to the suppressive actions of exocytosed PMN granules [157] and ROS [151]. This gradual process likely allows for efficient elimination of microbes before suppression takes place. However, CLP with its atypical immune signaling load likely triggers premature suppression. This is supported by our studies and others. Six hours post-CLP, Die-P PMNs generate little ROS via stimulation by opsonized bacteria, and they show limited capacity to generate ROS following PMA stimulation (Figure 2-4, Figure 2-5). NOX-derived ROS signaling results in the activation of anti-inflammatory Nrf2 [151]. It is likely that the events that led to the exhaustion of NOX activity (i.e. phagocytosis, extracellular PAMPs, chemotactic agents) produced sufficient ROS to trigger Nrf2. It is important to keep in mind that PMN NOX ROS can be released extracellularly, thereby
increasing the redox signaling of nearby cells, leading to their own Nrf2 induction. Meanwhile, the infection continues to spread and results in further pro-inflammatory signaling and as this increases in strength, so does the anti-inflammatory signaling. How likely is this scenario in our model of CLP?

Belikoff et al. showed that Die-P could be rescued by releasing the brakes on inflammation by blocking adenosine receptor 2B (A2BR). Importantly, it was shown that in the absence of A2BR signaling, macrophages demonstrated increased phagocytosis and cytokine production following CLP. However, there was no difference observed for PMN phagocytosis. It may be that A2BR blockade helped to alleviate some of the PMN-mediated inhibition of macrophage function that is present following CLP [71]. In further support of too much inflammation triggering a suppressive phenotype, Osuchowski et al showed that low dose administration of dexamethasone (DEX), an anti-inflammatory corticosteroid, to Die-P mice resulted in increased survival [76]. In other studies, DEX has been shown to inhibit phagocyte ROS production and increase PMN survival [158-160]. It may be that DEX improves Die-P survival by preventing newly recruited peritoneal PMNs and macs from premature suppression via ROS signaling.

This may in part explain the transition from hyper-reactive BM phagocytes (Figure 3-1) to hypo-reactive cells in the periphery and peritoneum of Die-P mice. Their increased sensitivity to opsonized bacteria, and by extension, their increased sensitivity to circulating PAMPs and DAMPs may trigger premature ROS and degranulation.
Premature degranulation could have other effects besides impaired phagocytic activity. Die-P BM and peripheral phagocytes have increased surface expression of CD11b. Chishti et al reported that sepsis results in increased adhesion of neutrophils to the endothelium, owing to increased CD11b expression [161]. Increased activation of neutrophils predisposes them to increased degranulation upon pattern recognition receptor, i.e. Toll like receptor ligation [162-164], leading to increased vascular injury [165] which is prevalent in sepsis [166, 167]. This also fits with unpublished data from our lab that shows decreased heart rate and pulse distension in mice that die from CLP. This may also fit with our recent study that documented renal injury prior to death from CLP [92], as neutrophil degranulation has been shown to be involved in animal models of glomerulonephritis [168].

**Acute vs. Chronic Sepsis**

Taken together, the aforementioned studies and ours provide a plausible explanation as to why mice die from too much inflammation, and yet not enough inflammation. However, our studies dealt with acute phase mortality, whereas most clinical mortality occurs during the chronic phase. This is in part due to the advances in clinical care that has led to patients surviving the hyperinflammatory phase and entering a protracted state of immunosuppression [109]. However, our studies show that mice are indeed immunosuppressed during this hyperinflammatory phase. Are patients similarly immunosuppressed during the acute phase, and thanks to source control and early goal
directed therapy (EGDT), they stay alive to be immunosuppressed a little longer? Animal studies seem to suggest so.

A previous study by our lab showed that cecal resection, a form of source control, at 8hr post-CLP saved all Die-P mice that were followed out to 21 days post-CLP [75]. A similar study where resection was performed on mice still alive by day 4 showed that some mice succumbed to infection following resection, suggesting a state of immunosuppression as the infectious source was removed [70]. It may be that Live-P and Die-P differentially reprogram global immunity for immune-vigilance or suppression following CLP. In further support of this is a study by Delano et al that performed a mild form of CLP that resulted in approximately 10% mortality with the majority of deaths occurring within 3 days of CLP. Survivors can loosely be considered Live-P equivalents. When these survivors were challenged with pneumonia 7 days later, they showed similar mortality to sham mice [114]. In the same vein, Pastille et al showed that CLP resulted in sustained immunosuppression of dendritic cells (DC), and that this was due to changes in the DC progenitors in the bone marrow [169]. Would Die-P mice similarly reprogram their marrow for sustained immunosuppression if they were rescued from their acute fate? These studies need to be performed, but there is additional reason to believe sustained immunosuppression would develop. Circulating factors are known to influence myelopoiesis and enhance neutrophil function [170]. We showed in chapter 4 that Die-P fluids result in phagocytic suppression, and bone marrow alterations became evident by
24hr post-CLP. It may be that these alterations would produce a sustained state of immunosuppression, as is the case for humans.

Although our studies provide strong evidence for phagocyte suppression preceding mortality, they do not suggest how this comes to be. Given that mice receive the same inoculum, why do some mice go on to die while others do not? Although mice may receive similar cecal inoculums, their cecal contents may differ in the presence and/or prevalence of certain bacteria. Silva et al showed that a particular strain of *E. coli* present in mice impaired macrophage phagocytosis during CLP and that this was due to FcR:*E. coli* interactions [63]. Similarly *Bacteroides fragilis* and *Faecalibacterium prausnitzii* are commensal bacteria that modulate immune responses by encouraging tolerance, as evidenced by the development of intestinal inflammation without them [171, 172]. Perhaps an element of chance exists in which Die-P immune cells encounter tolerance-promoting commensals first, while Live-P mice have the good fortune of recognizing non-tolerance inducing commensals first. In this situation, inflammatory processes would be suppressed in Die-P, allowing increased bacterial dissemination before a more robust inflammatory response could begin.

Another possibility, and one with more direct evidence to support it, is that Die-P mice have circulating inhibitory IgG that is present prior to CLP. Moitra et al. showed that mortality could be predicted prior to performing CLP [173]. This was performed by collecting plasma samples from mice several days prior to CLP, and using them in an ex-
vivo bacterial killing assay. Opsonization of bacteria with plasma samples from mice that would go on to die from subsequent CLP, resulted in less macrophage mediated bacterial killing, as compared to mice that survived CLP. Preliminary studies suggest that the plasma factor responsible is an inhibitory IgG. Similar to tolerance-inducing commensals, iIgG may work to suppress unwanted inflammation against the healthy microbiota. In the context of CLP, this would result in non-survivors beginning their response from an immunosuppressed state, thereby facilitating bacterial dissemination and the subsequent SIRS/CARS surge. As many patients who become septic are already immunosuppressed, it is likely that our acute phase model recapitulates human sepsis more than we appreciate.
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