THE INDIVIDUAL AND UNIQUE ROLES FOR EOSINOPHILS AND INTERLEUKIN-5 IN THE INNATE IMMUNE RESPONSE TO SEPSIS

by

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A DISSERTATION

Presented to the Department of Molecular Microbiology and Immunology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

July 2011

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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ACKNOWLEDGEMENTS

I would first like to thank the members of my thesis committee for all their help and encouragement throughout this process: Dr. David Jacoby, Dr. David Lewinsohn, Dr. David Parker, and Dr. Ann Hill. They are all intelligent and kind mentors whose unique perspectives and guidance helped shape my research.

I would like to give a special "shout-out" and continued gratitude to my mentor Dr. Jeff Gold. Dr. Gold is incredibly intelligent and a gifted scientist, one whom I am lucky to have worked with and been trained by. His recall ability for scientific articles and research is infinite; his ideas and wisdom are endless. But more than this, his demeanor and personality were what stand out most in my mind: his humor is well placed and off-the-wall; his respectfulness toward others is enviable; and his understanding and kindness are unrivaled. Dr. Gold has always been respectful to me and my ideas, and always treats me like a peer, rather than a student. His encouragement has helped me grow immensely as a scientist. He has what seems to be an endless supply of patience and was never frustrated by my frequent and repeated barrage of questions or my scientific and technical failures. His presence in the lab lightened the mood, and brought endless hours of discussion on politics, basketball, and weird YouTube videos. Most importantly, he has taught me the importance of thinking on my feet, and to remember the value of this quote in science:

"Whenever you fall, pick something up." --Oswald Avery

Working in a small lab has many advantages. Everyone collaborates on his or her projects, so much so that it genuinely feels like a team effort. Erin Danielson and Ann Kelly were instrumental in this effort; they made managing the lab while doing multiple experiments seem simple and effortless. I cannot thank them enough for their assistance, teaching me various techniques vital to my research as well as maintaining and genotyping the large number of transgenic animals used in this thesis, performing ELISAs, culturing bacteria, and eosinophil isolation. In my case, not only did I have the help and support of these women, but also an ear to listen, and a shoulder to cry on. I cannot thank Erin Danielson and Ann Kelly enough for their helpful advice and caring nature.

I am also incredibly grateful that the Gold lab shared space with the wonderful labs of Dr. Jacoby and Dr. Fryer. This friendly and collaborative atmosphere allowed me to meet so many bright, kind, and funny scientists. We shared many triumphs and failures as scientists, but through it all were jokes, laughs, and helpful advice. I cannot count the number of times I have told others how much I love and appreciate everyone I work with. You are an exceptional, unequivocal group of people.

On a slightly different note, I would also like to thank my favorite cytokine—IL-5. We have had our ups; we have certainly had our downs. But, we have had a very interesting five years. I hope I've helped give you a different sort of name in the scientific community and that one day you go on to do bigger and better things.

I would like to thank my large and wonderful family. I am fortunate enough to have had two mothers and two fathers in my lifetime; four parents to look to for guidance. When one of them faltered or couldn't offer specific advice, there was always another one there to pick up where they left off. My mom is one of the sweetest, most caring people I've ever met. Regardless of what I did, she was there to support me whenever I needed her. My step-dad was always pushing me to achieve more than I thought I could, and he has helped make me an appropriately skeptical scientist. My dad and step-mom always listened when I needed to talk, and never forgot to tell me how proud they were of my accomplishments. My sister and brother were endlessly entertaining (though sometimes unintentionally). I love seeing the amazing people they are becoming and look forward to growing old with them.

Finally, I would like to thank my number one supporter and partner-in-crime. Michael, you have been my greatest asset during my time as a graduate student. You are there to listen to me, to distract me, to keep me focused, to feed me when I was "too busy to eat" and to give me wine when I was "too busy to sleep". You are the most thoughtful and kindest person I have ever met. I know I can always count on you when I need you, and that you're even there when I don't.

You said it best—together we are unstoppable.

Thank you all for being amazing and wonderful people!

PUBLICATIONS

Manuscripts

Linch, S. N., Danielson, E. T., Kelly, A. M., and J. A. Gold. IL-5 Augments the Innate Immune Response during Polymicrobial Sepsis. [manuscript in preparation].

Linch, S. N. and J. A. Gold. The Role of Eosinophils in Non-Parasitic Infections. *Endocr Metab Immune Disord Drug Targets*. 2011 Jun 1;11(2):165-72.

Linch, S. N., Kelly, A. M., Lee, J.J. and J. A. Gold. Mouse Eosinophils Possess Potent Antibacterial Properties *in vivo*. *Infect Immun*. 2009 November; 77(11); 4976-82.

Abstracts and Presentations

Linch, S. N., Danielson, E. T., Kelly, A. M., and J. A. Gold. IL-5 augments macrophage function in polymicrobial sepsis. *Am J Respir Crit Care Med,* 2010; 181: A1802

Linch, S. N., Danielson, E. T., Kelly, A. M., Lee, J. J., and J. A. Gold. The Effect of IL-5 on Macrophages and PMNs in Sepsis. *Am. J. Respir. Crit. Care Med,* 2009; 179: A1024. [Oral Presentation]

Manzer, S. N., Kelly, A. M., Lee, J. J., and J. A. Gold. Antibacterial Properties of Eosinophils *in vivo*. *Proceedings of the American Thoracic Society* [Poster Session B56, 19 May 2008]

Manzer, S. N., Kelly, A. M., Lee, J. J., and J. A. Gold. Role of IL-5 in Polymicrobial Sepsis. *Proceedings of the American Thoracic Society* [Poster Session A11, 18 May 2008]

ABSTRACT

Sepsis is defined as the systemic inflammatory response to infection, with a mortality rate in excess of 25%. It is associated with an acquired impairment in innate immune function, which is typified by reduced neutrophil bacterial killing, reduced antigen presentation and cytokine production by monocytes, and apoptosis of both immune and non-immune cells. Furthermore, sepsis is associated with eosinopenia. Treatment options for patients consist of antibiotics and supportive care, highlighting the need for means to improve patient mortality.

A recent study indicated that IL-5 levels are elevated in sepsis survivors compared to non-survivors. However, because sepsis is associated with eosinopenia, these two observations present a paradox concerning the function of IL-5 and eosinophils in sepsis. Data presented in this thesis reconcile these observations, and show individual and unique roles for eosinophils and IL-5 in sepsis.

Data presented in Chapter 3 provide evidence that isolated mouse eosinophils have potent anti-bacterial properties *in vitro* against *P. aeruginosa*. *In vivo*, IL-5 transgenic mice, which have a profound eosinophilia, had improved bacterial clearance in Pseudomonas peritonitis. Improved bacterial clearance following adoptive transfer of eosinophils, as well as evidence of impaired bacterial clearance in mice with a congenital eosinophil deficiency, established that this antibacterial activity was eosinophil specific. Eosinophils mediated this antibacterial effect through the release of cationic secondary granule proteins, as purified eosinophil granules had

potent antibacterial properties *in vitro* and significantly improved bacterial clearance *in vivo*.

Data in Chapter 4 demonstrate that IL-5 has an eosinophil-independent protective role in sepsis. IL-5 transgenic mice with congenital deficiency of eosinophils still had a marked improvement in survival and bacterial clearance following sepsis. Loss of IL-5 was detrimental to survival and bacterial clearance during sepsis, and prophylactic or therapeutic administration of IL-5 improved survival in mice. IL-5 levels were elevated in septic patients, and higher levels were associated with improved outcomes. Interestingly, administration of IL-5 induced neutrophil recruitment into the peritoneal cavity, suggesting an effect of IL-5 on additional myeloid cells.

In Chapter 5, data demonstrate novel expression of the IL-5Rα on neutrophils and monocytes/macrophages both in mice and in humans with sepsis. In addition, expression of this receptor was unique to sepsis and waned as patient morbidity improved. IL-5Rα expression was inducible *in vitro* through stimulation with bacteria and bacterial derived products, providing a possible mechanism for this expression *in vivo*.

Data presented in Chapter 6 demonstrate that IL-5 stimulation of neutrophils does not affect bacterial killing, or spontaneous cell death. However, IL-5 stimulation of macrophages results in increased STAT-1 nuclear translocation, increased cytokine production, increased phagocytosis and bacterial killing, and prolonged survival.

Finally, macrophages are necessary for these protective effects *in vivo*. These data collectively suggest a novel and protective role for IL-5 in sepsis.

Taken together, these data suggest that treatment with eosinophil granules or IL-5 may be viable immunomodulatory therapies for septic or bacteremic patients. Moreover, these data suggest that anti-IL-5 or anti-IL-5Rα therapies may have detrimental effects in patients and predispose them to infection.

CHAPTER 1:

INTRODUCTION

INTRODUCTION

This thesis addresses the role of the innate immune response in sepsis and how it can be manipulated to improve outcomes using two animal models of sepsis. Specifically, the role for eosinophils in improving bacterial clearance will be examined, as well as the unique ability of Interleukin (IL)-5 to impact the effector functions of neutrophils and macrophages in polymicrobial sepsis.

I. SEPSIS: EPIDEMIOLOGY, DEMOGRAPHICS, CAUSATIVE AGENTS AND CURRENT THERAPY

This Section provides background on the epidemiology and characteristics of human sepsis in the United States. Patient population characteristics and clinical manifestation of the disease will be discussed, as well as the causes of sepsis and current methods of treatment.

A. Significance and Epidemiology

Sepsis is defined as the systemic inflammatory response by the host to infection (Bone et al., 1992). It is a major encumbrance financially to the healthcare system in the United States, costing approximately $17 billion each year. Furthermore, it is the leading cause of mortality in the Intensive Care Unit (ICU), and occurs in more than 750,000 patients in the United States each year (Angus et al., 2001). Mortality in sepsis ranges from 25-70% depending on the severity of disease, and several

studies indicate that incidence rates are rising between 2-9% each year (Angus et al., 2001; Annane et al., 2003; Annane et al., 2005; Martin et al., 2003).

It was not until 1992, at a conference held by the Society of Critical Care Medicine and the American College of Chest Physicians, that disease severity was divided into several defined categories based on clinical observations. These categories are as follows (progressing from least to most severe): Systemic Inflammatory Response Syndrome (SIRS), sepsis, severe sepsis, septic shock, and Multiple Organ Dysfunction Syndrome (MODS) (Bone et al., 1992; Levy et al., 2003). The SIRS criteria are:

- Abnormal white blood cell (WBC) count defined as >12,000 or <4000 WBC/μL or >10% immature band forms
- Tachycardia defined as >90 beats/minute
- Hypothermic or febrile defined as <36 °C or >38.3 °C
- Respiratory rate of >20 breaths/minute or a $PaCO_2$ <32 mm Hg

Sepsis is defined as meeting two or more of the SIRS criteria in addition to evidence of infection; severe sepsis is defined as having sepsis with organ dysfunction, hypoperfusion, or hypotension; and septic shock is defined as sepsis with arterial hypotension, despite adequate fluid resuscitation (Table 1.1) (Bone et al., 1992; Levy et al., 2003). The adoption of a uniform set of criteria for categorizing patients allowed clinicians and investigators to assess disease epidemiology pro-and retrospectively, as well as interpret data from hospitals worldwide. Moreover, this

allowed for better organization of clinical trials, enabling assessment of mortality benefits in specific subgroups of patients. These clinical trails will be discussed in more detail in Section III.

B. Patient demographics

Typically, the incidence of sepsis peaks in two populations, the elderly (patients >60) and children, with the majority of patients being greater than 65 years of age (Angus et al., 2001; Annane et al., 2003; Cheng et al., 2007). Age alone is an independent predictor of mortality in patients (Martin et al., 2006). Numerous studies have examined mortality rates between men and women, however there are conflicting results regarding the impact of gender on survival (Adrie et al., 2007; Angus et al., 2001; Cheng et al., 2007; Crabtree et al., 1999; Schroder et al., 1998).

It is known that comorbidities, such as HIV infection, cancer, or autoimmune disease, occur in roughly half of all septic patients and affect the outcomes of sepsis (Angus et al., 2001; Cheng et al., 2007; Esper et al., 2006; Martin et al., 2003). These and numerous other studies indicate that factors including age, race, gender, genetics, and other comorbid conditions can all affect patient survival and outcomes in sepsis (Lin and Albertson, 2004; Sorensen et al., 1988). The heterogeneous nature of the disease further complicates the treatment of patients.

C. Causative Agents of Sepsis

Louis Pasteur first documented the presence of bacteria in the blood of septic patients in 1879. Since then several studies have documented that in 50-80% of cases, one or more pathogens can be isolated out of cultures from septic patients (Alberti et al., 2002; Annane et al., 2003; Heffner et al., 2010; Labelle et al., 2010). Three decades ago, Gram-negative bacteria were the most frequent organisms found in sepsis. However, the incidence of Gram-positive bacterial sepsis has become increasingly prevalent since the late 1980's and currently accounts for roughly 30-50% of all cases of bacterial sepsis (Martin et al., 2003). The most common causes of bacterial sepsis are *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Table 1.2) (Alberti et al., 2002; Annane et al., 2003; Annane et al., 2005; Martin et al., 2003).

Interestingly, the incidence of fungal infection increased more than 200% from 1979 to 2000; one reason for this is the rise of bone marrow and solid organ transplants and consequently the need for immunosuppression in these patients (Martin et al., 2003). For many patients, the most frequent site of infection is the lungs, followed by the abdomen and the urinary tract (Wheeler and Bernard, 1999).

One increasing concern in sepsis is the rise of antibiotic resistant species. The four most common causes of bacterial sepsis (listed above) have all developed antimicrobial resistance to varying degrees and at alarming rates (Aloush et al., 2006; Klevens et al., 2007; Moore et al., 2008; Neu, 1992; Ortega et al., 2009;

Paterson, 2006; Weinstein et al., 1983b). Despite high rates of antibiotic resistance, over the last thirty years clinical trials for new antimicrobials have decreased by roughly 75% (Boucher et al., 2009). The need for treatment modalities, in addition to antibiotics, is paramount for patients with bacterial infections. This will be discussed further in Section VI with regard to eosinophils and their highly cationic granule proteins.

D. Current Treatment for Sepsis

Currently, there is only one FDA approved drug for the treatment of sepsis, Activated Protein C. However, because this drug is only moderately efficacious on a subset of patients with severe sepsis and increases the risk of bleeding, it is not widely used in the United States (Abraham et al., 2005; Bernard et al., 2001; Eichacker et al., 2006; Laterre et al., 2007; Laterre et al., 2008; Warren et al., 2002). Typically, treatment for patients is only supportive care, which often includes intravenous fluids, mechanical ventilation, and antibiotics. However, inappropriate or delayed antibiotic usage further increases patient mortality by 15-40%, necessitating careful selection of antibiotics based on symptoms and blood, sputum, or other cultures from patients (Harbarth et al., 2003; Ibrahim et al., 2000; Kumar et al., 2009).

Recently, the implantation of early, goal-directed therapy in sepsis has proven effective at reducing the overall mortality of patients (Jones et al., 2008; Jones et al., 2007; Micek et al., 2006; Rivers et al., 2001; Shapiro et al., 2006; Trzeciak et al.,

2006). However, additional strategies are needed to improve patient morbidity and mortality. The potential to combine several therapeutic modalities to achieve a synergistic effect on patient survival is of particular interest to investigators. The persistently high mortality rate and lack of effective treatments for sepsis make it clear that more research and a better understanding of the disease are necessary to create successful therapeutic agents for patients.

Table 1.1: Clinical definitions of Sepsis

SIRS	Having two or more of the following: Temperature <36 °C or >38.3 °C Heart rate >90 beats per min Respiratory rate > 20 breaths per min or $PaCO_2$ <32 mm Hg White blood cell count >12,000 or <4,000/µl or >10% immature band forms
Sepsis	Having two more of the SIRS criteria and evidence of infection
Severe sepsis	Sepsis with organ dysfunction, hypoperfusion, or hypotension, and may include any of the following: Changes in mental status Urinary output <0.5 ml/kg for at least one hour Lactates >1 mmol/l Platelet counts <100,000/ml or disseminated intravascular coagulation (DIC) Acute lung injury or acute respiratory distress syndrome Cardiac dysfunction
Septic shock	Severe sepsis and sepsis-induced hypotension (e.g. systolic blood pressure <90 mm Hg or a reduction of >40 mm Hg from base line) despite adequate fluid resuscitation

*From (Annane et al., 2005; Levy et al., 2003; Riedemann et al., 2003).

Table 1.2: Pathogens found in septic shock patients

	Estimated Frequency
Gram-positive bacteria	**30-50%**
Methicillin-susceptible Staphylococcus aureus	14-24%
Methicillin-resistant S. aureus	5-11%
Streptococcus pneumonia	9-12%
Enterococcus spp.	3-13%
Gram-negative bacteria	**25-30%**
Escherichia coli	9-27%
Pseudomonas aeruginosa	8-15%
Klebsiella pneumonia	2-7%
Enterobacter spp	6-16%
Haemophilus influenzae	2-10%
Fungus	
Candida albicans	1-3%
Yeast	1%
Parasites	**1-3%**
Viruses	**2-4%**

* From (Alberti et al., 2002; Annane et al., 2003; Annane et al., 2005).

II. THE IMMUNOLOGIC RESPONSE IN SEPSIS AND BACTERIAL INFECTIONS

The immune response to sepsis has two important but distinct phases—an initial cytokine storm followed by immunosuppression, or immunoparalysis. These phases remain independent causes of mortality in patients, though most patients survive the initial hyperinflammatory response due to appropriate supportive care and antibiotics (Adrie and Pinsky, 2000; Hotchkiss and Karl, 2003; Russell, 2006; Wang and Deng, 2008). Death from the cytokine storm typically occurs within 24 hours after the onset of shock due to over-activation of the immune system (Annane et al., 2005; Pachot et al., 2006). Patients that survive the initial stage of sepsis often succumb to secondary infections or are unable to clear primary infections due to immunosuppression (Russell, 2006; Wang and Deng, 2008). Further, patients often have long-term defects in immunity due to the substantial antiinflammatory response in late sepsis (Benjamim et al., 2004). Effective strategies to reduce or inhibit immunosuppression would benefit septic patients enormously. This Section will discuss current knowledge of the immune response during sepsis, including the typical response to bacterial infection followed by highlighting hallmarks of immunosuppression and the innate immune response in sepsis. For the purposes of this thesis, focus on the typical innate immune response to bacterial infection will be emphasized, since both models used within are heavily involved in bacterial sepsis. These models will be discussed further in Section IV.

A. The Innate Immune Response to Bacterial Infection

Once a pathogen breaches the protective barriers of the body, it enters into either the bloodstream or into tissue. There, a pathogen is recognized by the innate immune system through pattern recognition receptors (PRRs) expressed as both extracellular and intracellular receptors in innate leukocytes including macrophages, neutrophils, dendritic cells, eosinophils, mast cells, and NK cells (Janeway and Medzhitov, 2002). These PRRs recognize pathogen associated molecular patterns (PAMPs), which are conserved throughout a variety of pathogens. Once pathogen recognition occurs, the recognizing cell secretes chemokines to draw in phagocytic leukocytes, most frequently neutrophils and macrophages. These two leukocyte subsets are the primary responders to bacterial infection and are vital to the innate immune system. Their three main roles are to clear the infection, initiate wound healing, and to provide cytokine and costimulation for an adaptive immune response (Janeway and Medzhitov, 2002). The initiation of an innate immune response to bacterial infection will be covered briefly in this Section, highlighting the general function and importance of this system. Great attention will be given to the roles of neutrophils and macrophages as they are at the center of the work presented in this thesis.

i. Initiation of the Innate Immune Response

The initiation of the innate immune response occurs within the first few hours following recognition of a bacterial pathogen. Pathogen recognition occurs through

expression of PRRs on host cells, as well as through the complement pathway. Neutrophils and macrophages are recruited to the site of infection where pathogen eradication and containment, wound healing, and clearance of apoptotic and cellular debris occurs. However, whether or not the innate immune response can control the infection, initiation of the adaptive response occurs.

Pathogen recognition via PRRs

There are four main classes of PRRs: the transmembrane bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs); and the intracellular Nod-like receptors (NLRs) and Rig-like receptors (RLRs) (Takeuchi and Akira, 2010). These receptors are widely expressed in numerous cell types including professional antigen presenting cells, i.e. macrophages and dendritic cells, but also on epithelial and endothelial cells in various tissues throughout the body. Signaling through these receptors induces an early innate immune response through the production of proinflammatory cytokines including IL-1 and tumor necrosis factor (TNF), as well as type I interferons (IFN), antimicrobial peptides, and chemokines (Janeway and Medzhitov, 2002; Takeuchi and Akira, 2010). These signals help orchestrate a concerted effort to eradicate pathogens efficiently while not exaggerating damage to the host and surrounding tissue.

TLRs have been studied extensively and are the most well characterized class of PRRs. While originally described in the *Drosophila*, more than 10 TLRs have been identified in mice and humans, each with a different function regarding pathogen

recognition and the subsequent immune response. They are transmembrane receptors containing a leucine-rich repeat on the extracellular portion, which serves to recognize PAMPs, and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain required to induce downstream signaling cascades. These receptors are localized to the cell surface and within cytoplasmic organelles, including endosomes and lysosomes (Akira et al., 2006). Their cellular localization is important for the recognition of a specific type of pathogen (Akira et al., 2006; Takeuchi and Akira, 2010).

TLRs signal through the use of multiple adapter proteins. Following pathogen recognition and TLR dimerization, these adapter proteins are recruited to the intracellular TIR domain, where they bind and recruit additional adapter proteins. These include myeloid differentiation primary-response protein 88 (MyD88), IL-1 receptor-associated kinases (IRAKs), and TNF-receptor-associated factor 6 (TRAF6) among others (Akira and Takeda, 2004). However, the main outcome of TLR signaling following bacterial recognition is activation of the transcription factor nuclear factor κB (NF- κB) and AP-1, resulting in transcription of proinflammatory cytokines TNFα, IL-1, IL-12 and IL-6 (Gerold et al., 2007; Martinon et al., 2009). NF-κB also regulates the expression of chemokines, adhesion molecules, cyclo-oxygenase 2, matrix metalloproteinases (MMPs), and inducible nictric oxide synthase (iNOS) (Li and Verma, 2002). TLR4 also induces the transcription of type I IFNs (IFNα and IFNβ) through the adapter TIR domain-containing adapter inducing

IFNβ (TRIF) and a family of transcription factors known as interferon regulatory factors (IRFs) (Takeuchi and Akira, 2010).

CLRs are another class of transmembrane PRRs. They are important for the recognition of carbohydrate moieties on viruses, bacteria and fungi, including mannose and β-glucans. CLRs are largely expressed on dendritic cells, but are also found on monocytes and neutrophils. However, many of the ligands for these receptors are still unknown (Geijtenbeek and Gringhuis, 2009). Most research on these receptors has been conducted using dendritic cells, so this will not be discussed in greater detail.

NLRs and RLRs are both classes of intracellular PRRs. While TLRs are membrane-bound, NLRs and RLRs are cytoplasmic proteins recognizing nucleic acids. RLRs are RNA helicases that recognize viral RNA and DNA, and induce upregulation of the IFN response. While these are important for recognition of viral infection, they are not believed to play a large role in bacterial recognition (Meylan and Tschopp, 2006; Takeuchi and Akira, 2010). NLRs recognize breakdown products of bacterial cell membranes and synergize with TLRs to induce proinflammatory cytokine production. Additionally, some NLRs, specifically those in the NLRP family, form a complex called the inflammasome, which is important in mediating early inflammation through IL-1β.

The Inflammasome

The inflammasome is important for activating caspase-1 and IL-1β. It is a large, multimeric complex composed of several subunits—either NALP3 or NALP1, and an adapter protein, which recruits pro-caspase-1 and binds to the pyrin domain on NALP3 (Davis et al., 2011; Dinarello, 2009). It is believed that the inflammasome is activated by direct recognition of both bacterial and viral PAMPs and danger associate molecular patterns, including extracellular matrix components, extracellular adenosine triphosphate (ATP) and UV radiation. Activation of the inflammasome and caspase-1 results in processing of the pro-forms of IL-1β, IL-18, and IL-33 (Dinarello, 2009). Without processing and cleavage, these pro-cytokines are not biologically active. The primary sources of IL-1β during infection are monocytes and macrophages. Upon binding to the receptor, IL-1β signals through the TIR domain, MyD88, and TRAF6 to cause activation of NF- κB, p38 and JNK. IL-1β is crucial for the local and systemic inflammation, through the production of IL-6 and fever, thereby initiating the acute phase response following recognition of infection (Dinarello, 2009).

The initial cytokine response to pathogen recognition

In addition to IL-1β, additional cytokines are produced in response to bacterial recognition. These cytokines are numerous and induced following a number of different stimuli, including lipopolysaccharide (LPS), formyl-methionyl-leucyl-

phenylalanine (fMLF), nucleic acids, and various bacterial byproducts, in addition to the organisms themselves.

TNFα is produced by macrophages following stimulation with bacterial products. It augments adherence of neutrophils and monocytes to the endothelium and subsequent migration into tissues, and triggers local production of additional proinflammatory cytokines (Tracey and Cerami, 1994). IL-6 is produced by monocytes, T cells, endothelial cells, and a number of other cell types in response to LPS, IL-1, TNF, granulocyte/macrophages-colony stimulating factor (GM-CSF), and various other stimuli. It induces proliferation of hematopoietic progenitors, induction of fever, and release of various hormones throughout the body (Van Snick, 1990). IL-6 also induces a number of proteins that limit inflammation (Annane et al., 2005). IL-12 is produced largely by macrophages in response to bacteria and bacterial products. It induces IFNγ production from natural killer (NK) and T cells, which in turn enhances phagocytosis and inflammation. It also facilitates Th1-type responses and induction of adaptive immunity (Trinchieri, 1995).

IL-1, TNFα, and IL-6 release activate the acute phase response. This response consists of transcriptional upregulation of numerous proteins causing fever, neutrophil release from the bone marrow, muscle catabolism, activation of complement, and coagulation pathways among others (Sriskandan and Altmann, 2008). The complement system serves as a way to directly lyse bacteria through the formation of pores in the outer membranes, and also as a means to opsonize pathogens for uptake by phagocytic cells.

This acute phase also induces production of antiinflammatory mediators to help achieve homeostasis and balance. This consists of IL-10 secretion and production of soluble cytokine receptors, which act as a sink for reducing circulating levels of proinflammatory cytokines. These include soluble TNF receptors and IL-1 receptor antagonists (Annane et al., 2005; Sriskandan and Altmann, 2008). IL-10 production inhibits numerous monocyte/macrophage functions including nitric oxide production and expression of costimulatory molecules, and thereby limiting T cell production of cytokines. It also inhibits chemokine production, further induces production of soluble receptor antagonists, and suppresses phagocytosis and bacterial killing (Moore et al., 2001). The end result of inflammatory cytokine production is to eliminate an infection and restore the host to a homeostatic state.

Chemokine secretion

Recognition of a pathogen through various PRRs results in the expression of numerous chemokines. Chemokines are a group of small proteins capable of recruiting and directing leukocytes to sites of infection, injury, or inflammation. These heparin-binding proteins are divided into several families based on structure; the two most studied being the CC family and the CXC family. Chemokines bind to their G-protein-coupled receptors on the surface of leukocytes, resulting in morphologic changes and cell motility. Circulating leukocytes roll along on the endothelium searching for an immobilized chemokine gradient using various chemokine receptors. Once a source is found, these cells firmly adhere to the

endothelium through adhesion molecules and migrate into tissues; this will be discussed in greater detail later in this Section.

Members of the CC family of chemokines possess multiple cysteine residues and the first two are adjacent to each other, hence the name CC. These chemokines attract monocytes to sites of inflammation, and include monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α and MIP-1β. MCP-1 (CCL2) and CCR2 knockout mice have defective monocyte recruitment following numerous stimuli, indicating the importance of MCP-1 in monocyte recruitment (Charo and Peters, 2003). Interestingly, CCR2 knockout mice or mice treated with a CCR2 antagonist, have improved survival in the Cecal Ligation and Puncture (CLP) model of polymicrobial sepsis likely due to reduced tissue injury and infiltration of neutrophils (Souto et al., 2011). These data suggest that while MCP-1 is important for monocyte recruitment and survival during certain bacterial infections, enhanced neutrophil recruitment by MCP-1 is detrimental to the host response in sepsis, indicating the importance of having a balance between pro- and antiinflammatory signals within the host.

The CXC family has one amino acid flanked on both sides by a cysteine residue. CXCL8, also known as IL-8, is the prototypical example of this family. Mice do not have IL-8, but instead have two functional homologs: keratinocyte-derived chemokine (KC) and MIP-2 (Olson and Ley, 2002). IL-8 is responsible for recruitment of neutrophils to sites of infection or inflammation and also activates monocytes to recruit them for vascular wound healing (Gerszten et al., 1999).

Epithelial cells secrete IL-8 rapidly after bacterial entry into the cell, signaling the innate immune response (Eckmann et al., 1993). Mice deficient in the IL-8 receptor (CXCR2) do not have neutrophil recruitment to the lung following LPS-inhalation (Reutershan et al., 2006). In addition, CXCR2 deficient mice have 100% mortality following *S. pneumoniae* infection in mice due to lack of neutrophil recruitment and bacterial growth (Herbold et al., 2010). These data indicate the importance of neutrophil recruitment to aid in bacterial clearance and survival through recognition of IL-8.

Secretion of TNF, IL-1, or the presence of LPS can trigger the production of MCP-1, MIP-1α, IL-8 and other members of these chemokine families by macrophages and endothelial cells (Bonecchi et al., 2000; Charo and Ransohoff, 2006). Circulating leukocytes roll along the endothelium, localize an inflammatory site following recognition of chemokines bound to the endothelial layer, and migrate into tissue where they are needed. This is described in detail below as it pertains to neutrophils and monocytes, which are important for the recognition and clearance of a bacterial infection.

Rolling, Adhesion, and Extravasation

Neutrophil and monocyte mobilization occurs following exposure to a number of different stimuli, including IL-8, platelet-activating factor (PAF), C5a, MIP-1α, leukotriene B_4, MCP-1, and fMLF (Imhof and Aurrand-Lions, 2004; Wagner and Roth, 2000). These chemoattractants activate neutrophils and monocytes, resulting

in an increase in intracellular calcium release (Sawyer et al., 1989). Additionally, TLR4-mediated production of type I IFNs results in iNOS leading to production of nitric oxide and consequently vasodilation. This vasodilation allows for slower blood flow, thereby allowing leukocytes to more readily attach to the vessel wall (Sriskandan and Altmann, 2008).

Neutrophils and monocytes circulate through the blood looking for an immobilized chemotactic gradient on the endothelial surface, indicating that their presence is required. L-selectin mediates binding of these cells to CD34 on the endothelium and facilitates extravasation (Imhof and Aurrand-Lions, 2004; Wagner and Roth, 2000). Interestingly, L-selectin is most abundant on neutrophils recently released from the bone marrow, in contrast to neutrophils that are already in the circulation. Shedding of L-selectin from the surface of neutrophils can be mediated by MMPs, occurs in non-inflammatory conditions, and is believed to facilitate their clearance from the circulation (Wagner and Roth, 1999; Wagner and Roth, 2000). P-selectin and E-selectin, when they are expressed on the endothelium, are bound by their appropriate ligands on the neutrophil or monocyte to capture these cells and slow their rolling along the endothelium. Firm adhesion is mediated through integrin expression, largely of Mac-1 integrin on the neutrophil and CD11a/CD18 (LFA-1) on the monocyte, but both cell types express Mac-1 and LFA-1 (Imhof and Aurrand-Lions, 2004; Wagner and Roth, 2000). Mac-1 and LFA-1 bind to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, which is induced by inflammatory cytokines (Hashimoto et al., 1994; Scholz et al., 1996). Binding

induces polarization of the cell toward the leading edge, and allows it to flatten out in order to cross the endothelium (Imhof and Aurrand-Lions, 2004; Wagner and Roth, 2000; Witko-Sarsat et al., 2000).

Finally, neutrophils and monocytes extravasate through the endothelial cell layer between endothelial cells. This is preceded by leukocyte-endothelial cell binding through CD31-CD31 interactions and downregulation of selectins (Wagner and Roth, 2000). Once in the tissues, leukocytes follow immobilized chemokine gradients to localize to the specific site of infection (Witko-Sarsat et al., 2000).

Neutrophils and monocytes/macrophages will be discussed in detail with regard to their role in bacterial infection.

Summary

The initiation of an inflammatory response to a bacterial pathogen is complex and involves a concerted effort by numerous cells, including endothelial cells and leukocytes, to induce production of pro- and antiinflammatory cytokines, chemokines, and acute phase proteins. The ultimate goal of this response is to clear or contain the pathogen, repair damaged tissue, and maintain homeostasis within the body. This is summarized in Figure 1. 1.

ii. Neutrophils

Differentiation and Homeostasis

Neutrophils were first described by the scientist Ilya Metchnikov in the late nineteenth century when he observed that "wandering mesodermal cells accumulated at the puncture site" of a starfish larvae (Segal, 2005). These cells make up 70% of the population of circulating granulocytes (Kantari et al., 2008). Neutrophils originate from the bone marrow as CD34+ myeloid progenitors where granulocyte-CSF (G-CSF) stimulates differentiation and proliferation of neutrophils. IL-4 or IL-3 has been shown to synergize with G-CSF *in vitro* for the production of neutrophils; however, G-CSF administration is sufficient for this to occur *in vivo* (Demetri and Griffin, 1991; Donini et al., 2007; Ema et al., 1990; Lieschke and Burgess, 1992). The release of neutrophils from the bone marrow occurs in a highly regulated fashion. Typically, in humans 10^{10} neutrophils are released from the bone marrow each day, with a half-life of 6-8 hours (Christopher and Link, 2007; Furze and Rankin, 2008). If neutrophils encounter a source or site of infection, their half-life is increased as a result of inflammatory cytokines and chemokines, including GM-CSF and IL-8. However, upon resolution of inflammation and even in the absence of it, neutrophils undergo programmed cell death which results in their phagocytosis by macrophages to clear apoptotic debris from tissues and prevent them from undergoing secondary necrosis, which becomes a very inflammatory stimulus.

Structure and granule content

Neutrophils are also known as polymorphonuclear granulocytes (PMNs) because of their irregularly shaped, multi-lobed nuclei and the presence of numerous cytoplasmic granules. There are four types of neutrophil granules, listed in order of their appearance as a neutrophil matures: primary or azurophil granules; secondary or specific granules; tertiary or gelatinase granules; and secretory granules. These latter two granules are only found in mature segmented neutrophils (Borregaard and Cowland, 1997). Interestingly, granule secretion begins with rapid secretion of the least abundant granules progressing to the most abundant (Faurschou and Borregaard, 2003).

Azurophil granules contain large amounts of myeloperoxidase (MPO), which serves as a marker of neutrophil presence and/or degranulation (Chertov et al., 2000). These granules contain smaller amounts of bactericidal/permeability increasing protein (BPI), defensins, elastase, and cathepsins. Cathepsin G and elastase are serine proteases with antimicrobial properties in addition to their ability to activate endothelial cells, epithelial cells, and macrophages (Chertov et al., 2000; Faurschou and Borregaard, 2003). Defensins are a class of cationic antimicrobial peptides, and can kill or inactivate bacteria, viruses, and fungi; however, these peptides are not found in mouse neutrophils. BPI is also antimicrobial in nature, and a portion of this peptide binds to the surface of neutrophils and macrophages to facilitate phagocytosis (Faurschou and Borregaard, 2003; Yang et al., 2004). Following neutrophil stimulation, azurophil granules are released in relatively small quantities

and thought to be most important for intracellular killing of phagocytosed pathogens (Faurschou and Borregaard, 2003).

Lysozyme and lactoferrin are the largest components of secondary granules, both of which are antimicrobial proteins (Yang et al., 2004). These two antimicrobial proteins are effective on a broad range of both Gram-positive and Gram-negative bacteria. Collagenase (MMP-8) is also found within these granules, and is important for degrading various components of the extracellular matrix (Faurschou and Borregaard, 2003). Proteins found in secondary granules are also important for generating the chemoattractant C5a through enzymatic cleavage and patients deficient in these neutrophil granules have abnormalities in chemotaxis (Sawyer et al., 1989).

Gelatinase granules, as the name suggests, contain mostly gelatinase (MMP-9). It is postulated that these granules are essential for neutrophil migration through basement membranes, as gelatinase breaks down the collagen found there (Borregaard and Cowland, 1997).

Secretory granules contain CD35, the complement receptor for C3b-mediated phagocytosis; these granules are the most rapidly mobilized granules in the neutrophil. Interestingly, the integrin Mac-1, the receptor for LPS CD14, Fc receptor CD16, and the receptor for fMLF, a bacterial peptide, are all found in the membrane of secretory granules (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003).

Degranulation

As mentioned above, secretory granules containing various transmembrane receptors are the first to be mobilized. This occurs very rapidly and in response to various stimuli including fMLF, LPS, TNFα, and shedding of L-selectin (Faurschou and Borregaard, 2003; Wagner and Roth, 2000). Fusion of secretory granules with the plasma membrane allows further recognition of bacterial products, phagocytosis, and adherence. The precise mechanisms and pathways involved in neutrophil degranulation are not fully understood. It is known that intracellular calcium flux precedes degranulation, and that calcium may induce interactions between soluble N-ethylmaleimide sensitive factor-attaching proteins (SNAPs) and SNAP receptor proteins (SNAREs), which are important for the fusion of vesicles with the plasma membrane (Burgoyne and Morgan, 2003). Research in this area of neutrophil biology is ongoing.

Chemokine and cytokine production

Once neutrophils have migrated to the site of infection, they secrete a number of different cytokines and chemokines to aid in recruitment of other innate effector cells. It is known that neutrophils secrete IL-8 and growth-related oncogene (GRO)-α (or MIP-2 and KC in mice), which in turn serves as a positive feedback loop for neutrophil recruitment (Cassatella, 1995; Kantari et al., 2008; Lapinet et al., 2000; Scapini et al., 2000). They also secrete various chemoattractant proteins for monocytes, including MIP-1α and MIP-1β. All of these chemokines can be released

following a variety of stimuli, including LPS, TNFα, fMLF, bacteria, fungi, viruses, and numerous other molecules (Cassatella, 1995; Kantari et al., 2008; Lapinet et al., 2000; Scapini et al., 2000); IL-8 seems to be the most promiscuous and prototypical cytokine produced by the neutrophil, and secretion of stored IL-8 occurs rapidly following recognition of any ligand or foreign molecule (Scapini et al., 2000).

Neutrophils also secrete various cytokines upon stimulation. Numerous reports indicate they can secrete TNFα, IL-1β, IL-12, and IL-1 receptor antagonist (IL-1Ra) (Bliss et al., 1999; Cassatella, 1995; Lapinet et al., 2000; Schroder et al., 2006; Sohn et al., 2007). Moreover, a few reports indicate they produce IL-6 and IFNγ (Melani et al., 1993; Riedemann et al., 2004; Yamada et al., 2011). However, these cells do not secrete the same levels of these cytokines compared to monocytes. Nevertheless, these cells outnumber monocytes/macrophages at the site of infection, and therefore must be considered a potent source of cytokines during infection (Cassatella, 1995; Kantari et al., 2008; Nathan, 2006; Scapini et al., 2000; Witko-Sarsat et al., 2000).

Bacterial clearance: Antimicrobial peptides and NETs

Neutrophils possess a number of different mechanisms for bacterial killing (Figure 1.2). First, killing can occur through degranulation and release of soluble antimicrobial peptides, including defensins, lactoferrin, lysozyme, and several serine proteases. These peptides are highly cationic, and these basic residues are responsible for their activity (Hancock and Diamond, 2000; Yang et al., 2004). These

peptides can bind to and neutralize bacterial endotoxin and disrupt cellular membranes, thereby causing direct lysis of bacteria (Hancock and Diamond, 2000; Yang et al., 2004).

However, the highly cationic nature of these peptides also causes destruction of bystander cells and the extracellular matrix. This requires a way in which to directly deliver these proteins in proximity to bacteria in order to contain them, while causing minimal damage to host tissues. One way in which the neutrophil accomplishes this is through release of nuclear DNA, called neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Recently discovered, these NETs enable neutrophils to deliver these granules in a meshwork of chromatin. NETs bind both Gram-positive and Gram-negative bacteria, causing cell death and degradation of virulence factors. Furthermore, NETs contain a high concentration of antimicrobial peptides and prevent bacterial dissemination (Brinkmann et al., 2004). However, one important consequence of NET release is in fact cell death, as the cell is extruding nuclear DNA. This is in contrast to the mitochondrial DNA released by eosinophils for this same purpose (Brinkmann et al., 2004; Yousefi et al., 2008). This will be discussed more in Section V regarding eosinophils.

Bacterial clearance: Phagocytosis

Finally, phagocytosis provides the neutrophil a manner in which to kill bacteria intracellularly. Phagocytosis occurs through binding bacteria via several different receptors. These include two main classes: Fcγ receptors, which bind to the Fc

region on IgG; and complement receptors including CD35 and Mac-1, which bind to microbes opsonized by complement components (Kantari et al., 2008). Using these receptors, opsonized pathogens enter the cell in a phagosome, where they can be destroyed by fusion with granules containing antimicrobial peptides and the respiratory burst through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, as described below.

Intracellular killing is largely accomplished through NADPH oxidase and MPO. NADPH oxidase dysfunction is also detrimental to pathogen killing and known to cause chronic granulomatous disease (CGD) in humans (Winkelstein et al., 2000). This oxidase is responsible for catalyzing the formation of superoxide anion and other reactive oxygen species (ROS), and is found in both the plasma membrane as well as the membrane of the phagosome; superoxide production, however, has only been detected within the phagosome (Quinn and Gauss, 2004; Segal, 2005). Electrons are transferred from the cytosol into the phagosome through NADPH oxidase, which is composed of multiple subunits found within the cytosol. Neutrophil stimulation results in translocation and assembly of these components in the membrane. The process of phagocytosis and activation of this oxidase is also known as the respiratory burst, as cells undergo a burst of oxygen consumption (Hampton et al., 1998; Nauseef, 2007).

MPO constitutes roughly 25% of the granule protein in the neutrophil, and catalyzes the oxidation of halides in a hydrogen peroxide-dependent manner (Segal, 2005). MPO knockout mice are highly susceptible to bacterial infections, as are

humans who lack this peroxidase (Lehrer et al., 1969; Segal, 2005; Winterbourn et al., 2000). While MPO does not kill bacteria by itself, it does synergize with NADPH oxidase to enhance the killing activity of ROS (Nathan, 2006; Segal, 2005). It appears that the function of MPO is to remove hydrogen peroxide from the phagosome, as hydrogen peroxide is known to cause inactivation of antimicrobial proteins (Segal, 2005).

Summary

Neutrophils circulate through the blood in search of inflammation or bacterial infection. Once they reach these sites, they are potent at killing bacteria through degranulation and phagocytosis. They also attract additional leukocytes through secretion of chemokines. Subsequently, even in the absence of inflammation, they undergo senescence and programmed cell death, allowing them to be phagocytosed and cleared by tissue macrophages. This is summarized in Figure 1.4.

iii. Monocytes/Macrophages

Monocyte Differentiation and Homeostasis

Monocytes constitute approximately 5-10% of all leukocytes in human blood and roughly 4% in mice (Auffray et al., 2009; Gordon and Taylor, 2005). They have large, irregularly shaped nuclei and originate in the bone marrow. Macrophage-CSF (M-CSF) is responsible for driving their differentiation from common myeloid progenitors into macrophages once monocytes enter into tissues. Monocytes also differentiate into dendritic cells, another important professional antigen presenting cell (APC), which migrate into tissues and lymph nodes. Dendritic cells are $CD14^-$ $CD16^-$ APCs vital for linking the innate and adaptive immune systems; this leukocyte will not be discussed in further detail in this thesis.

Monocytes are released from the bone marrow daily where they circulate for several days before migrating into tissues, either due to the presence of inflammation or as a homeostatic mechanism to replace dead and dying cells. They are morphologically heterogeneous in the blood, which is why they can often be confused with both lymphocytes and granulocytes. In the tissues, macrophages can live for weeks to months before they undergo programmed cell death and are replaced by new circulating monocytes (Soehnlein and Lindbom, 2010).

Monocyte subsets

In human blood, monocytes consist of primarily two subsets—the large $CD14^+CD16^-$ subset and smaller $CD16^+$ monocytes. $CD14^+CD16^-$ monocytes represent the majority of circulating monocytes (80-90%), and expresses high levels of CCR2 (MCP-1 receptor) and low levels of CX3CR1 (fractalkine receptor); this subset is known as resident monocytes. Resident monocytes produce IL-10 in response to LPS *in vitro*, rather than TNF or IL-1. Furthermore, this subset is more phagocytic than the $CD16^+$ subset (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). $CD16^+$ monocytes express low levels of CCR2 and high levels of CX3CR1. This subset produces TNF in response to LPS stimulation, and is also known as the inflammatory monocyte population (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). In addition, inflammatory monocytes are expanded during infectious disease, acute inflammation, and sepsis (Fingerle-Rowson et al., 1998).

$CD16^+$ monocytes in humans are further divided into two groups—$CD14^+$ monocytes, which also express CD64 and CD32 Fc receptors, and $CD14^{dim}$ monocytes, which lack these receptors. Interestingly, recent work has shown that the $CD14^{dim}$ monocyte population does not produce TNFα or IL-1 in response to LPS stimulation. In contrast, the $CD14^+CD16^+$ subset of $CD16^+$ cells is entirely responsible for production of these inflammatory cytokines. The exact function of $CD14^{dim}$ monocytes is not fully understood (Gordon and Taylor, 2005; Serbina et al., 2008).

In mice, monocytes are instead differentiated from other leukocytes by their expression of F4/80 and CD11b. Like human monocytes, they are subdivided based on expression of CCR2 or CX3CR1, and also Ly6c. Inflammatory monocytes in mice are $CCR2^{+}$ and $Ly6c^{+}$, whereas the resident monocytes are $CX3CR1^{+}$ and $Ly6c^{-}$ (Gordon and Taylor, 2005; Serbina et al., 2008). One important difference between these populations is that in human blood resident monocytes make up 80-90% of the population; in mice this population is roughly equal to the inflammatory monocyte subset (Serbina et al., 2008).

The $CCR2^{+}Ly6c^{+}$ inflammatory monocytes migrate into tissues where they then differentiate into macrophages, which are important for the clearance of microbes and wound repair. *In vitro* studies from both mice and human monocytes seem to indicate that the population of resident blood monocytes preferentially differentiates into dendritic cells and also tissue resident macrophages including Kupffer cells, alveolar and splenic macrophages, microglia, and osteoclasts. This population serves as a means to replenish these populations in tissues (Gordon and Taylor, 2005). There is continued debate whether tissue macrophages are terminally differentiated, and some evidence indicating that they do in fact proliferate (Mosser and Edwards, 2008; Ringheim, 1995). Many questions still remain regarding monocyte populations and their differentiation into tissue macrophages.

Structure and function

Monocytes possess an array of scavenger receptors capable of recognizing lipids and a number of microbial components. These receptors aid in phagocytosis of bacteria and apoptotic cells and recognition of infection. Moreover, expression of numerous PRRs allows monocytes to recognize microbial pathogens, both intra- and extracellularly. Once stimulated, they can produce large amounts of ROS, nitric oxide, complement components, and numerous cytokines including TNFα, IL-1β, IL-6, and IL-10. They also release numerous chemokines, including IL-8 and leukotriene B_4, to attract neutrophils to sites of infection and inflammation (Gordon and Taylor, 2005; Mosser and Edwards, 2008; Taylor et al., 2005).

In addition, once monocytes enter the tissues and differentiate into macrophages, they grow in size increasing in their number of mitochondria, lysosomal contents, and hydrolytic enzymes (Soehnlein and Lindbom, 2010). Here, macrophages are responsible for the phagocytosis and killing of microbial pathogens, and also serve as garbage collectors—to clean cellular debris and apoptotic cells from tissues. Depending on the tissue in which a macrophage is localized, as well as environmental cues, their function and phenotype can change dramatically.

Macrophage subsets and functional phenotypes

There are a number of macrophages subsets, many of which are named for the tissue in which they reside. These include: osteoclasts found in the bone; microglial cells found in the central nervous system; alveolar macrophages found in the lungs;

Kupffer cells found in the liver; histiocytes found in connective tissue; several types of splenic macrophages found in the spleen; and peritoneal macrophages found in the peritoneal cavity (Taylor et al., 2005). However, within the last two decades it has become abundantly clear that macrophages have very different functional responses to inflammatory stimuli and require different cytokines to induce their effector functions. Originally, two subsets were named—classically activated or alternatively activated macrophages. However, this classification implies that there is only one 'alternative' way to activate a macrophage, and this is in fact not the case. It has been proposed recently that there are three main types of macrophages (Figure 1.3): classically activated macrophages; wound-healing macrophages (previously known as alternatively activated); and regulatory or antiinflammatory macrophages (Gordon and Taylor, 2005; Mosser and Edwards, 2008).

Classically activated macrophages have enhanced microbicidal activity and production of proinflammatory cytokines compared to the other subsets. They are produced during an immune response to infection largely through the production of TNF and IFNγ, but are also produced following stimulation with TLR agonists that specifically induce production of TNF and IFNβ (Gordon and Taylor, 2005; Mosser and Edwards, 2008). Classically activated macrophages are an important source of proinflammatory cytokines including IL-1, IL-6, and IL-12. They produce ROS and nitric oxide to aid in bacterial killing, and have increased expression of major histocompatibility complex (MHC) class II and CD86, allowing for increased antigen presentation (Gordon and Taylor, 2005; Taylor et al., 2005). While this subset is

important for pathogen killing and containment, they also cause destruction of host tissue and contribute to autoimmune disease through production of IL-17 and IL-23 (Kolls and Linden, 2004; Mosser and Edwards, 2008). Their activation needs to be highly regulated to prevent the immunopathologies associated with their presence.

Wound-healing macrophages do exactly as their name suggests and are induced in response to IL-4 production, which is released rapidly following tissue injury (Loke et al., 2007). IL-4 shifts resident tissue macrophages toward a wound-healing phenotype, through expression of arginase, which allows them to make precursors of numerous extracellular matrix components, and chitinase, which binds to the extracellular matrix (Hesse et al., 2001; Kreider et al., 2007). Macrophage treatment *in vitro* with IL-4 and IL-13 reduces ROS and nitric oxide production, making them less efficient at killing pathogens than the classically activated macrophage. Moreover, these macrophages do not present antigen and have minimal production of proinflammatory cytokines (Edwards et al., 2006; Hesse et al., 2001). In fact, wound-healing macrophages are more susceptible to certain intracellular infections than their classically activated counterparts, and their increased presence leads to growth of the organism or a failure to clear it (Mosser and Edwards, 2008). Like classically activated macrophages, their activation must be controlled, as tissue fibrosis can occur if they are allowed to function unchecked. Evidence for this is seen during chronic schistosomiasis, where a decrease in wound-healing macrophages resulted in reduced tissue fibrosis in mice (Hesse et al., 2001).

The third class of macrophages is known as regulatory or antiinflammatory macrophages, due to their production of large amounts of IL-10. They are generated following stimulation with various Ig complexes, prostaglandins, glucocorticoids, apoptotic cells, and even IL-10 (Mosser and Edwards, 2008). To induce this phenotype, it is necessary to have a second signal, for example a TLR ligand, as a single signal on its own has minimal effect on the cell. In addition to producing large amounts of IL-10, regulatory macrophages highly express costimulatory molecules, indicating that they can present antigen (Edwards et al., 2006). Expansion of this cell type *in vivo* leads to elevated IL-10, reduced pathogen killing, and increased susceptibility of the host to disease (Mosser and Edwards, 2008).

One important consideration for these classification schemes is that macrophages still remain plastic and respond to environmental cues. What once was a wound-healing macrophage can become a regulatory macrophage while still maintaining expression of specific markers of the wound-healing macrophage. One such example is seen during cancer progression, where the initial enhancement of classically activated macrophages is to eradicate cancerous cells. However, local tissue damage and the tumor microenvironment develops into a more regulatory macrophage phenotype and high production of IL-10 (Pollard, 2008). Likewise, in obesity there is a switch from a predominant wound-healing macrophage phenotype to that of the classically activated inflammatory macrophage, leading to production of TNF and IL-6 in the adipose tissue (Lumeng et al., 2007). This plasticity seen during cancer and obesity is important to consider as some immunomodulatory disease strategies seek

to deplete a specific population of cells and may be detrimental to the host. Using environmental cues to induce functional and phenotypic changes may provide a more appropriate therapy for patients in various diseases where a specific population of macrophages is lacking or dominating.

Phagocytosis

Macrophage phagocytosis serves two important functions in the innate immune response: 1) it aids in pathogen containment and clearance; and 2) it is vital for clearance of apoptotic cells and other detritus left in the tissues following inflammation. Pathogen phagocytosis is accomplished through expression of a number of different cellular receptors including: scavenger receptors and TLRs, which recognize various lipo- and glycoproteins; Fc receptors including CD16 and CD32, which facilitate uptake of antibody-opsonized particles or organisms; and Mac-1, which assists in complement-mediated phagocytosis in addition to adherence. The importance of pathogen phagocytosis was discussed previously in the Initiation of the Innate Immune Response and Neutrophil portions of this Section and will not be discussed further here.

Phagocytosis of apoptotic cells, also known as efferocytosis, is an important process in regulating inflammation. It is a normal process occurring daily for tissue homeostasis and is very efficient. Normally, macrophage efferocytosis results in production of antiinflammatory mediators including IL-10. However, if apoptotic cells are not cleared they undergo secondary necrosis, which is highly inflammatory

(Fink and Cookson, 2005; Wesche et al., 2005). Neutrophils undergoing a secondary necrosis release numerous proteases, highly cationic peptides, and ROS that damage surrounding cells and tissue. This damage to bystander cells causes release of danger signals, which perpetuate the inflammatory response (Kono and Rock, 2008). Macrophage efferocytosis can occur through recognition of various receptors and molecules expressed by dying cells. Phosphatidyl serine (PS) is found on the cytosolic side of cellular membranes. When cells undergo apoptosis, this residue is exposed on the exterior of the cell, allowing internalization by macrophages using their PS receptor. Interestingly, the complement pathway and receptors have also been identified in the clearance of apoptotic cells, as have various scavenger receptors like CD36 and scavenger receptor A. Surfactant proteins A and D released by type II pneumocytes in the lung also enhance macrophage efferocytosis of neutrophils via the complement receptor calreticulin (Taylor et al., 2005). The precise mechanisms for phagocytosis of apoptotic cells depend on the receptor(s) involved, though there is still relatively little known about this process and about the subsequent digestion of these cells (Erwig and Henson, 2008; Fink and Cookson, 2005).

Summary

Monocytes circulate through the blood and migrate into tissues, where they differentiate into macrophages and secrete cytokines and chemokines to alert the immune system of infection. Macrophages are important for phagocytosis of pathogens and cellular debris found during infection and inflammation. The numerous different subtypes allow for highly specialized responses during inflammation. This is summarized in Figure 1.4.

B. The Pathophysiology of the Immune Response in Sepsis

The dysfunction of the immune system during sepsis is an important cause of mortality in patients, and the continued dysfunction of the immune system after resolution of sepsis leads to a lower quality of life and accelerated long-term mortality (Benjamim et al., 2004). This Section will discuss the current knowledge of the innate immune response during sepsis. It will highlight hallmarks of the cytokine storm as well as characteristics of immunosuppression in septic patients.

i. Innate Immunity and the Cytokine Storm

In sepsis, the initial recognition of a pathogen by PRRs results in a cascade of events triggering recruitment of leukocytes, production of pro-and antiinflammatory cytokines, chemokines, and activation of innate effector cells to induce microbial killing. This was described in detail earlier in Section IIA. Investigators initially believed that the major cause of death in sepsis was due to overactivation of the immune response, or the cytokine storm. However, patient studies revealed that while this phase is capable of causing mortality from sepsis, the frequency at which it occurs in patients is lower than originally estimated (Hotchkiss and Karl, 2003). Several studies have examined cytokine levels in septic patients to assess correlations with mortality. Elevated levels of IL-8 and MCP-1 correlate with increased likelihood of organ failure in sepsis (Bozza et al., 2007). Furthermore, elevated IL-10 and IL-6 levels are associated with increased mortality (Bozza et al., 2007; Kellum et al., 2007; Remick et al., 2002).

Elevation of numerous proinflammatory cytokines and chemokines in sepsis ultimately leads to enhanced neutrophil recruitment, degranulation, and production of nitric oxide and ROS. In particular, the release of nitric oxide by numerous cell types causes systemic vasodilation, increasing the accessibility of leukocytes to tissues, releasing proinflammatory mediators into potentially healthy tissues, and causing multiple organ failure (Hotchkiss and Karl, 2003; Russell, 2006). These studies indicate that there are numerous mediators of death and inflammation during the innate immune response to sepsis. However, clinical trials aimed at dampening the cytokine response have failed to improve mortality in patients. This will be discussed more in Section III.

ii. Immunosuppression in Sepsis

Patient studies indicate that the cytokine storm theory put forth by Lewis Thomas in the 1970's is accurate only in the first phase of infection (Adrie and Pinsky, 2000; Wang and Deng, 2008). The extent of the initial *cytokine storm* dictates the severity of the resulting *immunosuppression*; as mentioned previously, both of these physiologic states are capable of independently causing mortality (Hotchkiss and Karl, 2003; Hotchkiss and Nicholson, 2006; Martin et al., 2003; Russell, 2006).

Monocyte dysfunction

It is only within the last decade that the severity of immunosuppression in septic patients was appreciated. Numerous studies showed that monocytes isolated from septic individuals have reduced HLA-DR and CD86 costimulatory molecule

expression, causing impaired antigen presentation (Docke et al., 1997; Heumann et al., 1998; Lekkou et al., 2004; Monneret et al., 2004). Furthermore, the fewer monocytes present in the blood increases the risk of death from this disease (Ditschkowski et al., 1999; Haveman et al., 1999; Lekkou et al., 2004; van den Berk et al., 1997). Septic shock is also known to trigger monocyte apoptosis (Ayala et al., 1996), which explains the reduction in circulating monocytes in septic patients. These studies indicate that defects in monocyte function during sepsis are detrimental to the host.

Moreover, monocytes from septic individuals produce decreased levels of inflammatory cytokines including TNFα and IL-1β when treated with LPS, indicating deactivation (Docke et al., 1997; Lekkou et al., 2004; Piani et al., 2000; Wolk et al., 2000). However, when these monocytes are treated with the inflammatory cytokine IFNγ following LPS treatment, production of TNFα and IL-1β is restored, fostering the idea that the inflammatory state in septic patients potentiates the dysregulation of the immune response (Docke et al., 1997). In addition, when monocytes are treated with GM-CSF and LPS, TNFα and IL-1 production is enhanced over LPS treatment alone (Cannistra et al., 1988; Cohen et al., 1991). In sum, these data indicate immunosuppression associated with sepsis can be reversed by treatment with inflammatory cytokines.

Neutrophil dysfunction

Additionally, there is evidence of defective neutrophil function in septic patients. These primary responders to infections are in a suppressed or unreactive state and the inflammatory milieu in patients is the cause of much of this suppression. In particular, one study demonstrated that septic serum inhibits superoxide production from normal neutrophils, which is indicative of neutrophil deactivation (Zimmerman et al., 1989). Further, neutrophils isolated from both septic mice and patients exhibit reduced chemotaxis compared to control neutrophils in response to fMLF, IL-8, and C5a, all of which are known to have potent chemotactic properties (Arraes et al., 2006; Chishti et al., 2004; Solomkin et al., 1985; Wenisch et al., 2001). Treating control neutrophils with LPS, IL-1β, and IFNγ, to mimic the inflammatory environment in sepsis, resulted in a similar reduction in chemotaxis (Arraes et al., 2006). These data are consistent with the idea that neutrophils are deactivated by the initial cytokine storm, so that even when it subsides they remain paralyzed.

Other studies have shown that neutrophils from septic patients have reduced adhesion molecule expression, including CD11b, and reduced degranulation, both of which are important to effectively locate and fight infection (Brown et al., 2006; Chishti et al., 2004; Wenisch et al., 2001). Indeed, depletion of neutrophils at the onset of sepsis caused increased bacteremia and mortality in animals infected with either *S. aureus* or *Listeria monocytogenes* (Czuprynski et al., 1996; Hoesel et al., 2005; Verdrengh and Tarkowski, 1997). Interestingly, evidence suggests there is an increased incidence of immature neutrophils in septic patients, and these immature

cells have reduced phagocytosis and calcium signaling following stimulation (Taneja et al., 2008). Loss of neutrophils in septic patients is associated with increased mortality and treatment with G-CSF results in a massive increase in circulating neutrophils and reduced serious complications in patients (Nelson et al., 1998). These data indicate the importance of neutrophils in bacterial clearance during sepsis.

Apoptosis in sepsis

Apoptosis has been a major pathway implicated in the lethality of sepsis (Hotchkiss and Nicholson, 2006; Hotchkiss et al., 1999a). Additionally, granulocytopenia and lymphocytopenia are biomarkers of sepsis, and neutropenia alone is an independent predictor of mortality (Hotchkiss et al., 1999a; Weinstein et al., 1983a). Patient studies have shown that apoptosis is observed in leukocytes from septic patients at much greater levels than in non-septic ICU patients (Hotchkiss and Nicholson, 2006). In sepsis, apoptosis occurs at high rates in both lymphoid cells, including T and B cells, dendritic cells, monocytes, and neutrophils, as well as non-lymphoid endothelial and epithelial cells in the heart, intestines, and lungs (Wesche et al., 2005). Interestingly, mice deficient in caspase-12, a mediator of apoptosis, have improved bacterial clearance and 100% survival from CLP-induced sepsis (Saleh et al., 2006). Moreover, upregulation of the anti-apoptotic protein Bcl-2 (B cell lymphoma-2) leads to improvement in survival in CLP-induced sepsis (Hotchkiss et al., 1999b). These studies suggest that immunosuppression occurring in septic

patients is, at a minimum, partially reversed by improving survival of leukocytes, thereby augmenting the immune response in sepsis.

It is noteworthy that reduction in neutrophil apoptosis observed in sepsis may in fact be detrimental to host survival, as it can result in increased tissue damage due to the increased abundance of activated neutrophils (Jimenez et al., 1997). Specifically, neutrophil depletion studies or inhibition of neutrophil recruitment into the peritoneal cavity in the CLP model of sepsis demonstrated improved survival and reduced damage to the lung and liver (Walley et al., 1997; Wickel et al., 1997). As mentioned earlier, neutrophil depletion in mice at the onset of sepsis results in elevated bacteremia, indicating their importance for bacterial clearance. However, this study by Hoesel and colleagues showed there was no evidence of a reduction in organ damage at this time point. Further, when neutrophils were depleted 12 hours after the onset of CLP-induced sepsis (which is notably the time point at which immunosuppression is observed in this model), there was a sharp reduction in bacterial burden and reduced liver and renal dysfunction (Hoesel et al., 2005). These data support the notion that neutrophils represent a double-edged sword in sepsis—they are required early on for bacterial killing, but as they lose their effector functions their presence is associated with elevated organ dysfunction and increased bacterial burden.

Summary

Collectively, these data highlight the importance of the innate immune response in sepsis and illustrate the profound defects observed in patients. Moreover, it appears that the risk of death from this disease is increased when these defects are more pronounced. More effective therapies for patients and better understanding of this disease are paramount.

Figure 1.1: The inflammatory response to bacterial infection or sepsis

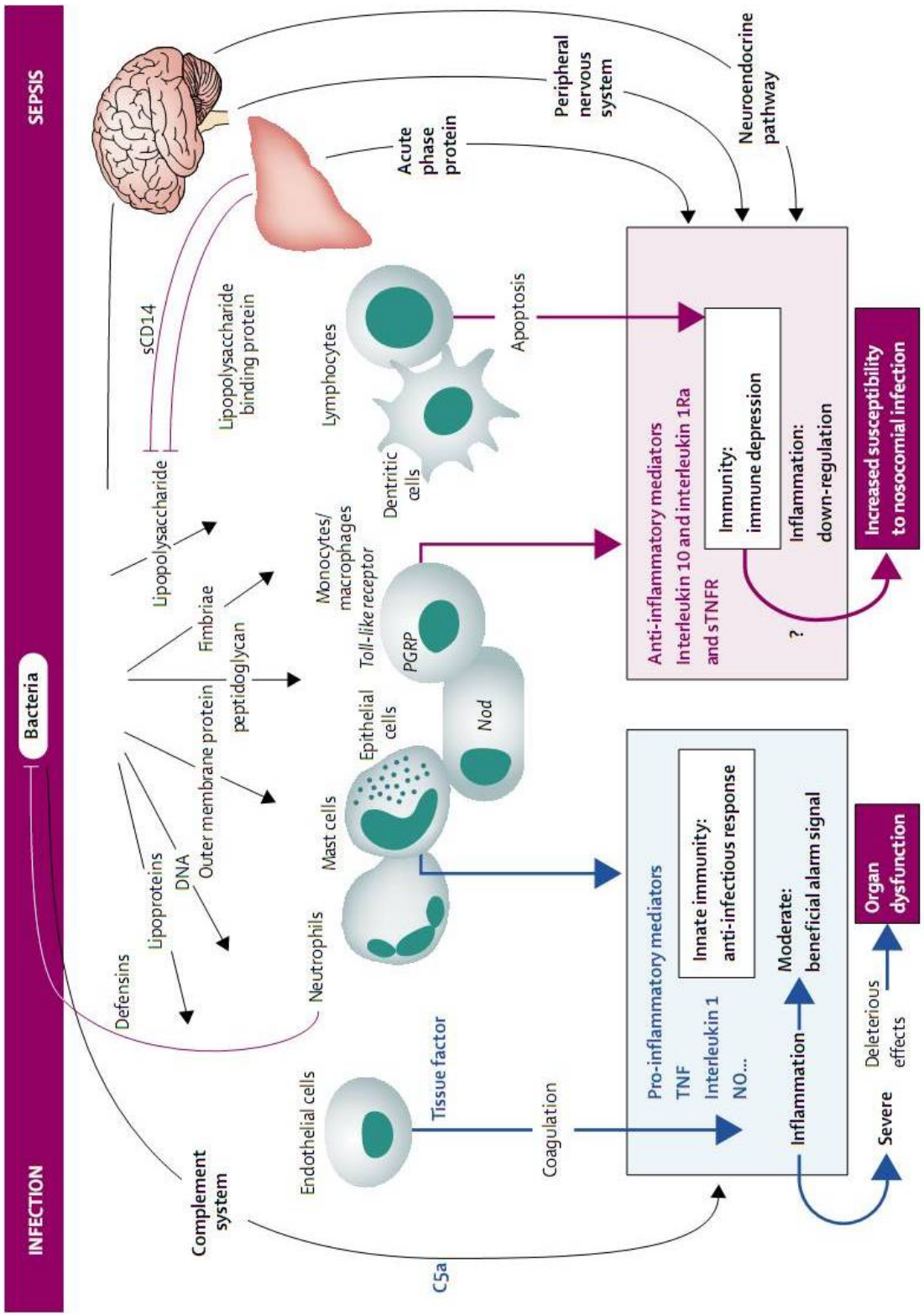

Figure 1.1: The inflammatory response to bacterial infection or sepsis

The inflammatory response to infection is complex, and involves a concerted effort by numerous cell types, both of the immune system as well as non-immune cells. The presence of bacteria releases foreign lipoproteins, DNA, and other bacterial components into the tissues and circulation. These are recognized by cells of the innate immune system, resulting in the recruitment of additional effector cells to sites of infection and the production of pro- and antiinflammatory cytokines and proteins to induce an immune response to ultimately clear the infection. This response affects multiple pathways and additional organ systems, including endothelial barriers and coagulation, the acute phase response by the liver, and induces changes in the neuroendocrine system. An overactive immune response often results in tissue damage leading to sepsis and organ dysfunction. The antiinflammatory response that is induced seeks to limit damage caused by the immune system. However, this can result in immunosuppression and susceptibility to secondary infections. Reprinted from the Lancet (Annane et al., 2005) with permission from Elsevier.

Figure 1.2: Neutrophil-mediated bacterial killing mechanisms

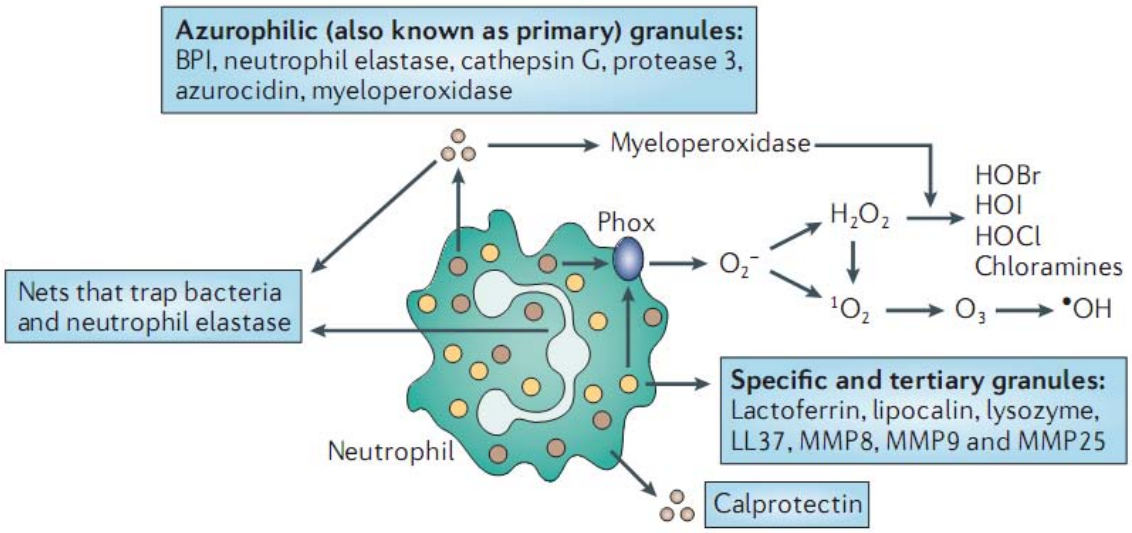

Figure 1.2: This figure demonstrates the various mechanisms neutrophils possess for killing bacteria: azurophilic granules, specific granules, and tertiary granules in addition to NETs and the respiratory burst. H_2O_2, hydrogen peroxide; HOBr, hypobromous acid; HOCl, hypochlorous acid; HOI, hypoiodous acid; O_2^-, superoxide; $^1O_2$, singlet oxygen; O_3, ozone; OH, hydroxyl radical; Phox, phagocyte oxidase (NADPH oxidase). Reprinted from (Nathan, 2006) with permission by Macmillan Publishers, Nature Publishing Group.

Figure 1.3: Functional classification of macrophage subsets

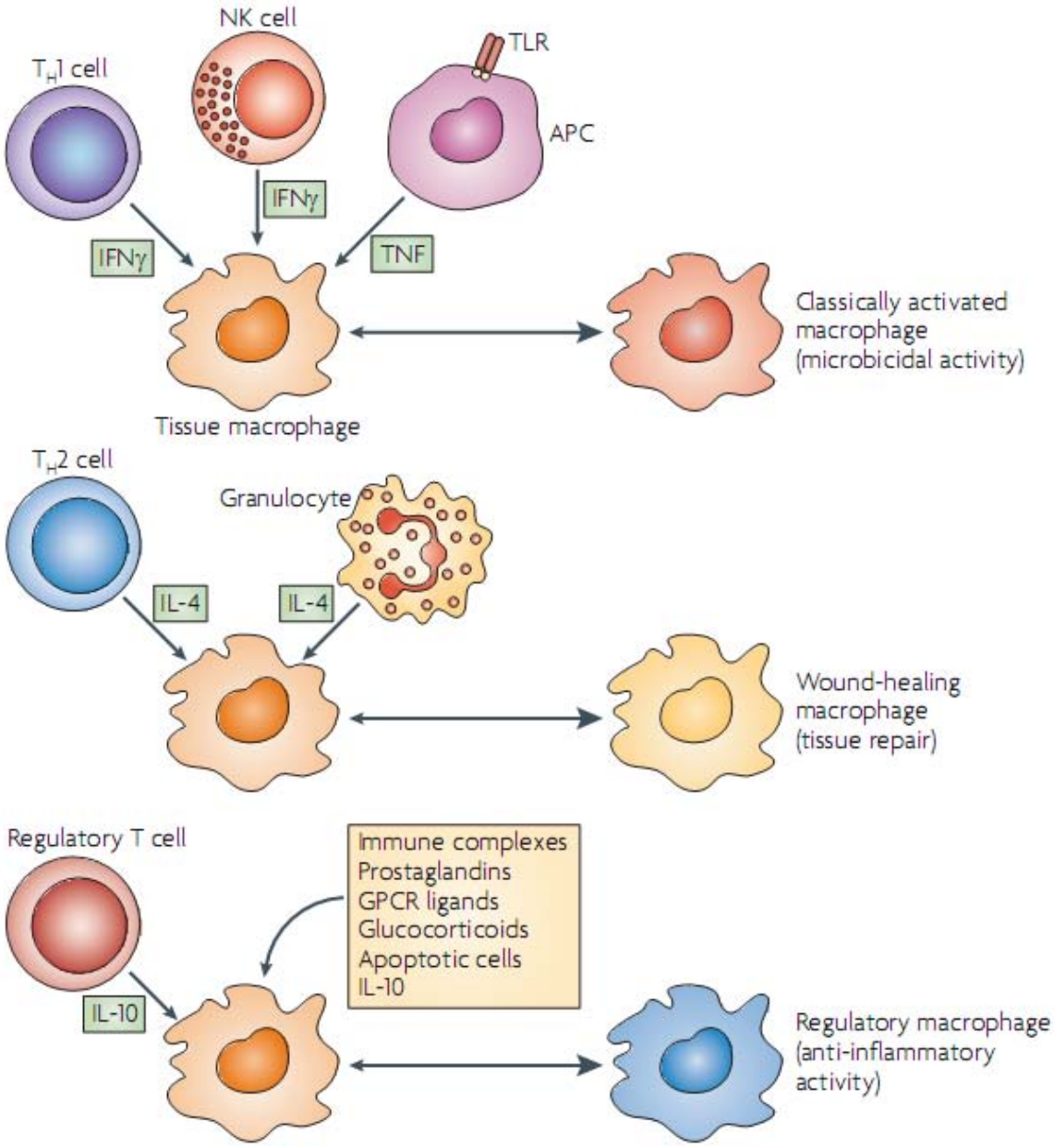

Figure 1.3: Functional classification of macrophage subsets

There are three main classes of macrophages based on their functional roles. Classically activated macrophages are induced by TNF and IFNγ production, and have potent microbicidal activity as well as enhanced production of proinflammatory cytokines. Wound-healing, or alternatively activated macrophages are induced by IL-4 and IL-13 release and are important in producing components of the extracellular matrix and clearing cellular debris through phagocytosis. Regulatory macrophages are produced in response to IL-10, apoptotic cells, glucocorticoids, and prostaglandins. They are an important source of IL-10, hence their antiinflammatory designation. Reprinted from (Mosser and Edwards, 2008) with permission by Macmillan Publishers Ltd, Nature Publishing Group.

Figure 1.4: Phagocyte interactions in inflammation

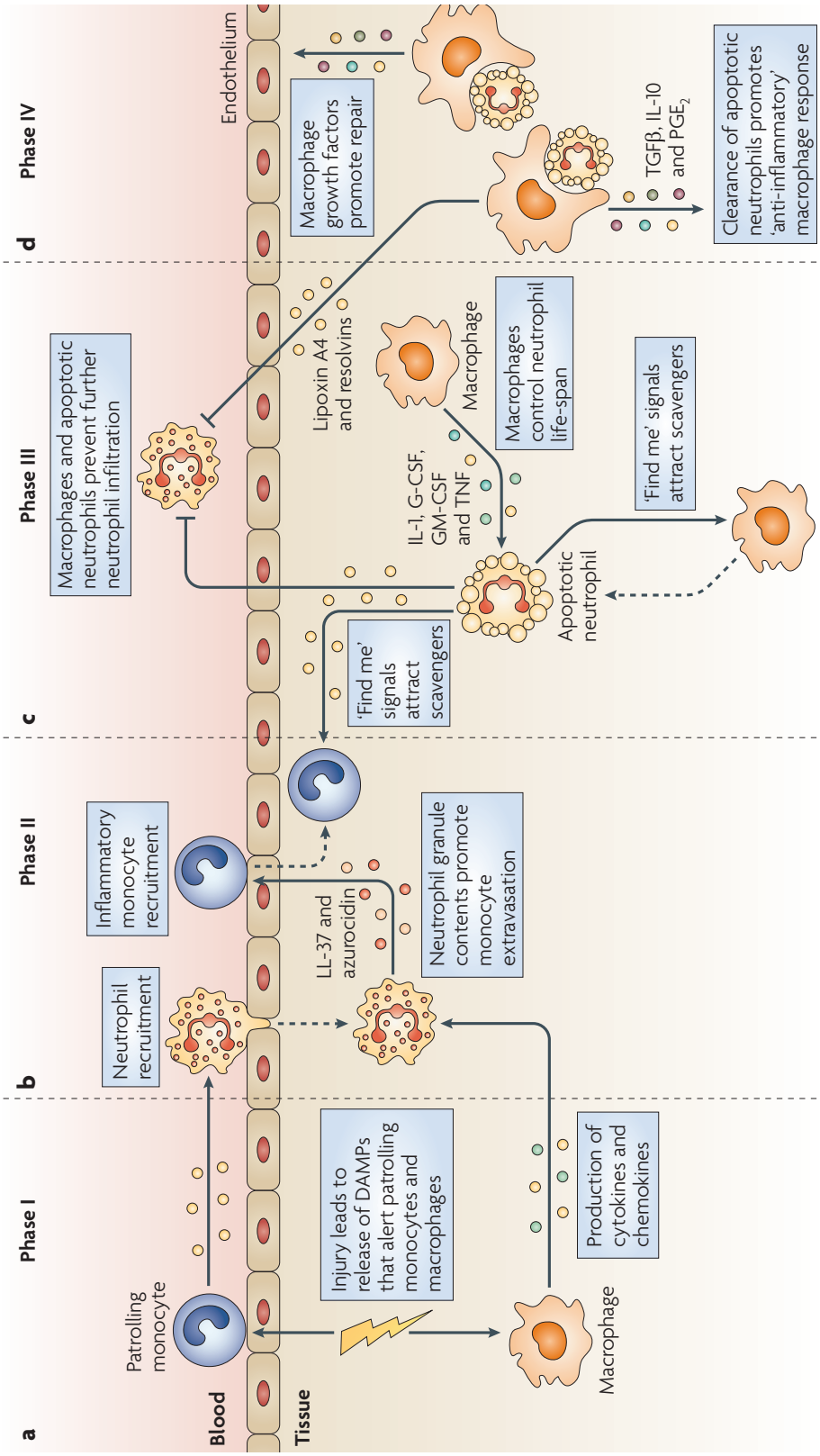

Figure 1.4: Phagocyte interactions in inflammation

a) Phase I constitutes the sensing of infection or inflammation in tissues by resident macrophages and monocytes in the blood. b) Phase II is the recruitment of neutrophils via chemokines to the site of infection, where they migrate into tissues and degranulate. This results in pathogen containment, tissue damage, and recruitment of additional monocytes. c) Phase III occurs when neutrophil senescence begins. This signal prevents influx of additional neutrophils, while allowing monocyte influx. It also calls in resident macrophages to ingest apoptotic neutrophils. d) Phase IV is when macrophage efferocytosis takes place resulting in the production of antiinflammatory mediators to prevent further influx of neutrophils and other leukocytes. It is also during this phase that wound healing by macrophages is initiated. PGE_2: prostaglandin E_2; TGFβ: transforming growth factor-β. Reprinted from (Soehnlein and Lindbom, 2010) with permission by Macmillan Publishers Ltd., Nature Publishing group.

III. THE FAILURE OF CLINICAL TRIALS IN SEPSIS

Over the last few decades, there have been tremendous efforts to find effective therapeutic modalities for septic patients. This Section will discuss the most notable failures of the past decades and the research that led up to these clinical trials. Table 1.3 summarizes the results of these trials, and several others not discussed in this thesis, which have all failed in septic patients. The failure of these promising agents led to a reevaluation of the most appropriate animal models of sepsis and a different approach to finding therapeutic interventions in sepsis. These models will be discussed in greater detail Section IV, and briefly below.

A. Endotoxemia is not Sepsis

Over the past 30 years, clinical trials were conducted largely based on a single model of animal sepsis, known as endotoxemia, induced by systemic administration of LPS, a cell wall component of Gram-negative bacteria. Endotoxemia results in rapid death in animals due to an overwhelming but transient production of inflammatory cytokines, known as the cytokine storm. Because it was known that patients also had robust production of inflammatory cytokines, endotoxemia led investigators to examine the benefits of dampening the initial cytokine storm (Adrie and Pinsky, 2000; Beutler et al., 1985; Cannon et al., 1990; Hamilton et al., 1992; Hesse et al., 1988). These clinical trials are discussed below.

B. The Pharmaceutical Graveyard: Failed drug targets in sepsis

Early research conducted using the endotoxin model showed promising results for drug targets in sepsis, through improved animal survival and reduction in the proinflammatory cytokine response. For example, administration of a recombinant IL-1Ra or anti-TNF antibodies protected mice from the lethality of endotoxemia (Beutler et al., 1985; Fischer et al., 1992; Ohlsson et al., 1990; Tracey et al., 1987). In addition, mice deficient in the co-receptor for LPS, CD14, do not respond to a lethal dose of LPS. Furthermore, animals treated with anti-CD14 antibodies have improved mortality following endotoxemia (Haziot et al., 1996; Schimke et al., 1998). However, when these drug targets were brought to clinical trials, IL-1Ra did not reduce mortality in septic patients, and high doses of anti-TNF antibodies actually increased mortality (Cohen and Carlet, 1996; Fisher et al., 1996; Fisher et al., 1994; Opal et al., 1997).

Anti-CD14 antibodies reduced the proinflammatory cytokine response and leukocyte activation in humans administered LPS, though it should be noted that *this is only when given prior to LPS injection* (Verbon et al., 2001). However, in a Phase 1 clinical trial, anti-CD14 failed to improve mortality, and may have increased it in groups receiving the highest doses (Reinhart et al., 2004). LPS injection induces different clinical features than human sepsis and therefore is not an accurate depiction of sepsis (Deitch, 2005; Hotchkiss and Karl, 2003). Moreover, the recent discovery of CD14 on non-myeloid cells, including endothelial and epithelial cells,

provide evidence that blocking CD14 may lead to unexpected complications in patients (Jersmann, 2005).

There have been numerous other candidates to target in septic patients. Corticosteroids, which are hormones released by the adrenal gland and thought to be antiinflammatory, have been examined as a therapy for septic patients. These trials have been largely negative, with no improvement in mortality and in some cases increasing it (Annane et al., 2002; Bone et al., 1987; Minneci et al., 2004). The inhibition of various components involved in coagulation, including treatment with antithrombin III or tissue-factor-pathway inhibitor, or the use of ibuprofen to inhibit prostaglandin production and inflammation have all failed to improve survival in patients, despite animal studies to the contrary (Abraham et al., 2003; Bernard et al., 1997; Warren et al., 2001).

The most successful FDA-approved therapy thus far in clinical trials is Activated Protein C, which acts on the coagulation pathway, inhibiting factors Va and VIIIa, and having antiinflammatory and antithrombotic effects (Abraham et al., 2005). Interestingly, this drug seems to increase the risk of bleeding and mortality in a subset of patients and is not widely used in the United States (Abraham et al., 2005; Bernard et al., 2001; Laterre et al., 2007; Laterre et al., 2008). Effectiveness of this therapy is currently being reevaluated (Gentry et al., 2009; Poole et al., 2009; Woodward and Cartwright, 2009).

The failure of numerous clinical trials and the discovery of immunosuppression in sepsis shifted the focus to augmenting the immune response in sepsis as a means to improve host control of infection and ultimately survival.

C. Augmenting the innate immune response is beneficial in sepsis

Investigators began to look at boosting the innate immune response to sepsis when the inhibition of proinflammatory cytokines failed. A trial investigating BPI, which binds and neutralizes LPS, as a therapy for meningococcal sepsis showed a trend toward improved survival, and reduced the number of amputations and length of hospital stay (Levin et al., 2000). Furthermore, administering septic and trauma patients GM-CSF, which mobilizes innate immune cells from the bone marrow, reduced the time spent on antibiotics, length of hospital stay, and infectious complications (Orozco et al., 2006). Treatment also shifted neutrophil presence from the lung into the blood, and restored the phagocytic function and superoxide production by innate effector cells (Presneill et al., 2002). In addition, G-CSF was successfully used as a prophylactic in surgical patients to prevent sepsis (Schneider et al., 2004; Weiss et al., 1995).

These trials indicate that boosting the innate immune response may be an effective strategy to reduce mortality and improve clinical outcomes of patients in sepsis. In Chapters 4, 5, and 6, the functional significance and effects of IL-5 as an immunomodulatory therapy in sepsis will be investigated.

Table 1.3: Results of Selected Clinical Trials and Meta-analysis Studies in Sepsis

Treatment	Results	References
Antiinflammatory agents		
TLR4 inhibitor	No decrease in mortality; failed to suppress IL-6	(Rice et al., 2010)
Anti-LPS	No decrease in mortality	(Ziegler et al., 1991)
IL-1β receptor antagonist	No decrease in mortality	(Fisher et al., 1994; Opal et al., 1997)
Anti-TNFα antibody	No decrease in mortality	(Abraham et al., 1998; Abraham et al., 1995)
Anti-TNF receptor antibody	No decrease in mortality	(Abraham et al., 2001)
Anti-coagulants		
Heparin	No decrease in mortality or multiple organ dysfunction	(Jaimes et al., 2009)
Antithrombin III	Decrease in mortality	(Wiedermann et al., 2006)
	No change in Mortality	(Warren et al., 2001)
Activated Protein C	Decreases mortality in subset of patients with severe sepsis; increased risk of bleeding; no change in mortality for patients with less severe sepsis	(Bernard et al., 2001)
	No decrease in mortality; increased risk of bleeding	(Abraham et al., 2005)
	Trend toward increased mortality; increased risk of bleeding	(Wiedermann and Kaneider, 2005)
Corticosteroids	Mixed results depending on dose: high dose increased or did not change mortality; low dose decreased mortality in subset of patients	(Annane et al., 2002; Bone et al., 1987; Minneci et al., 2004)
Intensive insulin therapy	Increased mortality	(Finfer et al., 2009)
	No change in mortality	(Van den Berghe et al., 2006)
Nitric Oxide Synthase inhibition	Increased mortality	(Lopez et al., 2004)

IV. CURRENT ANIMAL MODELS OF SEPSIS

The failure of clinical trials resulted in a new perspective on what constitutes sepsis in animal models. Clinical differences between the endotoxin model and human sepsis will be highlighted in this Section. In addition, the relevance of the CLP model will be addressed by examining numerous studies in both mice and humans with sepsis.

A. Disparities between patients and animal models

In the endotoxin model, mice develop a rapid but fleeting cytokine storm that peaks after 90 minutes and subsequently dies out approximately 8 hours after administration. However, cytokine levels persist much longer in human sepsis (Buras et al., 2005; Deitch, 1998; Deitch, 2005). While this model has the advantage of being easy to execute and highly reproducible, animals die rapidly after endotoxin administration, which is one feature not typical in human sepsis.

When clinical trials of therapeutic agents failed to improve mortality in humans, the accuracy of the endotoxin model came into question (Deitch, 2005; Hotchkiss and Karl, 2003; Remick et al., 1998). One of the major problems with this model is that in humans, 20-50% of the time a source of infection cannot be identified by culture (Alberti et al., 2002; Annane et al., 2005; Heffner et al., 2010; Hotchkiss and Karl, 2003). Additionally, when a causative agent can be identified it has more frequently been Gram-positive bacteria, which lack LPS, rather than Gram-negative bacteria (Hotchkiss and Karl, 2003; Martin et al., 2003; Sriskandan and Cohen, 1999). Failed

clinical trials in animals and humans forced investigators to reexamine the animal model of sepsis in order to provide a more accurate portrait of the human response. There are several models used today, and the two used in this thesis will be discussed in detail below.

B. Bacterial-induced Sepsis

In addition to the endotoxin model, another model used to study sepsis is the bacterial sepsis model. In this model, a single bacterial species is injected into animals intravenously, intraperitoneally, or subcutaneously. This model has been executed using a range of bacterial species including *E. coli* and *S. aureus*, two of the most common causes of sepsis (Buras et al., 2005; Deitch, 1998; Deitch, 2005). One benefit of using a single bacterial species is that bacterial killing is easily quantifiable following infection. Moreover, the mortality rate can easily be modulated by adjusting the number of bacteria injected or the inoculation route in order to meet the needs of the researcher. While this model provides a more accurate replication of the immune response to infection than the endotoxin model, it still only provides a glimpse into damage caused by a specific pathogen. Likewise, the route of infection can have very different results on the host response and mortality rates. For example, peritoneal infection typically results in recruitment of leukocytes to the site of infection and a local release of cytokines, whereas intravenous bacterial infection results in immediate damage to the vascular endothelium and subsequent escape of organisms into various tissues (Buras et al.,

2005). This model will be used in Chapter 3 to study the role of eosinophils in bacterial killing.

C. CLP is the most accurate model of human sepsis

CLP is a polymicrobial peritonitis model that involves the surgical isolation and ligation of the cecum so that normal bowel flow is not obstructed. The creation of one or more punctures releases fecal matter into the peritoneal cavity (Deitch, 2005; Wichterman et al., 1980). Mortality in the CLP model is easily manipulated depending on the needle gauge used and the number of holes created (Deitch, 2005; Wichterman et al., 1980). While the technique of CLP requires some time to reproduce accurately, the kinetics of the cytokine response in the CLP model more closely mimic the response in septic patients than the endotoxin model (Remick et al., 2000; Wichterman et al., 1980).

Animals exhibit a delayed peak of the cytokine storm, which continues rising eight hours after surgery (Remick et al., 1998). IL-6 peaks roughly 8 hours following CLP, in contrast to 4 hours in the endotoxin model. TNFα levels are nearly undetectable in CLP, similar to patient studies where levels are low and transient (Hamilton et al., 1992; Remick et al., 2000; Ward, 2004). Approximately 12 hours following CLP surgery, the cytokine storm shifts to an antiinflammatory state, evidenced by reduced TNFα, IFNγ, and IL-12 while levels of IL-10 reach their peak (Benjamim et al., 2000; Murphey et al., 2004; van der Poll et al., 1995).

Finally, CLP also induces leukocyte dysfunction similar to that observed in septic patients. It is known that lymphoid, endothelial, and epithelial apoptosis also occurs in CLP, and the ligated portion of the cecum provides a source of necrotic tissue (Ayala and Chaudry, 1996; Buras et al., 2005). All of these features make CLP experimentally tractable and more akin to human sepsis than endotoxemia. This model will be used in Chapters 4, 5, and 6 to study the effects of IL-5 in sepsis.

One downside to this model is that bacterial killing is more difficult to quantitate due to the presence of anaerobic as well as aerobic bacteria in the gut. Likewise, differences in gut flora occur in mice bred in different locations and can seriously impact mortality rates in the CLP model (unpublished data, Gold lab). Using littermate controls bred and housed in the same facility as transgenic or knockout animals can control for this effect. However, the polymicrobial nature of this model allows the investigator to the mimic human disease as a whole rather than the response to a single pathogen (Dejager et al., 2011; Echtenacher et al., 2001; Hubbard et al., 2005).

Interestingly, when investigators reexamined the therapeutic efficacy of agents that failed in clinical trials, they discovered that these agents also failed in the CLP model of polymicrobial sepsis. One study showed that the severity of sepsis increased in CD14-deficient mice when CLP was induced; this is contrary to results using the endotoxin model (Ebong et al., 2001). Another study showed that changes in LPS responsiveness, through alterations in TLR4 or LPS binding protein (LBP), did not improve mortality in CLP-induced sepsis, while results from the endotoxemia model

indicated that it did (Echtenacher et al., 2001). Likewise, depletion of TNF 8 hrs after CLP-induced sepsis increased mortality, while using the endotoxin model this therapy was protective (Echtenacher et al., 1990). In addition, administration of antiinflammatory IL-10 protects mice against endotoxin lethality, but has no effect on mortality from CLP (Gerard et al., 1993; Howard et al., 1993; Remick et al., 1998). The use of this more accurate model has led to a reevaluation of what constitutes the most appropriate therapeutic approach for septic patients.

Table 1.4: Advantages and Disadvantages of Animal Models of Sepsis

Sepsis Model	Advantages	Disadvantages
Endotoxemia	Simple and reproducible	LPS-mediated signaling is strictly TLR4-dependent
	Induced response is acute	Does not reflect all complex physiological human responses
	Highly controlled and standardized model	Rodents are endotoxin resistant, whereas humans are very sensitive
		Different hemodynamic response compared to human sepsis
		Variability in dose, toxin, and route of administration
Bacterial inoculum model	Presence of a single bacterial species allows insight into mechanisms of host response to pathogens	High doses induce and endotoxic instead of a septic shock, due to the presence of LPS after rapid lysis of the bacteria
		Does not reflect the diversity and combinations of infectious agents present in human sepsis
		Humans are not normally challenged with massive bacterial burden, but have a septic focus that intermittently and persistently challenges the body with bacteria
		Growth and quantification of bacteria is needed before administration
		Variability in bacterial load, route of administration and bacterial strain
CLP model	Simple procedure with easily adjustable mortality	Abcess formation required for animal survival
	Prolonged and lower elevation of cytokine release, as in humans	Variability in severity due to difference in experimental procedures
	Polymicrobial sepsis model	
	Uses the complete spectrum of host enteric bacteria	
	Recreates human sepsis progression with the presence of both hyper- and hypoinflammatory phases	
	Presence of an infectious focus	

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Figure 1.5: The Cecal Ligation and Puncture Model of Polymicrobial Sepsis

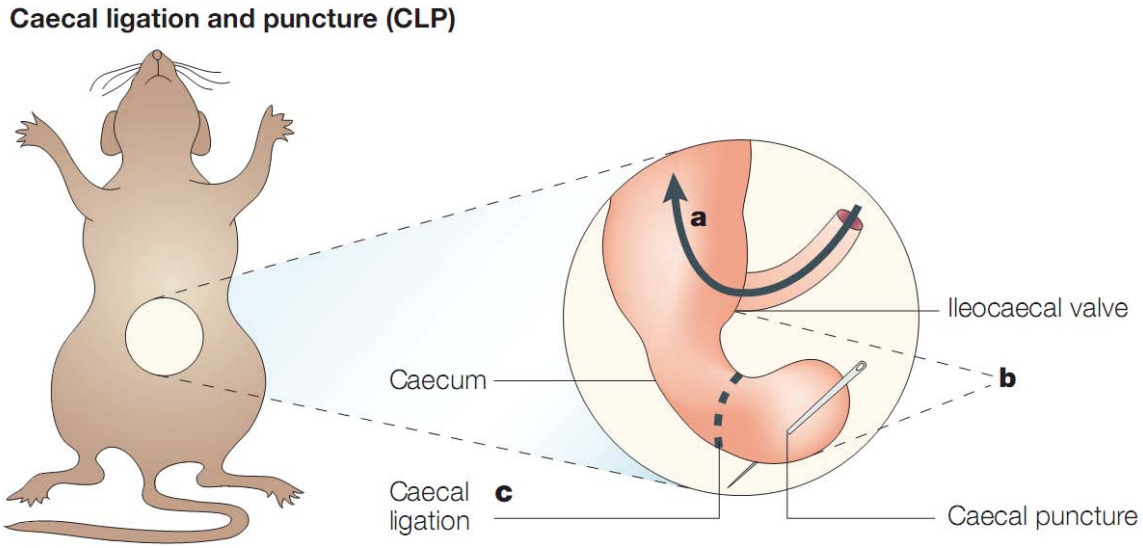


Figure 1.5: Schematic drawing of the CLP model. Briefly, a small incision is made in the abdominal cavity. The cecum is ligated and punctured through with a needle and returned into the cavity. The cavity is sutured closed, mice are fluid resuscitated and monitored for survival. Reprinted from (Buras et al., 2005) with permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery.

V. THE ASSOCIATION OF EOSINOPHILS, IL-5 AND SEPSIS

While eosinophils are traditionally associated with parasitic infections and allergic disorders, there is a small body of literature that suggests their involvement during bacterial infection or sepsis. Firstly, eosinopenia has been recognized as a marker of sepsis and acute infection for some time (Bass, 1975; Bass et al., 1980). During sepsis there is a loss of eosinophils and as patients recover, the presence of eosinophils in the blood returns; this loss can in fact be used as a marker of infection (Abidi et al., 2008; Shaaban et al., 2010; Venet et al., 2004). The more severe sepsis cases are associated with a more exaggerated loss of eosinophils (Figure 1.6) (Venet et al., 2004). However, the significance of these observations is poorly understood.

IL-5 is the primary hematopoietic cytokine controlling eosinophil growth, differentiation, and survival. Interestingly, a recent report documented elevated levels of IL-5 in patients who survived sepsis compared to those who did not, suggesting a protective effect for IL-5 in sepsis (Bozza et al., 2007). Nevertheless, if sepsis is associated with a loss of eosinophils, then the precise role for the protective effects of IL-5 in sepsis becomes unclear. This Section will discuss current knowledge of IL-5 and eosinophils, and data in this thesis will address the unclear association among eosinophils, IL-5, and sepsis.

Figure 1.6: Sepsis is associated with a loss of eosinophils, and increased eosinophils are found in survivors compared to non-survivors

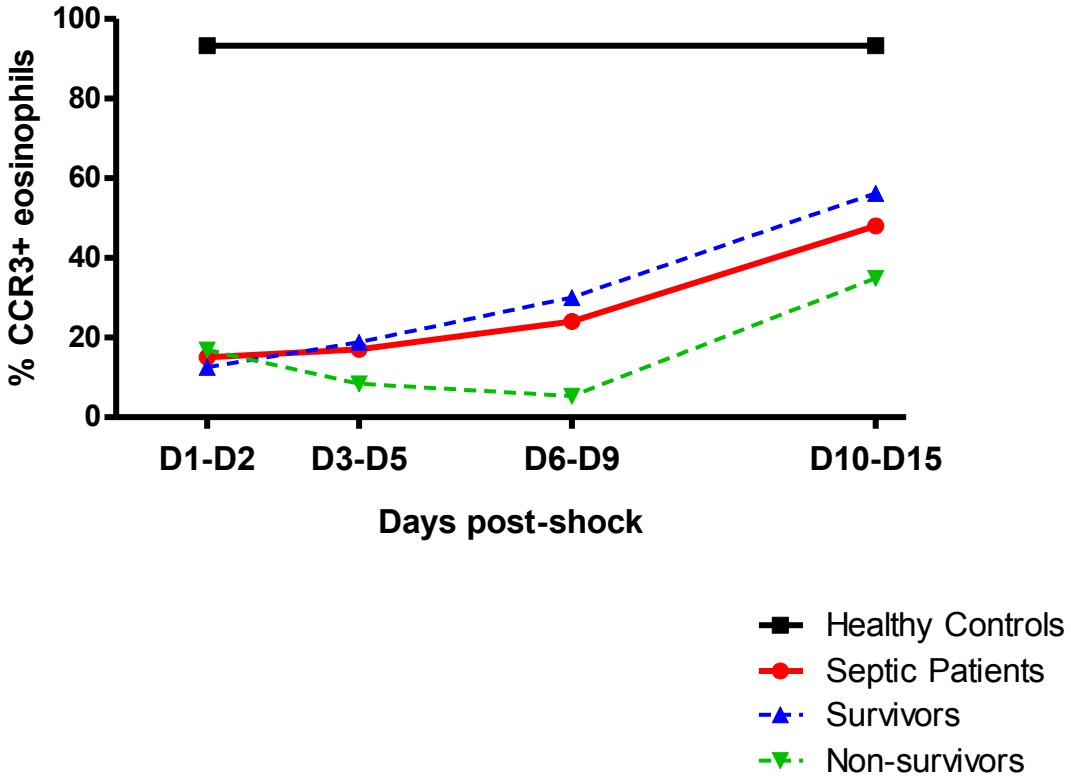


Figure 1.6: Data are expressed as median values; healthy control levels of eosinophils (black) were determined once at days 3-5. Septic patients (red) have significantly decreased levels of circulating eosinophils ($p<0.001$). Interestingly, septic patients who survive sepsis (dashed blue) have elevated levels compared to non-survivors (dashed green). Graph of data presented in (Venet et al., 2004) adapted with permission from Elsevier.

A. Eosinophils in innate immunity

Differentiation and homeostasis

Eosinophils are a granulocyte subset comprising 1-4% of all circulating leukocytes in humans. They are multifunctional effector granulocytes traditionally associated with parasitic infections and their role in the pathology of asthma and other allergic diseases. Eosinophils are generated in the bone marrow from pluripotent $CD34^+$ stem cells and are terminally differentiated. IL-5 in combination with IL-3 and GM-CSF are responsible for regulating eosinophil development (Lopez et al., 1986; Lopez et al., 1988; Rothenberg et al., 1988), though IL-5 is the most specific to eosinophils, and is known to trigger activation and prolong survival (Clutterbuck et al., 1989; Collins et al., 1995; Sanderson, 1992; Yamaguchi et al., 1988). Roughly 2 x 10^8 eosinophils/kg are turned out of the bone marrow each day and this is also driven by IL-5 in combination with eotaxin (Clutterbuck et al., 1989; Giembycz and Lindsay, 1999). They have a half-life of roughly 18 hours, though this increases in tissues where they can survive for days to weeks (Giembycz and Lindsay, 1999; Rothenberg and Hogan, 2006).

Structure and granule content

Eosinophils were initially discovered by Paul Ehrlich in 1879 due to the characteristic staining of their granules with eosin, a negatively charged fluorescein dye. Eosinophil granules are primarily divided into two categories: primary and secondary, with the latter representing the majority.

They are highly granular cells comprised of a crystalline core of Major Basic Protein (MBP), surrounded by a matrix of Eosinophil Peroxidase (EPO), and two ribonucleases—Eosinophil Cationic Protein (ECP) and Eosinophil-Derived Neurotoxin (EDN). In rodents, the latter two proteins are replaced by a family of proteins known as Eosinophil Associated Ribonucleases (EARs), with over eleven different family members described to date (Rothenberg and Hogan, 2006). Granule proteins known as Charcot-Leyden crystals are also present in the cytoplasm of eosinophils and have a very distinct needle shape, though their function is less clear (Dvorak et al., 1988). Though these are the most well characterized proteins to date, some labs have described the presence of additional proteins within these granules, including BPI and α-defensins (Calafat et al., 1998; Driss et al., 2009). While these granule proteins are capable of causing tissue damage and cytotoxicity, it is important to understand their function in, and contribution to, the eosinophilic response during infection.

Activation, chemotaxis, and degranulation

In the blood, eosinophils roll along the endothelial layer in the same manner as neutrophils, searching for chemoattractant gradients to lead them toward sites of infection or inflammation. These chemoattractants include eotaxins, leukotrienes, IL-5, GRO-α, MCP-2, and MIP-1α (Jose et al., 1994; Phillips et al., 2003; Rothenberg and Hogan, 2006). Activation of integrin very late antigen-4 on eosinophils, by binding to vascular cell adhesion molecule on endothelial cells, is important for firm adhesion and migration into tissues (Walsh et al., 1991). Eosinophil activation is

associated with changes in adhesion molecule expression, including upregulation of CD11b and CD69, and loss of L-selectin (Rothenberg and Hogan, 2006). Activation is also associated with intracellular calcium fluctuations, which are required for adhesion, chemotaxis, and degranulation in eosinophils. Following eosinophil activation, ultrastructural changes occur including loss of secondary granules and the MBP crystalline core through degranulation (Giembycz and Lindsay, 1999). There are several mechanisms for degranulation in eosinophils and all are tightly regulated (Giembycz and Lindsay, 1999; Hogan et al., 2008; Rothenberg and Hogan, 2006). Piecemeal degranulation is the process by which secondary granules in the cytoplasm fuse with the plasma membrane to release various granule proteins. Cytolysis and release of whole eosinophil granules occurs *in vivo*, frequently in eosinophilic diseases, and is characteristic of necrotic cells (Giembycz and Lindsay, 1999). The release of these highly cationic proteins is important for attacking parasites and bacteria, as well as aiding in tissue remodeling.

Functions in the immune response

Eosinophils have been traditionally associated with parasitic infections. However, the generation of both transgenic and knockout mice has allowed investigators to determine that while many parasitic infections are associated with eosinophilia, these cells are not required to kill most parasites or clear an infection, with the exception of *Strongyloides* sp. and filarial diseases (Anthony et al., 2007; Hogan et al., 2008; Rothenberg and Hogan, 2006). In fact, their role in parasitic diseases is still being debated. Since eosinophils are believed to be unnecessary to control the vast

majority of parasitic infections, several groups have postulated that eosinophils migrate to sites of infection and degranulate, and that this contributes to tissue remodeling and debris clearance for many parasitic diseases (Anthony et al., 2007). Evidence for this comes from biopsies taken from anti-IL-5 treated patients and the reduction in the deposition of extracellular matrix proteins in the bronchial basement membrane (Flood-Page et al., 2003). Eosinophil-deficient mice have reduced tissue remodeling in the lung, suggesting a role for eosinophils in this process (Humbles et al., 2004). Moreover, eosinophil accumulation in the lung during viral-associated asthma exacerbations suggests that aside from their classical anti-parasitic function, there are additional accessory roles for these cells (Phipps et al., 2007; Rosenberg and Domachowske, 2001).

There are numerous reports suggesting human eosinophils recognize bacteria and viruses. Specifically, eosinophils express numerous TLRs, and stimulation with TLR ligands including peptidoglycan (TLR2), flagellin (TLR5), and R837 (TLR7) induced release of IL-1β, IL-6, and IL-8 from eosinophils (Mansson et al.; Plotz et al., 2001; Sabroe et al., 2002; Wong et al., 2007). Additionally, recognition of β-glucans by eosinophils induced secretion of MCP-1, IL-8, and MIP-1α (Ahren et al., 2003; Yoon et al., 2008), and direct recognition of both bacteria and fungi induced IL-8 release from eosinophils (Ahren et al., 2003; Inoue et al., 2005). These data indicate that eosinophils recognize and respond to bacterial stimulation, and this results in chemokine and cytokine secretion giving them potential to greatly affect an immune response *in vivo*.

Eosinophils also produce various cytokines, including IL-2, IL-4, IL-6, IL-10, and IL-12, which are important in promoting T cell polarization (Th1/Th2), activation, and proliferation (Rothenberg and Hogan, 2006). This suggests eosinophils may provide an important link between the innate and the adaptive immune system. Further, growing evidence suggests eosinophils directly participate in the adaptive immune response. Several groups showed that eosinophils process and present antigen, and express costimulatory molecules (Bashir et al., 2004; Shi, 2004). Specifically, eosinophils presented soluble antigen to CD4 T cells (MacKenzie et al., 2001), and in culture they induced T cell proliferation and IFN-γ secretion following rhinovirus infection (Handzel et al., 1998). Moreover, blockade of various costimulatory molecules using neutralizing antibodies inhibited eosinophil-mediated T cell proliferation and cytokine secretion (Bashir et al., 2004). These data collectively suggest eosinophils provide a connection between innate and adaptive immunity, which may be an important but poorly recognized ability *in vivo*.

Bacterial clearance: Antimicrobial peptides, phagocytosis, and extracellular traps

In one of the earliest studies on antibacterial properties of eosinophils, Yazdanbakhsh *et al* demonstrated that human eosinophils phagocytose roughly 50% less *S. aureus* than neutrophils, but at a higher multiplicity of infection (MOI) there is no significant difference between these two cell types in bacterial killing (Yazdanbakhsh et al., 1986). While eosinophils are capable of phagocytosis, it is well known that in order for them to kill large parasites such as Strongyloides sp., they must use alternative mechanisms. Eosinophils rely on secreted mediators—

cytotoxic granules—to attack and kill parasites (Butterworth et al., 1979; McLaren et al., 1981; Specht et al., 2006; Wassom and Gleich, 1979).

Emerging data suggest that parasites are not the only organisms susceptible to the toxic effects of these highly cationic proteins. Increasing evidence indicates that eosinophil granule proteins can bind to and kill several different bacterial species, as well as fungi. A recent article showed that human eosinophil crude protein extracts alone have the ability to induce *E. coli* killing in a dose-dependent manner (Persson et al., 2001; Svensson and Wenneras, 2005). Likewise, several groups used purified granule proteins, MBP and ECP, or EPO, and showed that they all have significant bactericidal activity against *E. coli* and *S. aureus* (Jong et al., 1980; Lehrer et al., 1989; Watanabe et al., 1995). These data demonstrate the ability of human eosinophils to kill bacteria *in vitro*.

Recently, Yousefi and colleagues described another mechanism of antibacterial activity. They detected release of DNA from IL-5 or IFN-γ primed eosinophils following stimulation with LPS, and this DNA was bound to ECP and MBP as well as bacteria (Yousefi et al., 2008). Interestingly, this DNA was mitochondrial in origin unlike neutrophil NETs, which are comprised of nuclear DNA (Brinkmann et al., 2004). This method of mitochondrial DNA extrusion provides the eosinophil the distinct ability to survive following pathogen recognition and delivery of DNA, whereas neutrophil DNA extrusion is lethal to the cell. *In vivo*, release of DNA and MBP was detected in the ceca of IL-5 overexpressing, hypereosinophilic mice, which was absent in wild type controls. Additionally, IL-5 overexpressing mice had

decreased circulating bacterial burden and improved survival over wild type mice with CLP sepsis, further suggesting a protective role for eosinophils in bacterial disease (Yousefi et al., 2008). However, it is impossible to segregate the independent effects of IL-5 overexpression in this model, especially in light of the evidence for the protective role for IL-5 in human sepsis (Bozza et al., 2007). More definitive *in vivo* studies documenting a specific role for eosinophils in bacterial killing are necessary, and data presented in this thesis will provide strong evidence to support this role.

Summary

Eosinophils are multifunctional granulocytes that play a role in allergic diseases, as well as bacterial and viral killing though this is not widely appreciated. They release chemokines and cytokines to attract other leukocytes to sites of infection. Through degranulation and release of cytotoxic granules they participate in pathogen clearance and tissue remodeling. Their role in bacterial clearance needs to be further assessed *in vivo*.

Figure 1.7: How Eosinophils Combat Pathogens: Major Mechanisms

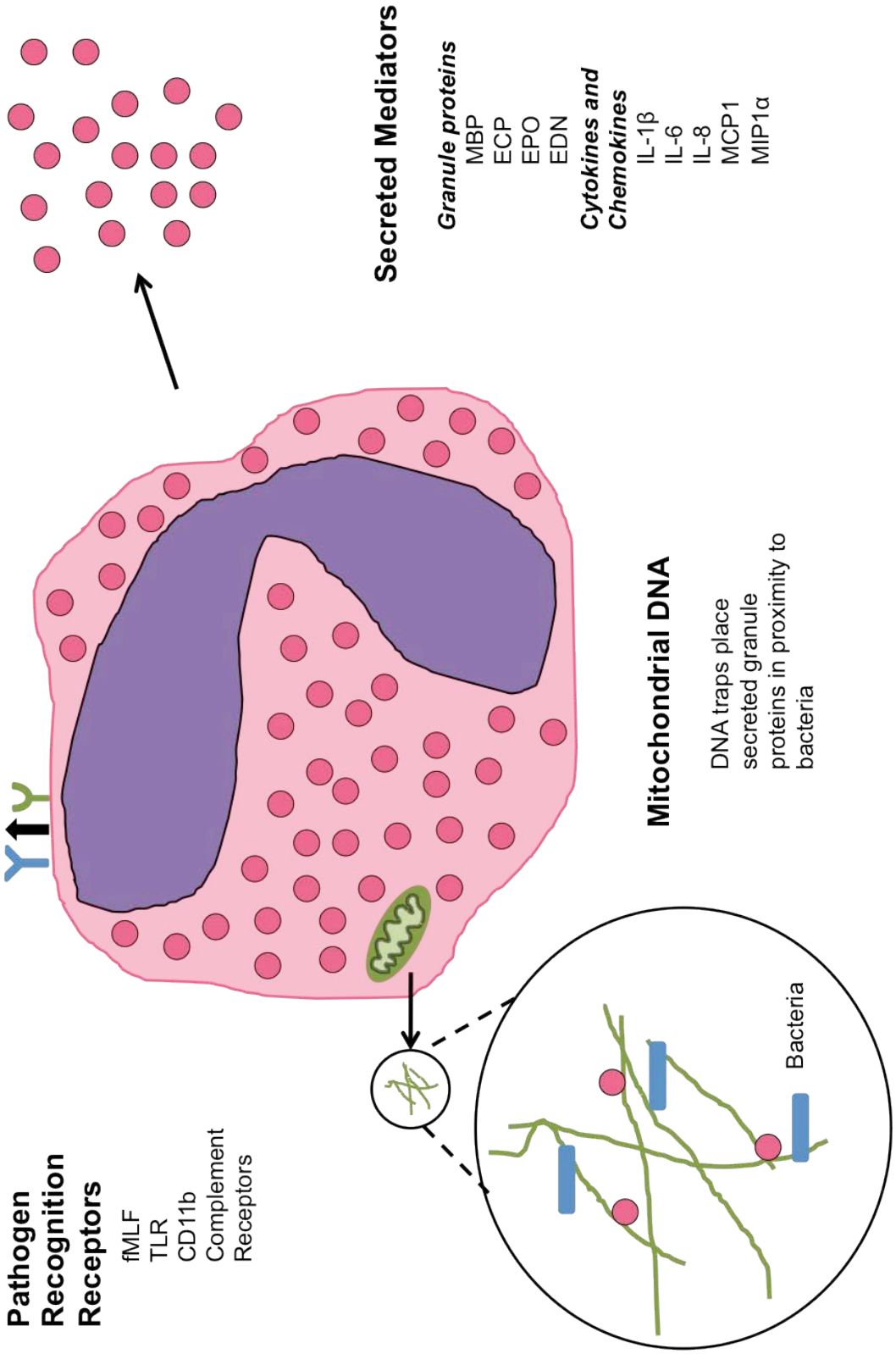


Figure 1.7: How Eosinophils Combat Pathogens: Major Mechanisms

Eosinophils possess a number of different mechanisms to assist in combatting pathogens. They secrete several highly cationic granule proteins in addition to cytokines and chemokines to attract additional leukocytes to sites of infection. They possess receptors to aid in pathogen recognition including fMLF receptor, TLRs, complement receptors, and CD11b. Finally, eosinophils can extrude mitochondrial DNA which provides a meshwork to put bacteria and cationic granule proteins in proximity to one another, to assist in pathogen degradation.

B. IL-5 and the IL-5 receptor

IL-5 production and function

IL-5 belongs to the hematopoietic growth factor cytokine family, and is highly homologous to IL-3 and GM-CSF. It was originally identified as a cytokine responsible for the growth and effector functions of eosinophils, as well as B cell differentiation into Ig-secreting cells (Adachi and Alam, 1998; Clutterbuck et al., 1989; Lee et al., 1997; Lopez et al., 1988; Moon et al., 2004). It is produced largely by T cells, but is also made by mast cells, NK cells, NKT cells and macrophages (Kurowska-Stolarska et al., 2008; Martinez-Moczygemba and Huston, 2003; Sakuishi et al., 2007; Warren et al., 1995). In addition, it is produced by airway epithelial cells and bone marrow stromal cells, which likely has negative effects in patients with asthma as it enhances eosinophil recruitment and survival in the lungs (Hogan et al., 2000; Salvi et al., 1999). Recently, it was shown that a novel population of non-T, non-B, c-kit^{+}FcRε^{-} cells (indicating that these are not mast cells) is a potent source of IL-5 during helminth infection, and also in response to IL-25 stimulation, suggesting that there are additional sources of this cytokine that may not be fully appreciated (Fallon et al., 2006; Fort et al., 2001).

In mice, IL-5 enhances immunoglobulin production and induces growth and proliferation of B cells. However, there have been conflicting results concerning human B cells (Adachi and Alam, 1998; Clutterbuck et al., 1987; Clutterbuck et al., 1989; Huston et al., 1996). IL-5 also induces innate mouse B-1 cell proliferation, Ig

production, and survival (Kopf et al., 1996; Moon et al., 2004). Additionally, IL-5 induces IL-2Rα expression on T cells and augments cytotoxic T cell generation (Adachi and Alam, 1998; Apostolopoulos et al., 2000). In particular, IL-5 is most well known for the role it plays in eosinophil development, as it stimulates the production of human eosinophils from the bone marrow (Clutterbuck et al., 1989). In addition, IL-5 induces integrin adhesion, survival through inhibition of apoptosis, and proliferation (Ochiai et al., 1997; Yamaguchi et al., 1988; Yousefi et al., 1996; Zhu et al., 2002). This role is exemplified in the eosinophil deficiency observed in IL-5 knockout mice, as well as the eosinophilia in IL-5 overexpressing mice (Kopf et al., 1996; Lee et al., 1997). However, IL-5 is not an absolute requirement for recruitment of eosinophils into tissues, as IL-5 knockout mice still have a residual population of these cells, though it should be noted that this population is not sufficient to mount an eosinophilic response to helminth infection (Matthaei et al., 1997; Robertson et al., 2000).

IL-5 receptor

The IL-5 receptor is composed of two subunits: the α subunit, which is the unique ligand portion localized to chromosome 3; and the β subunit, which is shared with the IL-3 and GM-CSF receptors and is localized to chromosome 22 (Martinez-Moczygemba and Huston, 2003; Miyajima et al., 1993; Tavernier et al., 1991). The β subunit is not sufficient to bind IL-5, but is required to induce intracellular signaling. The α subunit binds IL-5 with relatively low affinity, and heterodimerization with the β subunit forms the high-affinity complex (Adachi and Alam, 1998; Martinez-

Moczygemba and Huston, 2003). The IL-5Rα is typically found on eosinophils and B cells, though it has also been reported on airway epithelial cells and the promoter region for IL-5Rα is in fact active in myeloid cells (Andrew et al., 2003; Sun et al., 1995; Tavernier et al., 1991; Weber et al., 1996).

The structure of the receptor subunits is highly conserved for IL-3, IL-5, and GM-CSF. The extracellular domain has a homology module with two fibronectin type II domains connected by cysteine residues, and a WSXWS motif at the membrane proximal portion. The α subunit has a short intracellular domain, which is required for signaling through its constitutive association with Janus kinase (JAK)-2 (Bazan, 1990; Nicola, 1994; Takaki et al., 1993). There is also a soluble form of the IL-5Rα generated via alternative splicing that competes with the transmembrane-bound receptor for IL-5 binding, though it is not know whether it serves as a cytokine sink or a protective carrier of IL-5 (Martinez-Moczygemba and Huston, 2003; Tavernier et al., 1992). Moreover, the soluble form of the receptor can also be generated by cleavage of the membrane bound portion by proteases, including MMPs (Liu et al., 2002b). Surface expression of the IL-5Rα is mediated by binding to syntenin in endosomal compartments and may serve to stabilize IL-5R complexes; the precise function of this binding is unknown (Beekman et al., 2009; Martinez-Moczygemba and Huston, 2003). Following IL-5 binding, the receptor complex is endocytosed, and ubiquitinated. The signaling components of the β subunit are degraded by the proteasome, and the receptor complex (still bound to IL-5) is degraded in the lysosome (Martinez-Moczygemba et al., 2007).

IL-5 signaling

IL-5 signals following heterodimerization of the α-subunit (IL-5Rα) and common β-subunit (Martinez-Moczygemba and Huston, 2003). Ligand binding induces three major signaling pathways: the MAPK, the PI3K/Akt, and the JAK/STAT (signal transducers and activators of transcription) pathway (Adachi and Alam, 1998). In eosinophils, IL-5 signaling requires tyrosine kinase JAK2 and STAT1α; STAT5 and Lyn tyrosine kinase, although not required, are also activated in response to IL-5 signaling (Takatsu and Nakajima, 2008; van der Bruggen et al., 1995). Eosinophils are known to produce various inflammatory cytokines and chemokines following IL-5 signaling (Adachi and Alam, 1998; Clutterbuck et al., 1989). Activation of MAPK pathways are important for cell survival, growth, and proliferation (Martinez-Moczygemba and Huston, 2003). The role of the PI3K/Akt pathway is not fully established in eosinophils, but it does regulate the respiratory burst and IL-5-mediated mobilization from the bone marrow; this pathway also regulates cell survival in neutrophils and monocytes (Machida et al., 2005).

Effects of IL-5 on additional cell types

The role of IL-5 signaling in other cell types has not been well studied, as few other cell types have been shown to express the IL-5Rα. However, there are data that suggest eosinophils and B cells are not the only cells capable of responding to IL-5. Specifically, human neutrophils exhibit chemotaxis in response to IL-5 *in vitro* (Hakansson and Venge, 1994). In response to IL-5 treatment, airway smooth muscle

cells produce vascular endothelial growth factor; in addition, microglial cells and RAW264.7 cells, a macrophage cell line, proliferate in response to IL-5 (Ringheim, 1995; Wen et al., 2003). The effects of IL-5 in neutrophils and macrophages remain enigmatic. Moreover, it has not been established that these cells express the IL-5Rα and under what circumstances this occurs. This thesis will elucidate the effects of IL-5 in macrophages and neutrophils to further understand the role of IL-5 in sepsis.

Anti-IL-5 treatment

Currently, there are strategies aimed at reducing eosinophil recruitment into the airways in asthmatics, and also to reduce the eosinophilia observed in patients with eosinophilic disorders. This includes mepolizumab (anti-IL-5) and MEDI-563 (anti-IL-5Rα) therapies. Anti-IL-5 therapy has been effective at reducing the number of eosinophils present by roughly 55%, but it did not eliminate them nor did it provide a clinical benefit to asthmatic patients (Flood-Page et al., 2007; Kips et al., 2003; Leckie et al., 2000). It has been effective for patients with hypereosinophilic syndrome (HES) by relieving skin symptoms and reducing the number of eosinophils in skin biopsies (Rothenberg et al., 2008).

Since eosinophil and eosinophil precursors express IL-5Rα, additional therapies have been aimed at targeting eosinophils for antibody-dependent cell-mediated cytotoxicity (ADCC), which allows effector cells to destroy cells coated with antibodies. Anti-IL-5Rα therapy was effective in Phase I clinical trials at nearly eliminating eosinophils and basophils from the blood. However, a very interesting

side effect from this treatment was that 34% of patients had a decrease in WBC counts, and 23% had a decrease specifically in neutrophils. Of the 34% who had decreased WBC counts, there was an overall decrease in the absolute number of monocytes (20.5%) and neutrophils (13.3%) (Busse et al., 2010). This study suggests that either there are off-target effects of the anti-IL-5Rα antibody, or that neutrophils and monocytes express the IL-5Rα in some patients. In any case, these data document unanticipated effects of anti-IL-5Rα therapy. This therapy may in fact be dangerous in some patients and predispose them to infection by reducing their innate immune response and first line of defense.

Summary

IL-5 is the primary hematopoietic cytokine controlling eosinophil growth, differentiation, and survival. There are some reports indicating IL-5 has effects on other leukocytes, including neutrophils and macrophages. Moreover, anti-IL-5Rα therapy depletes a portion of neutrophils and monocytes, in addition to eosinophils, suggesting an as yet unappreciated role of IL-5 on these cells. This thesis will elucidate some of these functions in the context of polymicrobial sepsis.

Figure 1.8: Intracellular IL-5 signaling

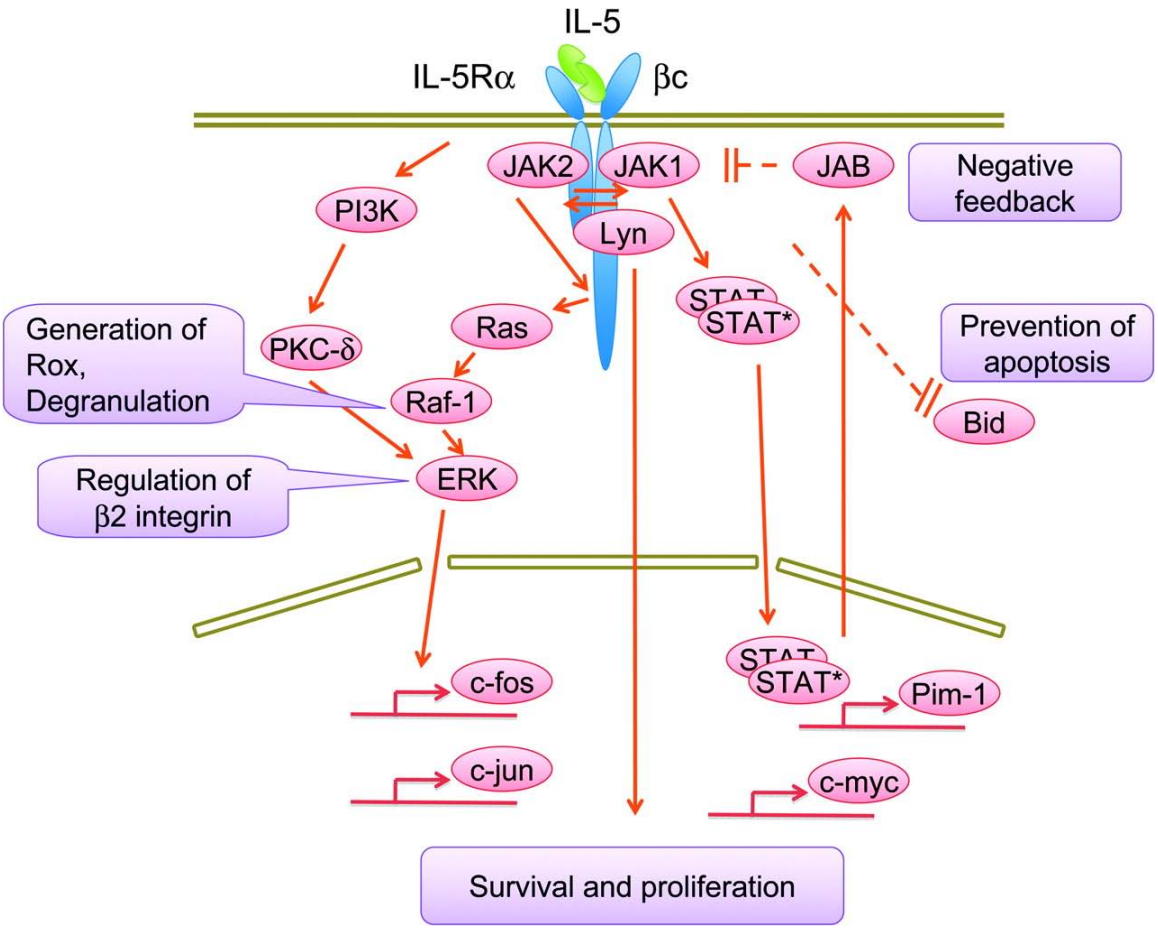


Figure 1.8: IL-5 binds to the IL-5Rα, which allows heterodimerization with the β subunit, allowing for JAK binding and phosphorylation. JAKs then phosphorylate a site on the β subunit allowing for STAT docking and phosphorylation. Once phosphorylated, the STATs translocate into the nucleus and induce gene transcription. STAT* includes STAT1, STAT3, and STAT5. Figure from (Kouro and Takatsu, 2009). Reproduced with permission from the Japanese Society for Immunology and Oxford Press.

VI. HYPOTHESIS AND RESEARCH GOALS

Sepsis is associated with an acquired impairment in innate immune function. This dysfunction is typified by reduced bacterial killing by neutrophils, reduced antigen presentation and cytokine production by monocytes, and apoptosis of both immune and non-immune cells. Currently, treatment options for patients consist of antibiotics and supportive care, highlighting the need for means to improve patient mortality.

A recent study indicated that IL-5 levels are elevated in sepsis survivors compared to non-survivors (Bozza et al., 2007). In addition, sepsis is associated with a loss of eosinophils, which is more pronounced in severe disease (Abidi et al., 2008; Weiner and Morkovin, 1952). *These two observations present a paradox concerning the function of IL-5 and eosinophils in sepsis.* The significance of eosinopenia in sepsis is not completely understood. Furthermore, the presence of elevated levels of IL-5 is poorly appreciated. In particular, since sepsis is associated with apoptosis of B cells and a loss of eosinophils (Abidi et al., 2008; Hotchkiss and Nicholson, 2006), then the presence of elevated IL-5 is puzzling given that these are the cells most affected by IL-5 stimulation.

This thesis will investigate both aspects of this disease in an attempt to understand the intricacies of the immune response during sepsis and bacterial infection. *The long-term goal of this work is to create new candidates for enhancing the immune*

response to bacterial infection, while gaining new insight to the interactions and functions of the innate immune system.

The beneficial role for eosinophils in sepsis was recently determined through the use of IL-5 overexpressing mice, in which roughly 40% of their circulating WBCs are eosinophils. While these animals are protected from the lethality of sepsis and have improved pathogen control, this study failed to establish whether IL-5 had an independent effect on this outcome *in vivo* (Yousefi et al., 2008).

The goal for the first portion of this thesis is to elucidate the independent role for mouse eosinophils in bacterial infection *in vivo*. Reports of the antimicrobial effects of eosinophil granule proteins will be further investigated. This thesis will definitively establish the unique role for eosinophils *in vivo* during bacterial infection, with the hypothesis that eosinophils kill bacteria specifically through release of cationic granule proteins. This work will assess the value of therapeutic administration of eosinophil granules as a viable adjuvant therapy for antimicrobial agents, which is especially important given the rise of antibiotic resistance.

The goal for the second portion of this thesis is to assess the importance of IL-5 and the IL-5Rα in sepsis. Anti-IL-5Rα treatment in human asthma studies demonstrated a reduction in neutrophils and monocytes following treatment. These data combined with evidence of elevated levels of IL-5 in survivors compared to non-survivors suggests a protective role for IL-5 and the IL-5Rα in sepsis. The hypothesis for this portion of the thesis is that IL-5 acts on neutrophils and macrophages to

promote resolution of sepsis and infection by enhancing their effector functions. Specifically, the effect of IL-5 will be measured on cellular activation, bacterial killing, phagocytosis, and survival in neutrophils and macrophages from mice. The role of IL-5 administration as an immunostimulatory therapy will be assessed in hopes of providing a therapeutic modality for patients.

CHAPTER 2:

GENERAL METHODS

I. ANIMAL AND HUMAN SELECTION CRITERIA

A. Rationale for the inclusion of both mice and humans

In order to assess the viability of therapeutic interventions in sepsis, and because both transgenic and knockout mouse strains are readily available, mice were included in these studies. However, due to the difficulty of modeling human sepsis in animals, this thesis utilizes patient samples taken at Oregon Health and Science University (OHSU) to examine changes in leukocyte populations and confirm the presence of IL-5 and IL-5Rα in septic patients.

B. Transgenic mouse strains

Female 8-12 week old C57BL/6 mice were purchased from Jackson Labs and allowed to acclimatize at a maximal-barrier specific-pathogen-free facility at OHSU for one week prior to use.

NJ.1638$^{+/-}$ and PHIL$^{+/-}$ mice were gifts from James J. Lee (Mayo Clinic; Scotsdale, AZ) and generated as previously described (Lee et al., 2004; Lee et al., 1997). Briefly, NJ.1638$^{+/-}$ mice have an IL-5 transgene containing the full length mouse IL-5 sequence inserted in the CD3δ promoter, resulting in constitutive overexpression of IL-5 by T cells and consequently a profound eosinophilia (Lee et al., 1997). By 4 months of age, 40% of their circulating leukocytes are eosinophils. In addition, circulating IL-5 levels reach approximately 400 pg/ml in these mice, which is physiologically relevant and observed during helminth infection (Lee et al., 1997).

These eosinophils are not activated and do not spontaneously degranulate, thus they provide an excellent source of eosinophils.

PHIL$^{+/-}$ mice have a diphtheria toxin A transgene inserted into the EPO promoter, which is specific to cells committed to the eosinophil lineage, thereby causing a congenital ablation of eosinophils (Lee et al., 2004). Loss of eosinophils in the PHIL$^{+/-}$ mice was confirmed in the blood and tissues where eosinophils are resident, including the uterus, small intestine, and bone marrow. Both NJ.1638$^{+/-}$ and PHIL$^{+/-}$ mice were backcrossed to the C57BL/6 background. NJ.1638/PHIL mice were generated by mating NJ.1638$^{+/-}$ mice with PHIL$^{+/-}$ mice at OHSU. Genotyping was performed as previously described (Lee et al., 2004; Lee et al., 1997). Double negative animals were used as littermate controls where appropriate.

IL-5$^{-/-}$ mice were also a gift of James J. Lee. IL-5$^{-/-}$ mice were generated by inserting a neomycin resistance gene into a cysteine codon required for the activity of IL-5 (Kopf et al., 1996).

Macrophage Fas-Induced Apoptosis (MaFIA) mice were purchased from Jackson Labs. These mice express a Fas receptor transgene under control of the colony stimulating factor receptor-1. Using a dimerization compound AP20187 (Ariad Pharmaceuticals), animals are depleted of macrophages (Burnett et al., 2004). AP20187 is administered at a dose of 10 mg/kg [60 µl of 20 mg/ml stock AP20187 in ethanol, 120 µl polyethylene glycol-400, 1020 µl 2% Tween-20 in water] per day, per mouse for 5 consecutive days. Three days afterward, sepsis is induced as

described below in Section IIa of the methods. MaFIA mice were injected with vehicle as a negative control for macrophage depletion.

In specified *in vivo* experiments, 1 μg recombinant mouse IL-5 was administered in 200 μl saline by intraperitoneal injection.

All animal experiments were in accordance with guidelines set by the Institutional Animal Care and Use Committee at OHSU. Experiments were performed using appropriately age-matched and sex-matched control mice and, in the case of transgenic animals bred at OHSU, with littermate control mice.

C. Patient selection criteria

Patients admitted to the Intensive Care Unit at OHSU were classified as septic according to Systemic Inflammatory Response Syndrome criteria. Patients meet at least two of the following requirements:

- Abnormal white blood cell count defined as >12,000 or <4000 WBC/μL
- Tachycardia defined as >90 beats/minute
- Hypothermic or febrile defined as <36 °C or >38 °C
- Respiratory rate of >20 breaths/minute or a pCO_2 <32 mm Hg

Blood samples were taken from patients within 24 hrs of admission. Based on patient survival and length of stay, samples were also taken at days 3 and 7 post-admission when possible. Patients were excluded from the study if they met any of

the following criteria: were pregnant, had a Do Not Resuscitate order, those with ongoing bleeding, or with heart disease.

II. ANIMAL MODELS AND SURGICAL PROCEDURES

A. CLP model of polymicrobial sepsis

Sepsis was induced via CLP in mice as previously reported (Chaudry et al., 1980; Gold et al., 2003; Wichterman et al., 1980). Animals are anesthetized using isofluorane (2.5% inhaled with 5% oxygen). The abdomen was shaved and disinfected. A laparotomy was performed, making a 1 cm incision in the midline of mice. The cecum was located, partially exteriorized, and ligated taking care to avoid the ileocecal valve, and as a consequence bowel obstruction. The cecum was punctured through and through with a needle and feces was extruded. The cecum was returned to the peritoneal cavity and the incision was closed with 3 to 4 surgical silk sutures. Mice received sterile saline (1 ml) subcutaneously for resuscitation. Sham-operated mice, in which the procedure was completed without ligation and puncture of the cecum, were used as a surgical control. No deaths resulted in the sham-operated mice (data not shown).

For NJ.1638$^{+/-}$ mice and animals treated with IL-5 either 4 hours before or 1 hour after CLP, one puncture was made in the cecum using a 19-gauge needle. For wild type animals treated with IL-5 after the onset of sepsis, IL-5$^{-/-}$ and NJ.1638/PHIL

mice, one puncture was made using a 22-gauge needle. For MaFIA mice, two punctures were made using a 19-gauge needle.

B. Peritonitis model using a single bacterial inoculum

Peritonitis was induced in mice by administering approximately 10^7 (in a volume of 200 µl) colony forming units (CFU) of either *P. aeruginosa* or *E. coli* into the peritoneal cavity of mice. Bacterial propagation and culture is outlined below in Section IIIf.

C. Bone Marrow isolation

Mice were euthanized by cervical dislocation. The skin was disinfected and removed from the hind legs. The musculature was excised and any remaining tissue was removed using sterile gauze. The femur was cut at the pelvic bone carefully, leaving epiphysis intact. The knee joint and ankle joint were severed and the feet were removed along with the fibula. The remaining bones were cut at the epiphyses and flushed with room temperature RPMI 1640 media using a 27-gauge needle and syringe. The suspension was homogenized by vigorous pipetting and strained through a 70µm sterile cell strainer. Suspensions were centrifuged at 400 x g, and resuspended in 5 ml calcium-free and magnesium-free Hank's Balanced Salt Solution (HBSS) for further processing.

D. Sample collection and preparation

Animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg), followed by exsanguination via axial bleeding. Plasma for cytokine analysis was taken by centrifuging samples at 14,000 RPM for 20 min. When necessary, whole blood was used to assess bacterial killing, which is described in detail below in Section IVf of the methods. Cervical dislocation was performed to ensure animals were deceased before further processing of tissues.

Peritoneal lavage was performed by disinfecting and cutting the skin away from the abdominal cavity, taking care not to puncture the viscera. The peritoneal cavity was then washed twice with 5 ml saline using a 21-gauge syringe. For cytokine measurement by Enzyme-Linked Immunosorbent Assay (ELISA) or bacterial killing assessment, the peritoneal cavity was washed once with 3 ml saline. These protocols are described in Section IV below.

Differential staining using Hemacolor® (EMD Chemicals) was used. Microscope slides were prepared by cytospin at 500 RPM for 5 min and allowed to air dry. Cells were fixed in methanol for 5 sec, followed by staining with the acidic dye eosin for 8 sec, and then the basic dye azure for 15 sec. Slides were rinsed with saline and mounted with cytoseal for long-term storage.

III. CELL CULTURE, IMMORTALIZED CELL LINES AND LEUKOCYTE ISOLATION

A. Cell lines and culturing media

RAW264.7 mouse macrophage cell line and THP-1 human monocytic cell line were purchased from ATCC, and maintained according to the manufacturer's instructions. RAW264.7 macrophages were grown in Dulbecco's Modified Eagle's Medium (DMEM). THP-1 monocytes were grown in RPMI 1640 media. Both media were supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone® (Invitrogen) before use. RAW264.7 cells were harvested by scraping.

B. Murine eosinophil and eosinophil granule isolation

Adult NJ.1638 female mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) followed by exsanguination via axial bleeding. Total blood was collected, diluted in saline with 2% heparin, and laid over a continuous Percoll™ (GE Healthcare) gradient at a density of 1.084 g/ml [45.5ml 10X HBSS, 7ml 1M HEPES pH7.4, 15ml 0.1N HCl, 301ml Percoll™, QS DI water], prepared according to previously published methods (Borchers et al., 2002). Gradients were centrifuged at 2000 x g for 45 min, brake off at 4°C. The buffy coat was collected and cells were washed twice with saline supplemented with 2% FBS. Cells were resuspended in saline supplemented with 10% FBS and sorted based on forward and side scatter characteristics using the Vantage cell sorter (BD Biosciences). Cells were counted by differential staining (Section IId above in methods), resuspended to the desired

concentration and assessed for viability using trypan blue dye exclusion. The cell viability was >95% and purity for eosinophils was >98% for all studies.

Eosinophil granule extracts were purified by the laboratory of James J. Lee, Mayo Clinic. Upon arrival, frozen extracts were thawed and sonicated briefly (2-5 min) to release the granule contents. Protein concentration was determined using the bicinchonic acid assay (Pierce). The total granule protein per eosinophil was calculated based on the total protein in each vial divided by the number of eosinophils from which it was isolated. For all experiments, the amount of protein corresponding to 10^5 eosinophils was 180 μg.

C. Murine neutrophil isolation

Bone marrow neutrophils were harvested according to previous studies with a few modifications (Itou et al., 2006). Briefly, a discontinuous Percoll™ gradient was prepared by underlayering each of three solutions of Percoll™ with the following densities (1.071, 1.084, 1.092 g/ml). The Percoll™ Solutions were prepared as follows: 9 parts Percoll™ was diluted with 1 part 10X HBSS (without calcium and magnesium) to make a 100% Percoll solution (with a density of 1.123 g/ml). 100% Percoll was diluted with 1X HBSS to achieve the appropriate densities using a densitometer.

Bone marrow was isolated according to the protocol above in Section IIc. This solution was laid on top of the discontinuous gradient, which was prepared by starting with the 1.071 g/ml Percoll™ solution and under-laying the 1.084 g/ml

solution and finally the 1.092 g/ml solution. Gradients were centrifuged at 1500 x g for 30 min, brake off at room temperature. The interface layer between 1.084 and 1.092 g/ml was collected and cells were washed twice with supplemented RPMI 1640 media. Cells were counted by differential staining (Section IId above in methods), resuspended to the desired concentration and assessed for viability using trypan blue dye exclusion. The cell viability was >95% and purity of neutrophils was >85% in all studies.

Thioglycollate-elicted neutrophils were isolated according to established protocol with modifications (Itou et al., 2006). Briefly, 1.25 ml of 3% brewer's thioglycollate was administered intraperitoneally to male C57Bl/6 mice. Four hours later, their peritoneal cavities were lavaged with 5 ml saline twice and neutrophils were collected. Cells were counted by differential staining (Section IId above in methods), resuspended to the desired concentration and assessed for viability using trypan blue dye exclusion. The cell viability was >95% and purity was >90% in all studies.

D. Murine macrophage isolation

Thioglycollate-elicited macrophages were isolated according to established protocol (Zhang et al., 2008). Briefly, 1.25 ml of 3% brewer's thioglycollate was administered intraperitoneally to C57Bl/6 mice. Four days later, their peritoneal cavities were lavaged twice with 5 ml saline and macrophages were collected. Cells were counted by differential staining (Section IId above in methods), resuspended to the desired

concentration and assessed for viability using trypan blue dye exclusion. The cell viability was >95% and purity was >90% in all studies.

E. Human neutrophil and peripheral blood mononuclear cell isolation

Blood was collected from healthy volunteers in ethylenediaminetetraacetic acid (EDTA) treated tubes to prevent clotting. Blood was diluted with saline at a ratio of 1:1, and laid on top of room temperature Ficoll-Paque™ PLUS (GE Healthcare) again in a 1:1 ratio. Gradients were centrifuged at 1500 x g for 25 min, no brake, room temperature. The interface layer containing peripheral blood mononuclear cells (PBMC) was removed along with all remaining Ficoll above the interface. This layer was washed twice with RPMI 1640 media and plated in 10 cm petri dishes. One hour later, non-adherent cells were removed by gentle washing. The remaining cells were allowed to differentiate into monocytes for 4 days in RPMI 1640 supplemented with human serum.

The neutrophil and red blood cell pellet was washed once with saline, followed by lysis in 10 ml ammonium chloride for 5 min on ice, with periodic agitation. Lysing was repeated a second time when necessary. Cells were washed with saline and resuspended in supplemented RPMI 1640 media. Cells were counted by differential staining (Section IId above in methods), resuspended to the desired concentration, and assessed for viability using trypan blue dye exclusion. The cell viability was >95% and purity was >95% in all studies.

F. Bacterial culture and propagation

Initial growth curves were made for each bacterial species according to standard methods, in order to correlate optical density (OD) with CFU. *P. aeruginosa* (Boston 41501; ATCC) and *E. coli* (a clinical isolate from OHSU; a generous gift from the laboratory of Dr. Fred Heffron) were grown shaking in LB broth [5g NaCl, 10g Tryptone, 5 g Yeast extract, deionized water to 1 L vol, sterilized by autoclaving] at 37°C until a concentration of 10^9 CFU/ml was achieved. This was determined by measuring the OD (600 nm for *E. coli*; 650 nm for *P. aeruginosa*) and comparing these values with CFU found on the growth curves (Figure 2.1).

Figure 2.1: Bacterial Growth Curve

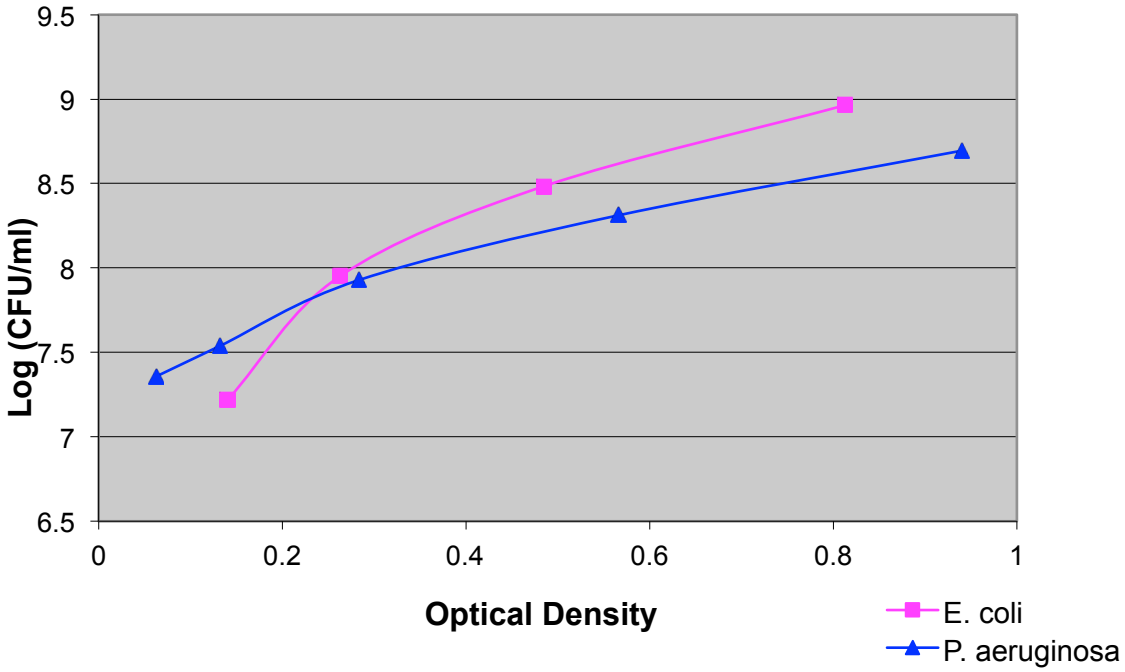


Figure 2.1: *P. aeruginosa* or *E. coli* was grown shaking in LB broth at 37°C overnight. Cultures were diluted 1:25 and samples were taken at several time points for serial dilution and plated on LB agar. Optical densities of the samples were also taken at 650 nm (for *P. aeruginosa*) and at 600 nm (for *E. coli*). Colonies were enumerated 24 hrs later. Colony counts were plotted against optical density to obtain a growth curve.

IV. *IN VITRO* ASSAYS FOR CELLULAR FUNCTION

A. Transcriptional activation by Western blot

Cytoplasmic and nuclear extracts were obtained using a nuclear extract kit and the protocol supplied by the manufacturer (Active Motif). Protein concentration was determined using the bicinchonic acid assay and normalized for each sample (Pierce). Western blot was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, and non-specific binding was blocked using blocking buffer [5% solution of dry milk in PBS/T (0.01% Tween-20)] for 2 hours at room temperature. Membranes were probed using anti-STAT-1 (Cell Signaling Technologies), anti-IL-5Rα (Santa Cruz Biotech), or anti-MBP (gift from James J. Lee, Mayo Clinic) overnight at 4°C, followed by probing with the appropriate horseradish peroxidase (HRP) -conjugated secondary antibody at a dilution of 1:2000 (depending on the species of the primary antibody). Proteins were detected using ECL Plus detection reagents (GE Healthcare).

B. Cytokine production by ELISA

Sandwich ELISA was performed to test for the presence of various cytokines in plasma and peritoneal lavage fluid. All ELISA reagents were purchased as pre-coated Quantikine® Colorimetric ELISA Kits (R&D Systems). Manufacturer's instructions were followed. Briefly, samples or standards were diluted in sample buffer and added to pre-coated wells for 2 hours, room temperature. All wells were

washed using the provided wash buffer five times. The secondary detection antibody conjugated to horseradish peroxidase (HRP) was added for 2 hours, room temperature. Wells were washed again using the wash buffer five times. The substrate solution (a mixture of tetramethylbenzidine and hydrogen peroxide) was added and incubated for 30 min, room temperature, protected from light. This solution turns blue in the presence of the HRP-conjugated antibody. The reaction was stopped after 30 min using the hydrochloric acid stop solution provided. Optical density was measured for each well using a spectrophotometer at 450nm and 570nm. Background readings at 570nm are subtracted from total values, and total cytokine concentration can be calculated for each well based on the standard curve generated.

C. Receptor expression by flow cytometry

IL-5Rα and other surface receptors were fluorescently stained and analyzed using flow cytometry. Non-specific binding in mouse peritoneal and bronchoalveolar lavage (BAL) samples was blocked using anti-CD16/32. This step was not necessary in blood samples from both mice and humans due to the presence of serum antibodies. Murine samples were stained with the following lineage markers: Ly6g (Gr-1) for neutrophils, CD11b and/or F4/80 for monocytes/macrophages in mice, and CCR3 for eosinophils, all combined with forward and side scatter allowing for accurate identification of specific populations (Borchers et al., 2002; Lagasse and Weissman, 1996; Thurau et al., 1996). Human neutrophils were identified by forward and side scatter, and expression of CD14; human monocytes were identified

by forward and side scatter, and CD16 expression. Graphs were made using FlowJo software (Treestar).

D. Activation by intracellular calcium release

Calcium imaging was performed using time-lapse fluorescent microscopy (Hallett et al., 1999). Briefly, thioglycollate-elicited macrophages or neutrophils were isolated (as described above in Section III) and allowed to adhere to 25 mm glass coverslips as follows: macrophages ($4x10^6$/ml) adhered overnight in RPMI (phenol red free) supplemented with 10% FBS; neutrophils ($4x10^6$/ml) adhered to poly-d-lysine coated coverslips for one hour. Cells were loaded with 5 μm Fluo-4, 2 μm probenecid and 0.2% Pluronic Acid (Invitrogen) for 40 min at 37°C, in the dark. Coverslips were washed twice with saline and resuspended in 50 μl calcium-free and magnesium-free HBSS (no phenol red). Cells were then treated with media, fMLF, or recombinant mouse IL-5, and time-lapse images were taken with MetaMorph software (Molecular Devices) using a Nikon Eclipse TS100 inverted microscope and Cool Snap camera (Photometrics) under a 60X objective lens. Responding cells were defined as those that released intracellular calcium within 90 sec following treatment, which was determined by increase in fluorescence intensity of Fluo-4. Briefly, Fluo-4 crosses the cellular membrane into the cytosol, where it is cleaved, thereby preventing the dye from passing through the cell membrane again. The dye is then free to bind calcium, resulting in increased fluorescence within the cytosol (Gee et al., 2000). Positivity was defined using fMLF as a positive control (Brown and Roth, 1991; Chandler et al., 1983; Maudsley and Morris, 1987). Buffer

alone was used as a negative control. Three samples per condition were imaged (~75 cells/experiment) for at least three experiments.

E. Bacterial killing by neutrophils, macrophages, and eosinophils

Thioglycollate-elicited neutrophils or bone marrow neutrophils were isolated as described in Section IIIc in the methods; thioglycollate-elicited macrophages were isolated as described in Section IIId. Eosinophils were isolated as described in Section IIIb. Cells were resuspended to 10^6/ml in RPMI supplemented with 2% FBS. Neutrophils and macrophages were treated with either 1 μg/ml of recombinant mouse IL-5 or saline and added to a 96-well plate. *P. aeruginosa* or *E. coli* was resuspended to 10^7/ml in RPMI supplemented with 2% FBS, and added to wells alone with control-treated cells or cells stimulated with IL-5. After one hour, cell suspensions were removed and centrifuged at 10000 x g for 5 min on a table top mini-centrifuge. The supernatant was removed and the cell pellet was lysed using 0.1% Triton-X in water. Viable bacteria were plated by serial dilution onto LB agar and cultured at 37°C. Colonies were enumerated 24 hours later. Plates that did not have between 30-300 CFU were excluded due to high variability on the lower end, and difficulty counting on the upper end.

F. Phagocytosis by macrophages

RAW264.7 macrophages were seeded at 10^6 cells per chamber in 2-chamber microscope slides (Nalgene) and allowed to adhere overnight at 37°C. Macrophages were treated for 1 hour with 1 μg/ml of recombinant mouse IL-5 or saline control.

As a control, some samples were treated with 10 μg/ml cytochalasin D, which inhibits phagocytosis. Alexafluor 488-labeled *E. coli* bioparticles (Invitrogen) were resuspended in dimethlysulfoxide (DMSO) to achieve an approximate CFU of 10^9/ml, diluted in supplemented RPMI 1640 media (no phenol red), and added to individual chambers to achieve an MOI of 10. Macrophages were allowed to phagocytose the bioparticles for 20 min. Cells were washed four times with saline and fixed for 30 sec using methanol. VECTASHIELD HardSet mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) was used to stabilize fluorescence, visualize the nucleus, and prevent photobleaching. Slides were visualized using a Nikon Eclipse E400 upright microscope using the 60X objective. Images were taken using a Cool Snap camera and analyzed using MetaMorph software. Background fluorescence was subtracted from each image, and total fluorescence intensity for each cell was quantified for a total of 200 cells per experiment, for at least three experiments

G. Apoptosis and survival by immunostaining

Bone marrow neutrophils, thioglycollate-elicited neutrophils, or thioglycollate-elicited macrophages were treated with 1 μg/ml recombinant mouse IL-5 or saline. Neutrophils were stained at 0, 2, 5, 9, 18, 24, and 48 hours following IL-5 stimulation. Macrophages were stained 24, 48, and 72 hours following IL-5 stimulation. Survival was initially assessed by staining using Annexin-V and propidium iodide (PI) and analysis by flow cytometry. However, a recent report indicated that these two dyes should not be used on adherent cells or on large or

granular cell types, as staining frequently results in overestimating levels of PI staining (Rieger et al., 2010).

Due to problems with non-specific binding, the LIVE/DEAD fixable violet dead cell stain (Invitrogen) was also used to assess viability, along with antibodies to IL-5Rα, CD11b, and Ly6g. Cells were centrifuged at 300 x g for 5 min and washed once with saline. The cell pellets were resuspended in saline and 1 µl LIVE/DEAD violet stain, which was diluted as per the manufacturer's instructions, followed by incubation in the dark at 4°C for 30 min. Samples were washed once with saline, resuspended in 100 µl saline, and antibodies were added according to methods listed above in Section IVc of the methods. Samples were washed once with saline and fixed using 3.7% paraformaldehyde for 15 min. Cells were analyzed by using the LSRII flow cytometer and FlowJo (Treestar) software.

V. MATERIALS AND REAGENTS

All antibodies used for flow cytometry were purchased from BD Biosciences. Antibodies used for Western blot were purchased from Santa Cruz Biotechnology and Cell Signaling Technologies. All ELISA reagents were purchased from R&D Systems. All chemicals were purchased from Sigma Aldrich and Fisher Scientific. Cell culture media (RPMI 1640, DMEM, and HBSS, penicillin/streptomycin) were purchased from Invitrogen.

VI. STATISTICS

The majority of data were analyzed using GraphPad Prism software and the student's unpaired t-test to compare two means. Survival curves were analyzed using the log-rank test. One-way ANOVA with Bonferroni's multiple comparisons test was used to compare means across groups where indicated. Chi square analysis was used to assess the presence or absence of bacteria in blood samples from mice, and used where indicated. Significance was defined as *$P<0.05$, **$P<0.01$, and ***$P<0.001$. All error bars represent the SEM.

CHAPTER 3:

EOSINOPHILS POSSESS ANTI-BACTERIAL PROPERTIES IN PSEUDOMONAS PERITONITIS

Abstract

Sepsis is associated with a loss of eosinophils. Moreover, the majority of sepsis cases are caused by bacterial infection. Recently, numerous studies have documented the presence of Toll-Like Receptors on the surface of eosinophils, suggesting these leukocytes may participate in the recognition and killing of viruses and bacteria. However, the significance of this role in the innate immune response to sepsis or bacterial infection is largely unknown. These data demonstrate that isolated mouse eosinophils had potent anti-bacterial properties *in vitro* against *P. aeruginosa*. *In vivo*, IL-5 transgenic mice, which have a profound eosinophilia, demonstrated improved bacterial clearance in Pseudomonas peritonitis. Improved bacterial clearance following adoptive transfer of eosinophils, as well as evidence of impaired bacterial clearance in mice with a congenital eosinophil deficiency, established that this antibacterial activity was eosinophil specific. The data presented in this Chapter also demonstrate that eosinophils mediated this antibacterial effect in part through the release of cationic secondary granule proteins. Specifically, purified eosinophil granules had antibacterial properties *in vitro* and administration of eosinophil granule extracts significantly improved bacterial clearance *in vivo*. These data suggest a potent and yet underappreciated antibacterial role for eosinophils and eosinophil granules *in vivo*. Moreover, these data suggest that administration of eosinophil-derived products may represent a viable adjuvant therapy for septic or bacteremic patients.

Introduction

Sepsis, defined as the systemic inflammatory response to infection, is currently the leading cause of death in the ICU (Angus et al., 2001; Hotchkiss and Karl, 2003). It affects more than 700,000 people each year in the U.S. and costs over $17 billion annually (Angus et al., 2001; Hotchkiss and Karl, 2003). The majority of cases are caused by bacterial infections (Martin et al., 2003). Despite maximal supportive care and antimicrobial therapy, mortality remains in excess of 25% and the use of inappropriate antibiotics further increases mortality by up to 15% (Harbarth et al., 2003; Ibrahim et al., 2000; Kumar et al., 2009; Pittet et al., 1996). These observations highlight the need for novel antimicrobial adjuvant therapies for patients, especially with the increasing prevalence of antibiotic resistance (Angus et al., 2001; Hotchkiss and Karl, 2003; Opal et al., 1997; Pittet et al., 1996).

Innate immune responses are central to the containment of bacterial pathogens through non-specific PRRs present on innate effector cells like neutrophils and macrophages. Activation of innate immunity upregulates inflammatory cytokine production and recruitment of additional effector cells to sites of infection. However, in spite of an overwhelming cytokine response in sepsis, inhibition of either pathogen recognition through blockade of TLRs or proinflammatory cytokines, such as TNFα or IL-1β, has failed to improve survival (Abraham et al., 1998; Cohen and Carlet, 1996; Fisher et al., 1994; Opal et al., 2004; Opal et al., 1997; Remick et al., 1995; Remick et al., 1998). In addition, patients with neutropenia or congenital defects in the innate response have increased susceptibility to, and

mortality from, severe bacterial infections (Donini et al., 2007; Mills and Quie, 1980; Ottonello et al., 1995). Finally, recent studies suggest that sepsis is associated with acquired impairment of innate immune responses as evidenced by reduced bacterial killing by neutrophils isolated from septic patients (Solomkin et al., 1985; Zimmerman et al., 1989). Collectively, these data highlight the importance of the pathogen control function of the innate immune response in sepsis.

Recent studies have focused on augmenting innate immune responses in order to improve host defense and clinical outcomes in sepsis. For example, administration of granulocyte-macrophage colony stimulating factor to septic patients increased neutrophil recruitment and enhanced monocyte activation resulting in increased pathogen control and bacterial clearance (Orozco et al., 2006; Presneill et al., 2002; Rosenbloom et al., 2005). Investigators have also focused on neutrophil-derived products such as BPI, a dominant component of neutrophil granules, which possesses both endotoxin neutralizing capabilities, as well as potent nonspecific bactericidal activity against multiple bacterial species (Gazzano-Santoro et al., 1992; Weiss et al., 1992). A randomized controlled trial of BPI treatment for meningococcal sepsis demonstrated a significant improvement in morbidity, a decrease in the number of infectious complications, and a trend toward decreased mortality in the treatment group (Levin et al., 2000). Thus, these studies suggest either granulocyte activation or administration of granulocyte-derived products may enhance the innate immune response and provide a viable adjuvant therapy for sepsis or bacterial infection.

Eosinophils are another granulocyte subset recently implicated in the innate response to bacterial infections. Recent data documented expression of multiple TLRs on *human* eosinophils (Nagase et al., 2003; Wong et al., 2007). As a result, human eosinophils recognize and are activated by multiple bacterial species *in vitro* (Borelli et al., 2003; Persson et al., 2001; Svensson and Wenneras, 2005; Wong et al., 2007). Once activated, these cells secrete cytotoxic granule proteins, including MBP, ECP, and EPO, all of which have antibacterial properties *in vitro* (Ishihara et al., 2003; Lehrer et al., 1989; Rothenberg and Hogan, 2006). However, little is known about the role of eosinophils *in vivo* during bacterial infection. Interestingly, multiple observational studies over several decades showed that the number of circulating eosinophils inversely correlates with disease severity in patients with sepsis or severe bacterial infections (Bass, 1975; Bass et al., 1980; Setterberg et al., 2004; Weiner and Morkovin, 1952). Conversely, it was recently shown that hypereosinophilic mice have improved survival in a mouse model of polymicrobial sepsis (Yousefi et al., 2008). The mechanism of this protection remains enigmatic; therefore, the goal of this study was to fully define the antibacterial properties of mouse eosinophils, both *in vitro* and *in vivo*, and specifically address the role of eosinophil granules in the host response to bacterial infection.

Methods

Eosinophil isolation from NJ.1638 mice

See General Methods Section Ia, IId and IIIb

Eosinophil granule protein preparation

See General Methods Section IIIb

Bacterial killing and cytokine analysis

See General Methods Section IVb and IVe

Pseudomonas peritonitis model

See General Methods Section IIb, IId and IIIf

CLP model of polymicrobial sepsis

See General Methods Section IIa

Results

Mouse eosinophils kill *P. aeruginosa in vitro*

Recent data indirectly indicate that eosinophils improve bacterial clearance and survival in mice using the polymicrobial sepsis model CLP (Yousefi et al., 2008). In order to determine if mouse eosinophils directly possess antibacterial properties, eosinophils were isolated from IL-5 transgenic NJ.1638 mice. These mice constitutively express elevated levels of IL-5—the primary hematopoietic growth factor controlling eosinophil maturation and survival—under control of the CD3δ promoter, resulting in constitutive IL-5 expression by T cells and a profound eosinophilia (Lee et al., 1997). Isolated eosinophils were incubated for one hour with *P. aeruginosa in vitro* at an MOI of 10. Bacterial viability assessment demonstrated that eosinophils killed approximately 40% of the bacteria present (Figure 3.1), indicating that mouse eosinophils, like human eosinophils, do have antibacterial properties.

NJ.1638 mice have improved bacterial clearance and increased survival in Pseudomonas peritonitis

In order to determine if eosinophils participated in bacterial clearance *in vivo*, *P. aeruginosa* was administered intraperitoneally (i.p.) to NJ.1638 mice or littermate controls. Eosinophils comprise roughly 40% of the circulating leukocytes in these

mice by 4 months of age, compared to littermate control mice, which have approximately 1% eosinophils (Lee et al., 1997); using these mice will provide strong evidence for the role of eosinophils in bacterial infection *in vivo*. NJ.1638 mice had improved survival following Pseudomonas peritonitis compared to littermate controls (Figure 3.2). Furthermore, this improvement in survival was associated with a 79% decrease in bacterial burden in peritoneal lavage (Figure 3.2) and peripheral blood cultures (data not shown) 18 hours following infection. Interestingly, the decrease in bacterial burden was not associated with a significant difference in IL-6, IL-10, IL-12, IL-1β, or TNFα in the blood or peritoneal lavage fluid compared to littermate controls (Figure 3.3). These results suggest that improved survival in this model is mediated by enhanced bacterial clearance, and not through dampening or altering the inflammatory response. The expansion of eosinophils and enhanced bacterial clearance further suggest a role for these cells in bacterial infection.

Eeosinophils are protective in Pseudomonas peritonitis by enhancing bacterial clearance

Since IL-5 is known to enhance B cell differentiation and proliferation of other cell types (Lee et al., 1997; Liva and de Vellis, 2001; Ringheim, 1995), and because of the increased presence of B cells in NJ.1638 mice, the next step was to identify the specific effects of eosinophils in the absence of IL-5 overexpression. To determine

this, eosinophils were isolated from NJ.1638 mice and adoptively transferred into the peritoneal cavity of wild type C57Bl/6 mice. There are two reasons eosinophils were used from NJ.1638 mice: wild type mice lack a significant population of eosinophils; and the number of mice necessary for this experiment would have made it technically and financially difficult. Eosinophils from these mice are not activated at baseline and do not spontaneously degranulate. One hour following adoptive transfer of eosinophils, animals were infected i.p. with Pseudomonas. Mice that received adoptively transferred eosinophils had a 95% reduction in bacterial burden in the peritoneal lavage fluid 18 hours post-infection compared to vehicle-treated controls (Figure 3.4). These data confirm that eosinophils possess antibacterial properties *in vivo*.

To assess whether eosinophils are necessary for enhanced bacterial clearance *in vivo*, *P. aeruginosa* was injected i.p. into eosinophil-deficient PHIL transgenic mice (Lee et al., 2004). PHIL mice have a diphtheria toxin transgene expressed under control of the EPO promoter. When EPO is expressed during eosinophil development, the diphtheria toxin is produced which halts protein synthesis, thereby causing cell death (Lee et al., 2004). PHIL mice had increased bacterial burden in the peritoneal lavage fluid compared to littermate controls 18 hours following infection (Figure 3.5). Furthermore, 60% of PHIL mice had bacteria present in the blood compared to 0% of littermate controls (Chi-square analysis; $p=0.04$). Levels of IL-6 and IL-10 in the blood and peritoneal lavage fluid, as well as IL-12 in the blood, were not statistically different between PHIL mice and littermate

controls. There was a significant elevation of IL-12 in the peritoneal lavage of PHIL mice compared to littermate controls, however levels locally were very low in both groups compared to IL-12 levels in the circulation (Figure 3.6). These data directly address the ability of eosinophils to participate in bacterial infections *in vivo*.

Purified eosinophil granule extracts directly kill bacteria and enhance bacterial clearance when administered *in vivo*

There are two main ways for eosinophils to participate in bacterial clearance. The first is through release of inflammatory cytokines, in order to call in other innate effector cells, thereby providing an indirect manner of killing. The first possibility was tested in IL-5 overexpression mice and in PHIL mice following Pseudomonas infection, and no change in cytokine production was found *in vivo*. The second way for eosinophils to participate in bacterial killing is through release of cationic granule proteins, which directly lyse and kill bacteria. Previous studies indicate that human eosinophils degranulated in response to bacteria *in vitro* (Borelli et al., 2003; Ishihara et al., 2003; Lehrer et al., 1989; Persson et al., 2001; Svensson and Wenneras, 2005). In order to determine if the mechanism of antibacterial activity observed in our peritonitis model was due to the release of eosinophil cationic granule proteins, eosinophil granules were purified (by James J. Lee, Mayo Clinic) and soluble extract prepared by sonication. Granule protein extract was incubated with *P. aeruginosa in vitro* at varying doses. Eosinophil granule proteins directly killed *P. aeruginosa* in a dose-dependent manner (Figure 3.7). The dose of granule proteins equivalent to 10^5 eosinophils (180 μg) showed an equivalent level of killing

(approximately 40%) compared to *in vitro* studies, suggesting that eosinophil antibacterial activity is largely mediated through eosinophil granule proteins. Moreover, MBP was detected by Western blot in supernatants (data not shown) from *in vitro* eosinophil bacterial killing experiments, confirming the release of eosinophil granules following bacterial recognition.

To address the ability of eosinophil granules to directly enhance bacterial clearance *in vivo*, granule proteins were injected into the peritoneal cavity of wild type mice one hour following i.p. infection with *P. aeruginosa*. Interestingly, mice treated with eosinophil granules had an 82% reduction in bacterial burden in the peritoneal cavity compared to those treated with vehicle control (Figure 3.8). Increased bacterial clearance was again not associated with significant difference in IL-6, IL-10, or IL-12 in the blood or peritoneal cavity (Figure 3.9). These results indicate that administration of eosinophil granule proteins was sufficient to mediate bacterial killing *in vivo*.

Treatment with eosinophil granule protein extract improves survival in a polymicrobial model of sepsis

Collectively, these data indicate that eosinophils and eosinophil granule proteins augment bacterial clearance *in vivo*. In particular, a recent study demonstrated that adoptive transfer of eosinophils improves survival in the CLP model of polymicrobial sepsis (Yousefi et al., 2008). One important consideration is that the

therapeutic injection of eosinophils in septic patients would be highly difficult. However, administration of granule protein is more feasible and has been examined previously (Levin et al., 2000). To assess whether eosinophil granule administration is a viable therapeutic strategy in polymicrobial infection, wild type mice were administered eosinophil granule protein extract one hour after CLP-induced sepsis. Indeed, eosinophil granule protein treatment prolonged survival in mice compared to vehicle-treated controls (Figure 3.10). These data indicate the useful ability of eosinophil granule proteins to enhance antibacterial killing *in vivo*, and may prove useful for this purpose clinically.

Summary

Taken together, these data confirmed the antibacterial properties of eosinophils *in vitro*, and provided novel evidence for their role in bacterial killing *in vivo*. It appears this activity was largely mediated through release of granule proteins, and these proteins were sufficient *in vivo* to enhance bacterial clearance and prolong survival in a polymicrobial model of sepsis.

Figure 3.1: Mouse Eosinophils kill *P. aeruginosa in vitro*

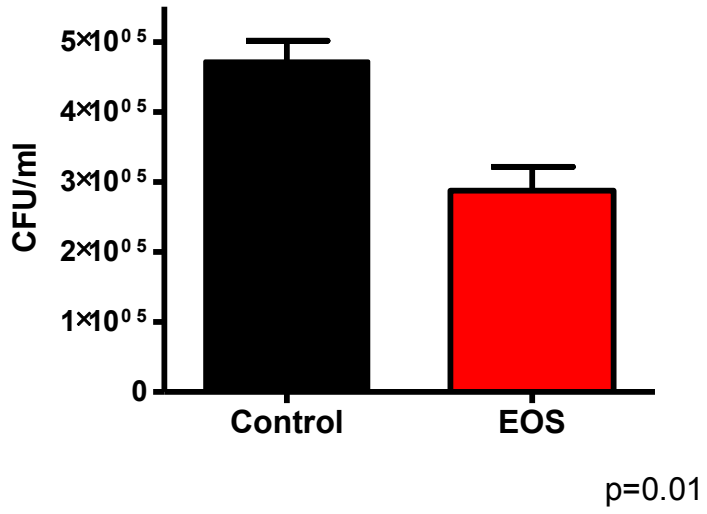


Figure 3.1: Eosinophils (10^6/ml) isolated from NJ.1638 mice or PBS was incubated at an MOI of 10 with *P. aeruginosa* for 1 hr. Black bar represents bacteria treated with PBS control; red bar represents bacteria incubated with eosinophils. Data represent three independent experiments. Error bars represent means +/-SEM. ** P=0.01

Figure 3.2: NJ.1638 mice have improved survival and increased bacterial clearance in *P. aeruginosa* peritonitis

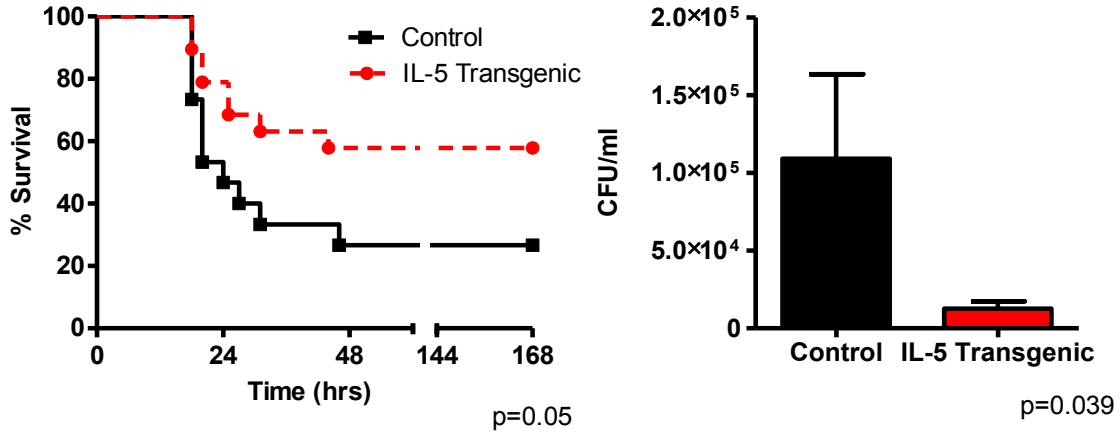
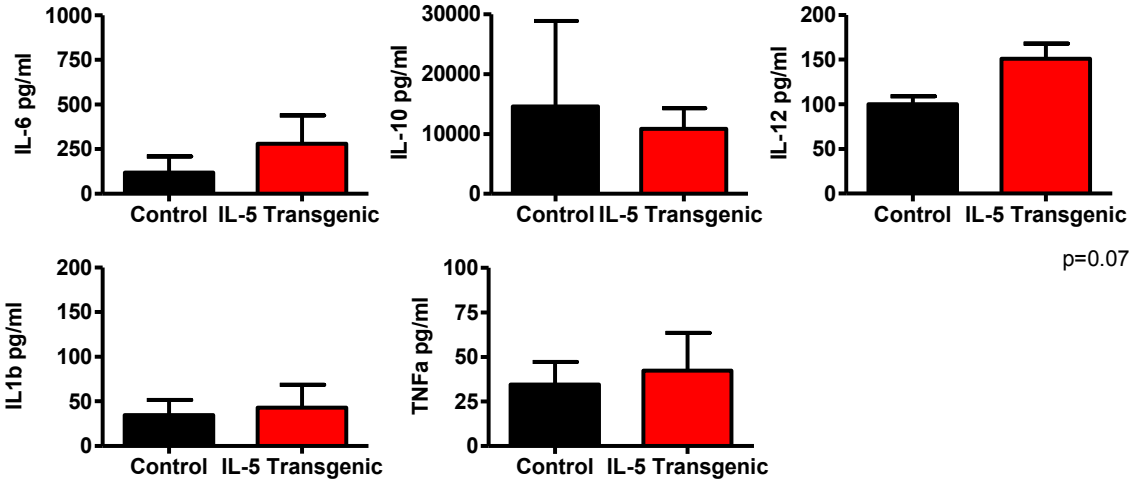


Figure 3.2: Survival curve (graph on left) of NJ.1638 (n=19) or littermate controls (n=16) following i.p. infection with 10^7 CFU *P. aeruginosa*. Data were analyzed using the Log-rank test. *P=0.05. NJ.1638 (n=15) or littermate controls (n=15) were infected i.p. with 10^7 CFU *P. aeruginosa*. At 18 hrs post-infection, animals were sacrificed. Quantitative colony counts of the peritoneal lavage fluid were made by serial dilution (graph on right). Bars represent means +/- SEM. *P=0.039

Figure 3.3: NJ.1638 mice have no significant difference in inflammatory cytokine production in *P. aeruginosa* peritonitis

A. Plasma



B. Peritoneal Lavage Fluid

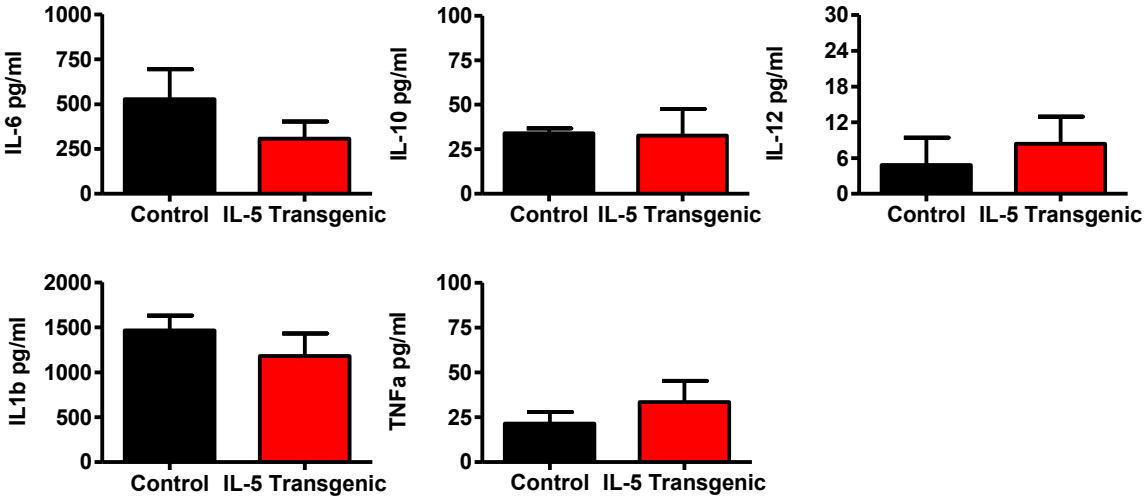


Figure 3.3: NJ.1638 mice have no significant difference in inflammatory cytokine production in *P. aeruginosa* peritonitis

NJ.1638 (n=15) or littermate controls (n=15) were infected i.p. with 10^7 CFU *P. aeruginosa*. At 18 hrs post-infection, animals were sacrificed. IL-6, IL-10, IL-12, IL-1β, and TNFα were measured in plasma and peritoneal lavage fluid by ELISA. Samples were run in duplicate. Bars represent means +/- SEM. Data were analyzed by student's t-test. All P values are non-significant ($p>0.05$)

Figure 3.4: Mice receiving Adoptively transferred Eosinophils have reduced bacterial burden in *P. aeruginosa* peritonitis

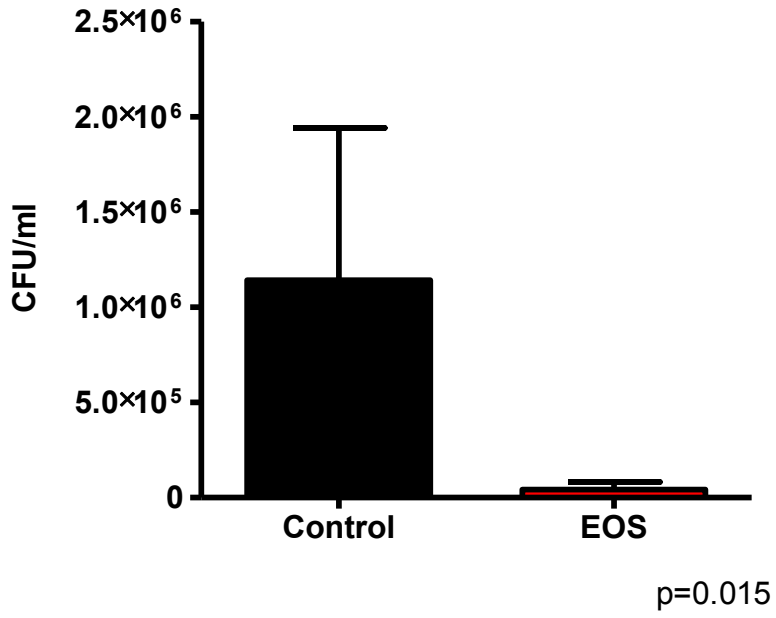


Figure 3.4: C57Bl/6 mice were injected i.p. with either PBS (n=10) or $2x10^5$ eosinophils (n=9) isolated from NJ.1638 mice. One hour later, both groups were subjected to i.p. infection with 10^7 CFU *P. aeruginosa*. Animals were sacrificed 18 hrs post-infection. Quantitative colony counts of the peritoneal lavage fluid were made by serial dilution. Bars represent means +/- SEM. *P=0.015

Figure 3.5: PHIL mice have increased bacterial burden in *P. aeruginosa* peritonitis

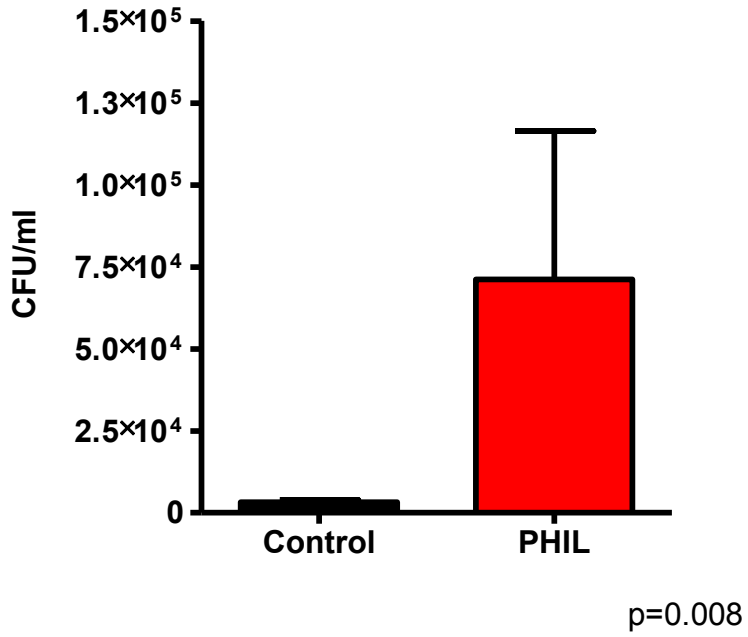
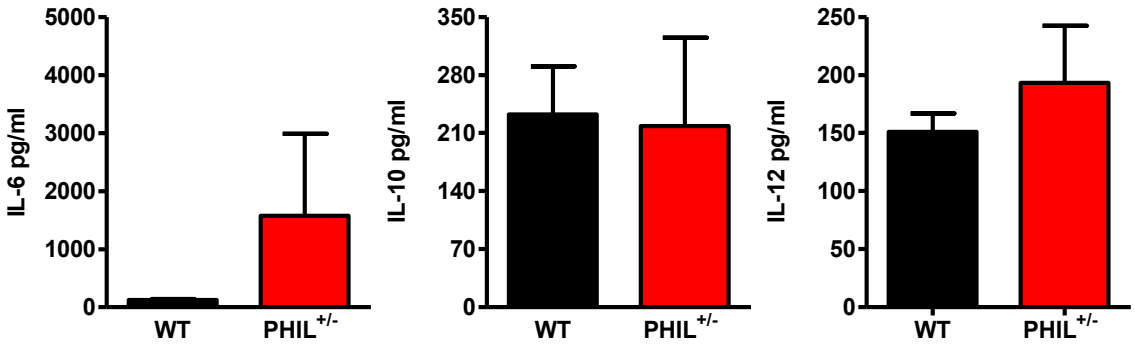


Figure 3.5: PHIL mice (n=5) or littermate controls (n=5) were subjected to i.p. infection with 10^7 CFU *P. aeruginosa*. Animals were sacrificed 18 hrs post-infection. Quantitative colony counts of the peritoneal lavage fluid were made by serial dilution. Bars represent means +/- SEM. ** P=0.008

Figure 3.6: PHIL mice have no significant difference in IL-6 or IL-10, but have increased IL-12 production at the site of infection in *P. aeruginosa* peritonitis

A. Plasma



B. Peritoneal Lavage Fluid

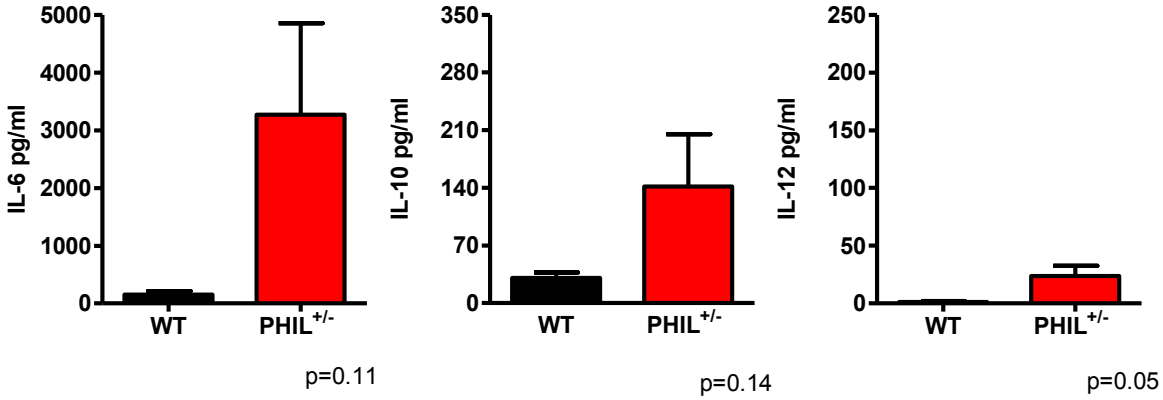


Figure 3.6: PHIL mice have no significant difference in IL-6 or IL-10, but have increased IL-12 production at the site of infection in *P. aeruginosa* peritonitis

PHIL mice (n=5) or littermate controls (n=5) were subjected to i.p. infection with 10^7 CFU *P. aeruginosa*. Animals were sacrificed 18 hrs post-infection. IL-6, IL-10, and IL-12 were measured in plasma and peritoneal lavage fluid by ELISA. Samples were run in duplicate. Bars represent means +/- SEM. Data were analyzed by student's t-test. *P=0.05 for peritoneal lavage IL-12. All P values not listed are non-significant (P>0.05).

Figure 3.7: Eosinophil Granules kill bacteria in a dose-dependent manner *in vitro*

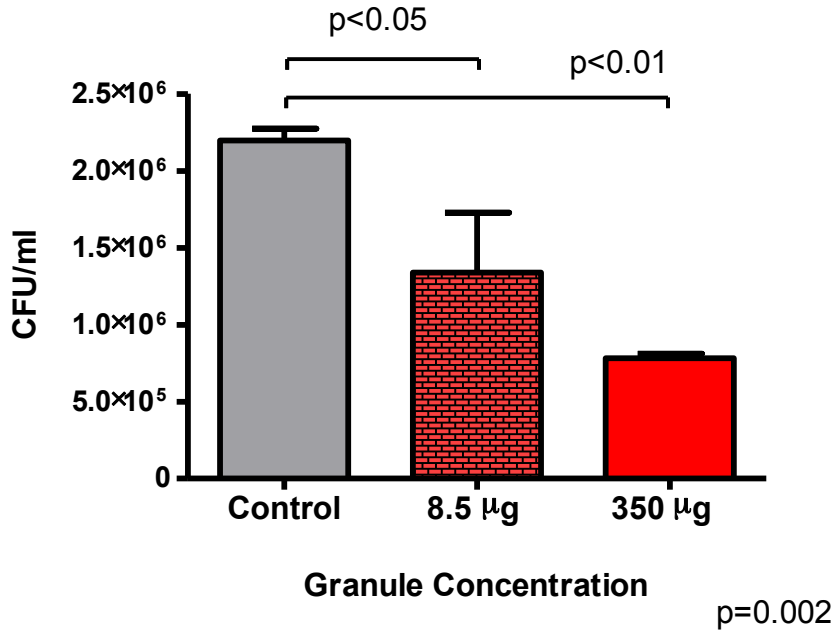


Figure 3.7: Eosinophil granules were extracted from eosinophils isolated from NJ.1638 mice (a gift from James J. Lee, Mayo Clinic). Eosinophil granule extract or vehicle control was incubated with 10^6 CFU *P. aeruginosa* for 1 hr. Quantitative colony counts were made by serial dilution. Data represent three independent experiments, and were analyzed using a one-way ANOVA with Bonferroni's multiple comparisons test. Bars represent means +/- SEM. 8.5 μg vs control * P<0.05; 350 μg vs control ** P<0.01; ANOVA comparison of all groups P=0.002

Figure 3.8: Eosinophil Granule-Treated mice have reduced bacterial burden in *P. aeruginosa* peritonitis

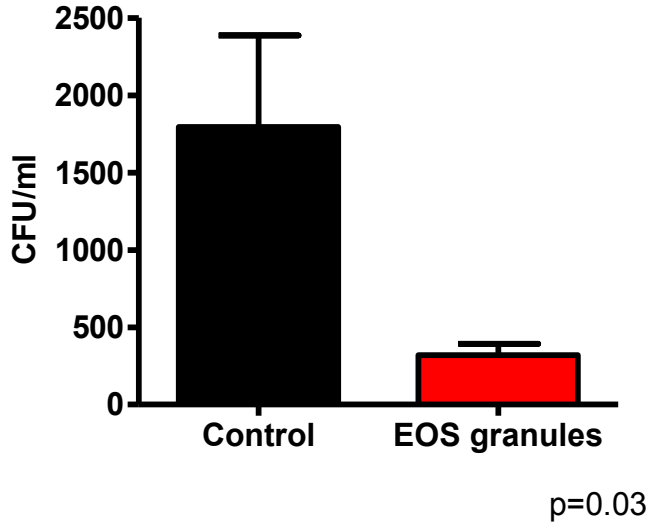
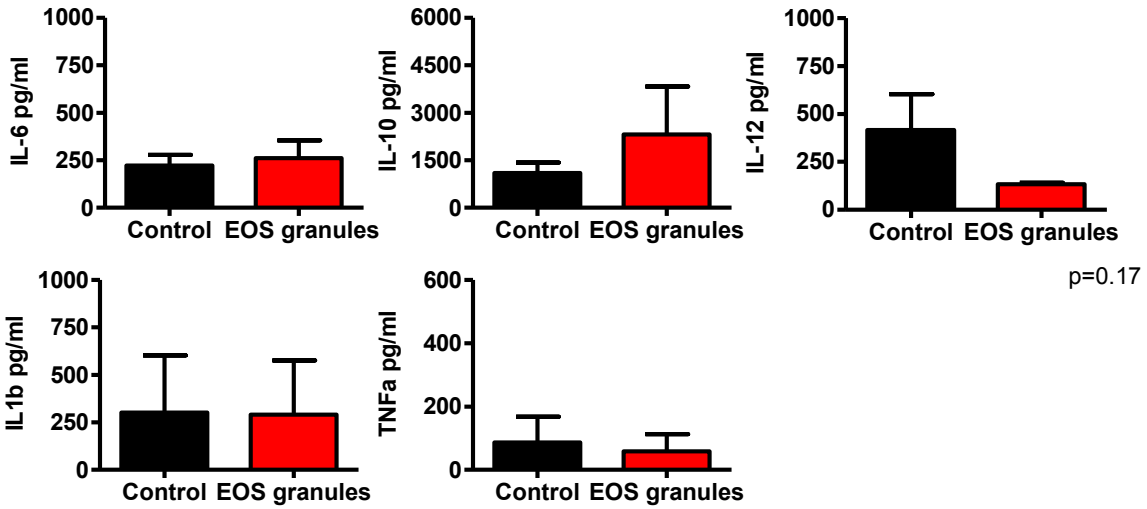


Figure 3.8: C57Bl/6 mice were administered i.p. either 180 µg eosinophil granule extract (n=9) or vehicle control (n=12) 1hr following i.p. infection with 10^7 CFU *P. aeruginosa*. Animals were sacrificed at 18 hrs post-infection. Quantitative colony counts of the peritoneal lavage fluid were made by serial dilution. Data were analyzed using student's t-test. Bars represent means +/- SEM. * P=0.03

Figure 3.9: Eosinophil Granule-Treated mice have no significant difference in inflammatory cytokine production

A. Plasma



B. Peritoneal Lavage Fluid

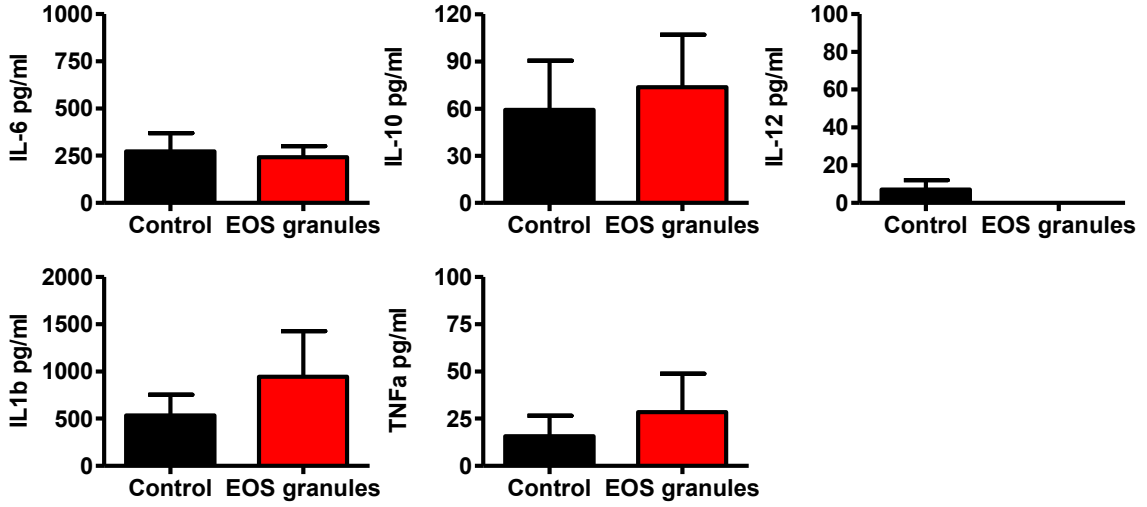


Figure 3.9: Eosinophil Granule-Treated mice have no significant difference in inflammatory cytokine production

C57Bl/6 mice were administered i.p. either 180 μg eosinophil granule extract (n=4) or vehicle control (n=7) 1 hr following i.p. infection with 10^7 CFU *P. aeruginosa*. Mice were sacrificed at 18 hrs post-infection. IL-6, IL-10, IL-12, IL-1β, and TNFα were measured in plasma and peritoneal lavage fluid by ELISA. Samples were run in duplicate. Bars represent means +/- SEM. Data were analyzed by student's t-test. All P values are non-significant ($p>0.05$)

Figure 3.10: Adoptive transfer of Eosinophil Granule Protein prolongs survival in the CLP model of polymicrobial sepsis

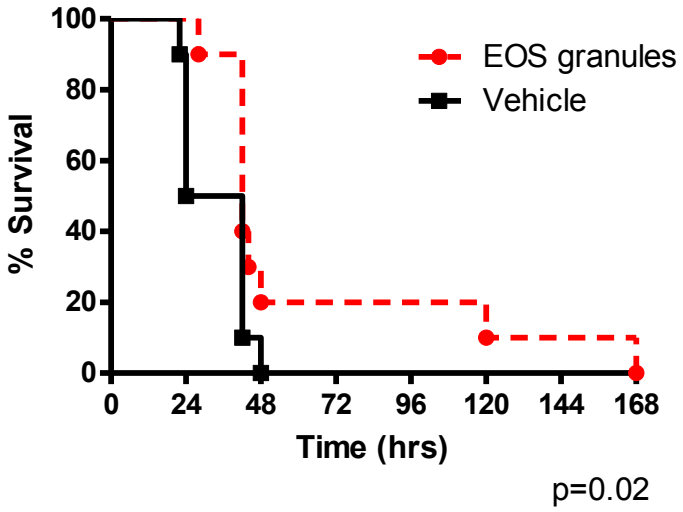


Figure 3.10: Survival curve of C57Bl/6 mice injected i.p. with either 180 µg eosinophil granule extract (n=10) or vehicle control (n=10). One hour later, both groups were subjected to CLP. Data were analyzed using the Log-rank test. *P=0.02

Discussion

Understanding the host innate immune response to sepsis is essential for creating new and effective immunomodulatory therapies for patients. The findings presented in this Chapter not only suggest eosinophils are beneficial in host defense against bacterial infection, but also administration of eosinophil granule proteins is a viable therapeutic strategy to improve host control of bacterial infections.

Eosinophils are considered essential to host responses following parasitic infections. In addition, eosinophils appear to be a critical component of Th2 mediated allergic responses, including those associated with asthma (Jacobsen et al., 2008; Lee et al., 2004). Mounting evidence suggests human eosinophils have a potentially important role in innate immune responses to viral and bacterial infections (Phipps et al., 2007; Rosenberg and Domachowske, 2001; Svensson and Wenneras, 2005). This role likely stems from the ability to recognize and kill bacteria through the expression of multiple TLRs, including TLR-2, -4, -5, and -9 (Wong et al., 2007).

The results presented here indicate that mouse eosinophils also have potent antibacterial properties. Specifically, mouse eosinophils had antibacterial activity against *P. aeruginosa*, a highly antibiotic-resistant species important in the clinical setting. These data confirm the work of Persson et al and Lehrer et al, which demonstrated human eosinophils are capable of killing bacteria *in vitro*, specifically *E. coli* and *S. aureus* (Lehrer et al., 1989; Persson et al., 2001).

After establishing that mouse eosinophils possess antibacterial properties *in vitro*, a mouse model of Pseudomonas peritonitis was used to show the biological significance of this activity *in vivo*. IL-5 overexpressing NJ.1638 mice, in which 40% of the circulating leukocytes are eosinophils, had improved bacterial clearance in this model of peritonitis. Additionally, the antibacterial activity observed was highly specific to eosinophils as adoptive transfer of these cells into wild type mice recapitulated this activity and further established the antibacterial role for eosinophils *in vivo*. The specificity of the antibacterial role for eosinophils is confirmed by impaired bacterial clearance in PHIL mice, which have a congenital eosinophil deficiency.

Recent data showed that adoptive transfer of eosinophils rescues mice from the lethality of polymicrobial sepsis (Yousefi et al., 2008). However, the exact mechanism has yet to be determined. The data presented in this Chapter suggest this protection was through an antibacterial mechanism. Likewise, improved survival in the absence of inflammatory cytokine modulation suggests that the increase in bacterial clearance was highly specific, though the possibility remains that other inflammatory pathways are affected by eosinophils *in vivo*. It should be noted that other studies demonstrated the hypereosinophilic state induced by ovalbumin sensitization impairs Pseudomonas clearance in the lung (Beisswenger et al., 2006). One possible explanation for this apparent discrepancy may be the induction of other Th2 cytokines in the ovalbumin model, including IL-4, which has recently been shown to promote bacterial growth *in vivo* (Hultgren et al., 1998).

Another explanation is that in the lung, eosinophils cleave surfactant protein-D through release of MMP-9, which may hinder bacterial clearance and mask any potential effect on killing (Okada et al., 1997; Weathington et al., 2010). This also explains data showing NJ.1638 mice have increased mortality and reduced bacterial clearance in Pseudomonas pneumonia (unpublished data from J. A. Gold). These studies combined with data in this Chapter suggest there are tissue-specific effects for eosinophil-mediated bacterial killing, and that such killing may be protective in the peritoneal cavity but detrimental in the lungs. Further studies are necessary to assess this hypothesis.

Given that adoptive transfer of eosinophils is a difficult therapeutic strategy and is of limited use in patients in the ICU, this study investigated whether eosinophil-derived products could mediate a similar antibacterial effect *in vivo*, as demonstrated with the use of neutrophil-derived BPI in meningococcal sepsis (Levin et al., 2000). These data suggest that the bacterial killing afforded by eosinophils was in a large part due to degranulation and release of cytotoxic granule proteins. These results support previous data, which showed that EARs possess antibacterial properties against *E. coli*, and that purified EPO, another granule protein, can kill *Mycobacterium tuberculosis in vitro* (Borelli et al., 2003; Ishihara et al., 2003).

These findings advance previous knowledge to establish a similar role for mouse eosinophil granules *in vitro* and the ability of eosinophil granule proteins to enhance bacterial clearance *in vivo*. Moreover, the ability of these granule proteins to prolong

survival in the CLP model of polymicrobial sepsis provides a unique rescue therapy that can be exploited by physicians to augment bacterial clearance without altering inflammatory cytokine production. While the possibility cannot be excluded that contaminating components like mitochondrial DNA also mediate bacterial killing, as shown recently by Yousefi and colleagues, the method used for granule isolation makes this possibility unlikely. Furthermore, these data provide evidence of a unique antibacterial activity afforded by granule proteins alone.

While these data demonstrate an important role for eosinophils in bacterial clearance *in vivo*, further studies are necessary to fully define the role of specific granule proteins, MBP, ECP, etc., to determine the active component(s) in these granules. It may be that a combination of these proteins acts synergistically to enhance bacterial clearance or they may in fact have detrimental effects on the host response that were not measured in these experiments. Based on numerous studies, it is probable that MBP and EPO play a prominent role in bacterial clearance (Borelli et al., 2003; Klebanoff and Shepard, 1984; Lehrer et al., 1989; Svensson and Wenneras, 2005). To assess the unique contribution of each protein, MBP, EPO or MBP/EPO knockout mice could be used to assess any defect in bacterial killing by eosinophils *in vivo* (Denzler et al., 2001; Denzler et al., 2000). The description of BPI as a component of eosinophil granules implicates this protein as another potential candidate due to its known antibacterial activity *in vivo* (Calafat et al., 1998). These possibilities need to be investigated to precisely determine the role for specific eosinophil granules in bacterial killing.

In summary, this Chapter provides evidence that eosinophils and eosinophil granules play a beneficial but underappreciated role in the innate immune response to bacterial infections. These data suggest that the use of eosinophil-derived granule proteins may be an adjuvant antimicrobial therapy for patients with sepsis or other bacterial infections. In addition, these data suggest that patients receiving eosinophil-depletion therapies may experience potentially adverse effects on innate immune responses and resolution of bacterial/viral infections.

While these data provide a novel description for the role of eosinophils *in vivo* during bacterial infection, they do not address the protective role for IL-5 in sepsis despite the loss of eosinophils in humans with acute infection or sepsis (Abidi et al., 2008; Bass et al., 1980; Bozza et al., 2007; Shaaban et al., 2010). It seems that there are two possible explanations for this. The first is that elevated IL-5 is recruiting eosinophils into the tissues and out of the blood, so that while their numbers decrease in the blood, they increase in tissues. This may have important consequences for tissue remodeling and explain some of the tissue damage observed in septic patients. The second explanation is that expression of IL-5 in sepsis has an entirely different purpose than its typical effects on eosinophils. In addition to eosinopenia in sepsis, massive apoptosis of B cells occurs, leaving few cells known to respond to IL-5. Elevation of IL-5 may have effects on other leukocytes or non-immune cells, which are important and protective in sepsis. This second possibility will be explored in subsequent Chapters.

Data from this Chapter are published as:

Linch, S. N., Kelly, A. M., Lee, J.J. and J. A. Gold. Mouse Eosinophils Possess Potent Antibacterial Properties *in vivo*. *Infect Immun*. 2009 November; 77(11); 4976-82.

This work also resulted in the publication of an invited review:

Linch, S. N. and J. A. Gold. The Role of Eosinophils in Non-Parasitic Infections. *Endocr Metab Immune Disord Drug Targets*. 2011 Jun 1;11(2):165-72.

CHAPTER 4:

IL-5 IS PROTECTIVE IN SEPSIS IN MICE AND HUMANS, AND THIS PROTECTION IS EOSINOPHIL-INDEPENDENT

Abstract

Sepsis affects over 750,000 people each year in the US alone. Despite numerous advances in knowledge of this disease and of the immune system, therapeutic modalities have been unsuccessful. The discovery of immunosuppression in sepsis has shifted the focus of these therapies toward augmenting the immune response, and this strategy has shown some success. Studies documenting elevated levels of IL-5 in sepsis survivors and improved survival in septic mice overexpressing IL-5 suggest a novel and protective role for IL-5 in this disease. Data presented in Chapter 3 indicate that eosinophils killed bacteria and were protective *in vivo*. However the absence of eosinophils and elevation of IL-5 presents a paradox in sepsis. In this Chapter, it becomes clear that the protective role for IL-5 is in fact eosinophil-independent. IL-5 transgenic mice with congenital deficiency in eosinophils still had a marked improvement in survival and bacterial clearance following sepsis. Intriguingly, these mice have elevated numbers of neutrophils and monocytes in the blood. Loss of IL-5 was detrimental to survival and bacterial clearance during sepsis, and prophylactic or therapeutic administration IL-5 improved survival in septic mice. Furthermore, administration of IL-5 induced neutrophil recruitment into the peritoneal cavity, suggesting an effect of IL-5 on additional myeloid cells. Finally, IL-5 levels were elevated in septic patients at OHSU and higher levels were associated with improved outcomes. These data collectively suggest a novel and protective role for IL-5 in sepsis. IL-5 treatment may be a viable immunomodulatory therapy for immunosuppressed patients.

Introduction

Sepsis is defined as the systemic inflammatory response to infection. Currently, it is the leading cause of death in the ICU with a mortality rate in excess of 25% (Angus et al., 2001; Hotchkiss and Karl, 2003). It remains an enormous financial burden in the US, costing over $17 billion annually (Angus et al., 2001; Hotchkiss and Karl, 2003). Despite maximal supportive care and antimicrobial therapy, mortality remains high due to the lack of efficacious therapeutic modalities.

Activation of the innate immune response is vital to combat infection. This results in the upregulation of inflammatory cytokine production and recruitment of additional leukocytes to sites of infection. However, despite an overwhelming cytokine response in sepsis, inhibition of the proinflammatory response has failed to improve survival in septic patients (Abraham et al., 1998; Cohen and Carlet, 1996; Fisher et al., 1994; Opal et al., 2004; Opal et al., 1997; Remick et al., 1995; Remick et al., 1998). Moreover, patients with neutropenia or congenital defects in the innate response have increased mortality from severe bacterial infections (Donini et al., 2007; Mills and Quie, 1980; Ottonello et al., 1995). Clinical studies from patients suggest that sepsis is associated with an acquired impairment of the innate immune response as evidenced by reduced bacterial killing by neutrophils from septic patients (Solomkin et al., 1985; Zimmerman et al., 1989).

Evidence of immunosuppression in sepsis led investigators to focus on augmenting the immune response as a means to improve survival. Administration of GM-CSF to septic patients lead to increased neutrophil recruitment and enhanced monocyte

activation, ultimately enhancing pathogen control and bacterial clearance (Orozco et al., 2006; Presneill et al., 2002; Rosenbloom et al., 2005). In another study, GM-CSF treatment restored monocyte HLA-DR expression and increased TLR-stimulated cytokine production *in vitro*. While this study was not powered to assess mortality as an end point, there was a small decrease in survival associated with GM-CSF (Meisel et al., 2009). These studies indicate the importance of the innate immune response and the benefit of immunostimulatory therapies in sepsis.

A recent study observed that IL-5 levels were elevated in septic patients who survived compared to non-survivors (Bozza et al., 2007). In addition, IL-5 overexpressing mice had improved survival in CLP sepsis, suggesting a protective role for IL-5 (Yousefi et al., 2008). However, IL-5 induces eosinophil recruitment and survival, and data presented in Chapter 3 indicate eosinophils enhanced bacterial clearance and survival in bacterial peritonitis. Since NJ.1638 mice exhibit a profound eosinophilia and eosinophils participate in bacterial clearance, it is unclear whether IL-5 has an eosinophil-independent role in sepsis. However, the loss of eosinophils in sepsis and the protective effects associated with IL-5 suggest a role for this cytokine in sepsis, though its precise function remains unknown.

Data presented in this Chapter indicate that IL-5 does indeed have a protective role in sepsis in the absence of eosinophils, as NJ.1638/PHIL mice had improved survival and bacterial clearance in CLP sepsis. Surprisingly, overexpression of IL-5 in the absence of eosinophils resulted in an increase in the absolute number of neutrophils and monocytes, suggesting pleiotropic effects of IL-5 on the innate immune system.

Loss of IL-5 was detrimental to the host in sepsis, and administration of exogenous IL-5 improved survival when administered both prophylactically and therapeutically to septic mice. Data from septic patients confirmed animal studies, and also demonstrated that elevated levels of IL-5 were associated with improved outcomes in sepsis. Furthermore, these data indicate that IL-5 had unappreciated effects on other myeloid cells during sepsis, specifically neutrophils and monocytes/macrophages. The next Chapter will clarify whether this is accurate, by examining IL-5Rα expression on these myeloid cells during sepsis. Collectively, data in this Chapter demonstrate the usefulness of exogenous IL-5 treatment as a means to improve mortality in sepsis. Further studies are necessary to assess the specific effects of IL-5 and the role it has on neutrophils and macrophages.

Methods

NJ.1638$^{+/-}$, PHIL$^{+/-}$, and IL-5$^{-/-}$ mice

See General Methods Section Ib

CLP model of polymicrobial sepsis

See General Methods Section IIa and IId

Bacterial clearance and cytokine analysis

See General Methods Section IVb and IVe

Neutrophil and Monocyte analysis by flow cytometry

See General Methods Section IVc

Human selection criteria

See General Methods Section Ia and Ic

Results

NJ.1638/PHIL mice have improved survival and increased bacterial clearance in CLP-induced sepsis, indicating eosinophil-independent effects of IL-5

Previous data from our lab showed that IL-5 overexpression in NJ.1638 mice was protective in the CLP mouse model of polymicrobial sepsis, and improvement in mortality was associated with increased bacterial killing (Yousefi et al., 2008). Data from Chapter 3 indicate that eosinophils possess potent antibacterial properties, both *in vitro* and *in vivo*.

Because overexpression of IL-5 in NJ.1638 mice leads to a profound eosinophilia and eosinophils have antibacterial properties, it was necessary to determine whether eosinophils alone were necessary to increase survival and bacterial killing in CLP-induced sepsis. To test this, IL-5 transgenic NJ.1638$^{+/-}$ mice were bred with PHIL$^{+/-}$ mice, which express the diphtheria toxin transgene under control of the eosinophil peroxidase promoter causing a congenital deficiency in eosinophils (Lee et al., 2004; Lee et al., 1997). NJ.1638/PHIL mice constitutively overexpress IL-5, but are deficient in eosinophils, thereby enabling accurate assessment of the independent effects of IL-5 in polymicrobial sepsis. Based on data in Chapter 3, it was expected that elevated IL-5 by itself would have little to no effect on bacterial clearance in the Pseudomonas peritonitis model. However, preliminary data using the *P. aeruginosa* infection demonstrated that this was not correct. Interestingly, bacterial clearance was increased in both the peritoneal lavage fluid (Figure 4.1), as

well as the blood (Chi square analysis P=0.06), of NJ.1638/PHIL mice suggesting that IL-5 overexpression affects bacterial killing independent of eosinophil-mediated bacterial killing shown in Chapter 3.

The next step was to determine if IL-5 was protective in the mouse model of polymicrobial sepsis, CLP. Using this sepsis model, NJ.1638/PHIL mice had improved survival over littermate control mice (Figure 4.2), suggesting an independent role for IL-5 in sepsis in the absence of eosinophils. Moreover, NJ.1638/PHIL mice had increased numbers of neutrophils and monocytes in the spleen and blood (Figure 4.3), suggesting that constitutive expression of IL-5 has unappreciated effects on other myeloid cell types. These data collectively indicate a protective role for IL-5 in the absence of eosinophils, possibly through effects on additional myeloid cell types.

IL-5 loss is detrimental to the host and results in decreased survival and bacterial clearance in the CLP model of polymicrobial sepsis

Since overexpression of IL-5 conferred a protective effect in CLP-induced sepsis independent of eosinophils, it was necessary to assess the unique effects of congenital IL-5 loss in this model using IL-$5^{-/-}$ mice (Kopf et al., 1996). IL-$5^{-/-}$ mice were generated by inserting a neomycin resistance gene into a codon for one of the cysteine residues required for IL-5 activity. These mice have normal numbers of eosinophils, and despite an initial defect in innate B-1 cell development, adult IL-$5^{-/-}$

mice have no defect in this population, antibody production, or in T cell and NK cell responses (Kopf et al., 1996). Since IL-5 had a protective role in the CLP model of sepsis, it was predicted that the loss of IL-5 would impair host defense and decrease survival. To determine this, a sub-lethal model of CLP was employed in order to detect a potential increase in mortality. Indeed, IL-$5^{-/-}$ mice had increased mortality following CLP (Figure 4.4), as well as decreased bacterial clearance in both the peritoneal lavage fluid and in the blood, compared to wild type control mice (Figure 4.5). These data confirm that IL-5 is protective *in vivo* during sepsis by improving survival and host defense as measured by bacterial clearance.

The effect of IL-5 loss on the inflammatory response, specifically through release of various cytokines, was also examined. Interestingly, IL-$5^{-/-}$ mice had a trend toward increased IL-6 and IL-10 compared to wild type controls, both in the peritoneal lavage fluid and in the blood 18 hours after the onset of sepsis; no significant difference was observed in IL-12 in either compartment at this time point (Figure 4.6). These data confirm that IL-5 improves survival in CLP sepsis by improving bacterial clearance and that congenital loss of IL-5 may affect levels of IL-6 and IL-10 during sepsis.

Recombinant IL-5 can be used as a prophylactic, but more importantly as a therapeutic, treatment to improve survival in the CLP model of sepsis.

The protective role of IL-5 overexpression in sepsis suggests that exogenous augmentation of this cytokine in sepsis may be a way in which to improve survival and patient outcomes. To provide proof of principle for this hypothesis, wild type C57BL/6 mice underwent CLP. Recombinant IL-5 was administered either 4 hours or 1 hour prior to CLP and animals were monitored for survival. All animals receiving IL-5 had reduced mortality in CLP-induced sepsis (Figure 4.7), suggesting that IL-5 can be used to improve survival in CLP-induced sepsis. Due to the rapid onset of mortality, a sub-lethal model of CLP was used in order to delay the time to IL-5 treatment in mice and assess the therapeutic benefits of treatment with this cytokine. Remarkably, delayed administration of recombinant IL-5 4 hours after inducing sepsis improved mortality in mice (Figure 4.7). These data indicate that IL-5 is useful as a rescue therapy in polymicrobial sepsis.

Recombinant IL-5 administration into the peritoneal cavity of mice induces neutrophil chemotaxis, with no effect on eosinophil recruitment.

Since IL-5 is a chemotactic factor for eosinophils, one possible explanation for the IL-5-mediated reduction in mortality is that IL-5 induced eosinophil recruitment, thereby increasing host defense and survival in sepsis. To examine this possibility and to assess leukocyte recruitment following treatment, wild type control mice

were administered IL-5 i.p.. Surprisingly, no change in eosinophil recruitment was observed in the peritoneal cavity of IL-5 treated mice. Instead, IL-5 treatment induced peritoneal neutrophil recruitment at 4 and 24 hours following administration (Figure 4.8), as determined by release of MPO into the peritoneal lavage fluid, a surrogate marker of neutrophil presence and degranulation. These data further suggest that IL-5 has unappreciated effects on other leukocytes. This hypothesis will be investigated in detail in Chapters 5 and 6.

IL-5 is elevated in human sepsis and is associated with improved outcomes in patients

In vivo mouse data presented in thus far indicate that IL-5 is beneficial and protective in sepsis. Furthermore, a previous study in humans found that IL-5 levels were greater in patients who survived sepsis compared to those who did not (Bozza et al., 2007). In order to assess the clinical implications of animal data and to confirm the study by Bozza and colleagues, levels of IL-5 were assessed in septic patients at OHSU. Circulating IL-5 levels were elevated in septic patients compared to healthy controls (Figure 4.9a). In particular, increased levels of IL-5 were associated with improved outcomes in septic patients. This was evidenced by elevated levels of IL-5 in patients who survived sepsis compared to non-survivors (Figure 4.9b), and also in patients who did not require mechanical ventilation compared to those who did (Figure 4.9c). This finding suggests the effects of IL-5

observed in the CLP model of sepsis are not species specific, and that research from this model may effectively translate into human sepsis.

Summary

Taken together, the data in this Chapter demonstrate that endogenous IL-5 is protective in mice and humans with sepsis. Moreover, both prophylactic and therapeutic administration of IL-5 improved survival in mice. Surprisingly, IL-5 administration induced recruitment of non-eosinophilic leukocytes into the peritoneal cavity. These data indicate a non-traditional role for IL-5 through effects on additional myeloid cells. This will be examined further in Chapters 5 and 6.

Figure 4.1: NJ.1638/PHIL mice have increased local bacterial clearance in Pseudomonas peritonitis

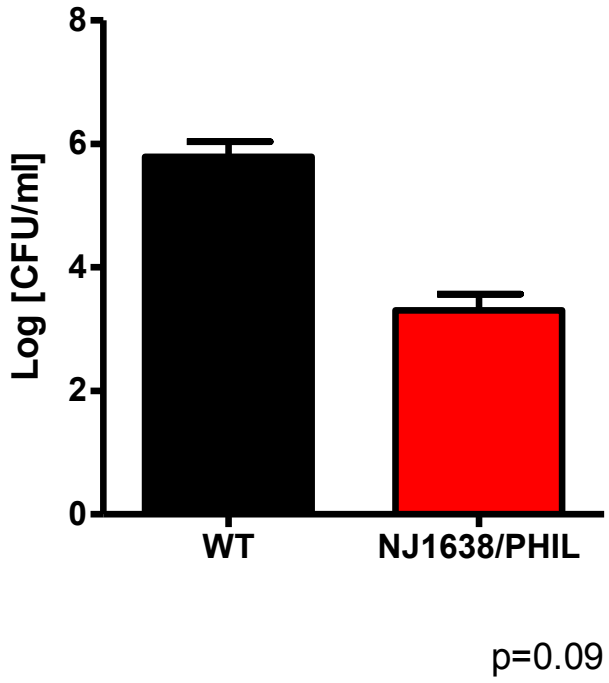


Figure 4.1: NJ.1638/PHIL mice (n=4) or littermate controls (n=5) were subjected to i.p. infection with 10^7 CFU *P. aeruginosa*. Animals were sacrificed 18 hrs post-infection. Quantitative colony counts of the peritoneal lavage fluid and blood were made by serial dilution. Data were analyzed by student's t-test. Bars represent means +/- SEM. T-test performed on Peritoneal lavage fluid P=0.09; Blood cultures were analyzed using Chi square analysis assessing the presence or absence of bacteria in the blood. P=0.06

Figure 4.2: NJ.1638/PHIL mice have improved survival following CLP-induced Sepsis

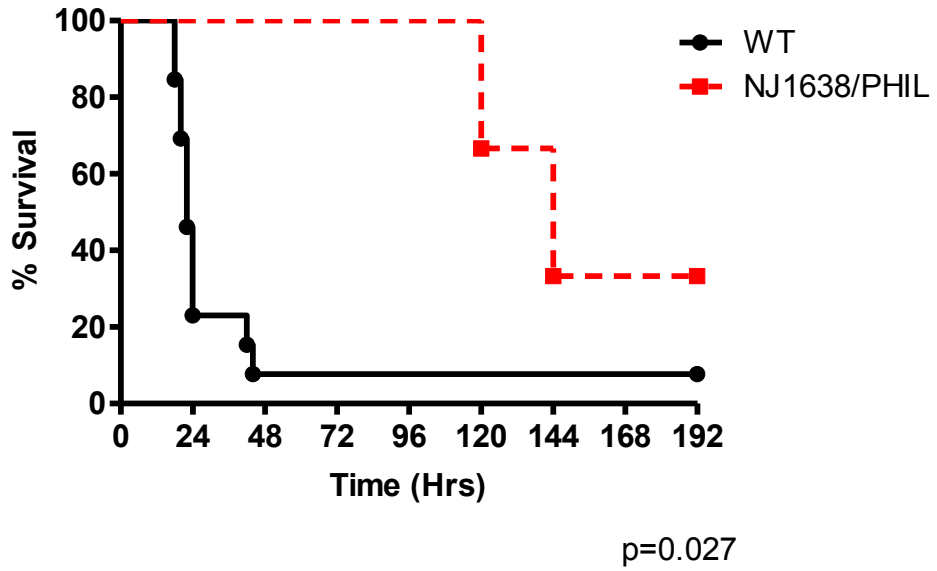


Figure 4.2: Survival curve of NJ.1638/PHIL mice (n=4) or littermate controls (n=10) following CLP. Mice were monitored for survival for 7 days. No additional animals died after this time point. Data were analyzed using the Log-rank test. *P=0.027

Figure 4.3: NJ.1638/PHIL mice have elevated numbers of Neutrophils and Monocytes in the blood and spleen

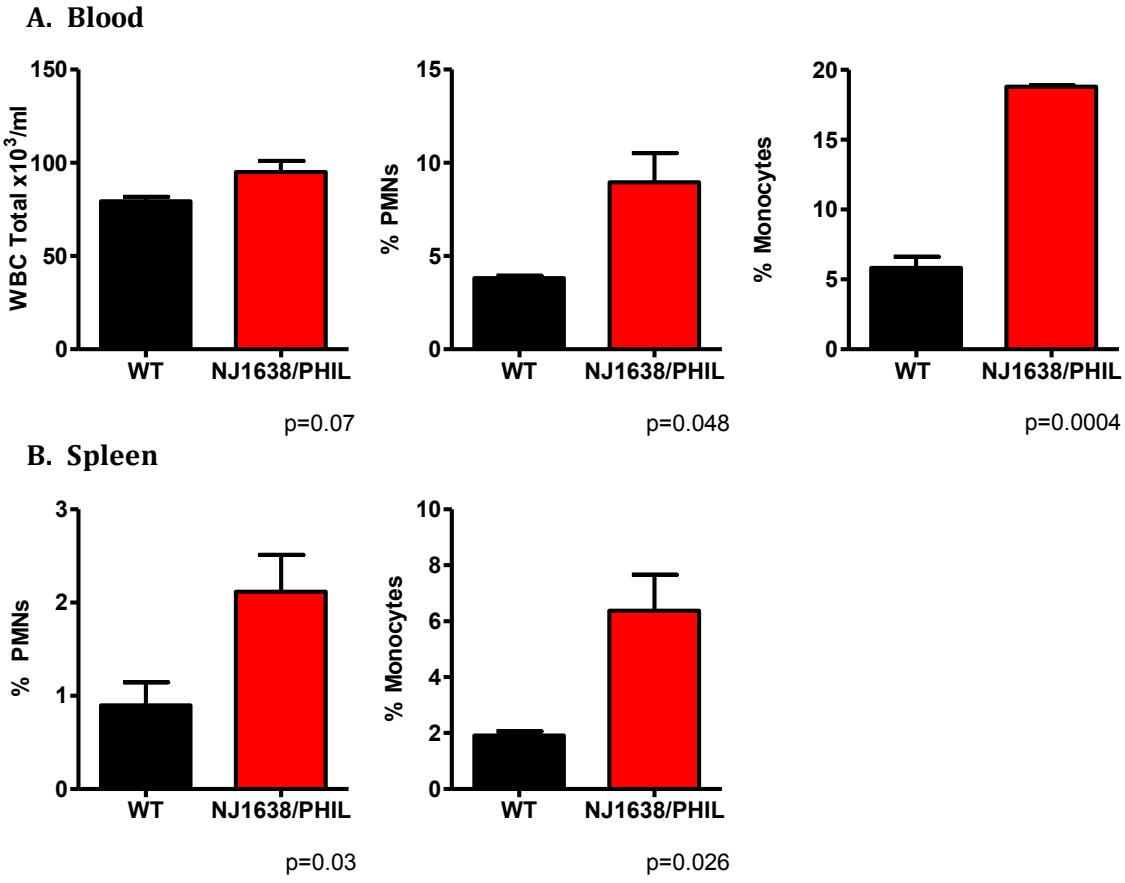


Figure 4.3: Blood was taken from NJ.1638/PHIL or littermate control mice and stained for Ly6g or CD11b. The percentages of neutrophils (PMNs) or monocytes were determined in the blood (Panel A) and the spleen (Panel B) using these markers combined with forward and side scatter by flow cytometry. Total WBC were quantified by differential staining. Data were analyzed by student's t-test. Bars represent means +/- SEM. Blood: PMNs *P=0.048; Monocytes ***P=0.0004. Spleen: PMNs *P=0.03; Monocytes *P=0.026. Total WBC P=0.07

Figure 4.4: IL-5$^{-/-}$ mice have increased mortality following CLP-induced Sepsis

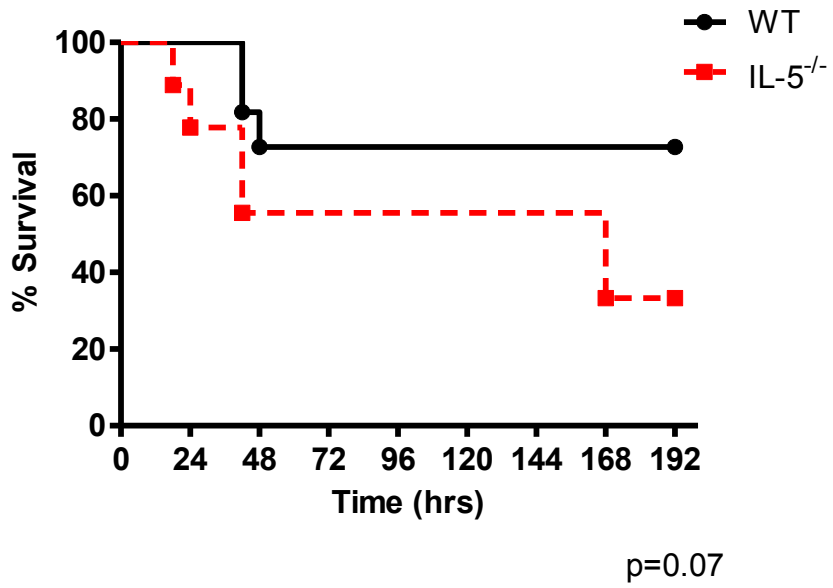


Figure 4.4: Survival curve of IL-5$^{-/-}$ mice (n=9) or C57Bl/6 control mice (n=11) following CLP. Animals were monitored for survival for 7 days. No additional animals died after this time point. Data were analyzed using the Log-rank test. P=0.07

Figure 4.5: IL-5$^{-/-}$ mice have reduced bacterial clearance and increased tissue damage following CLP-induced Sepsis

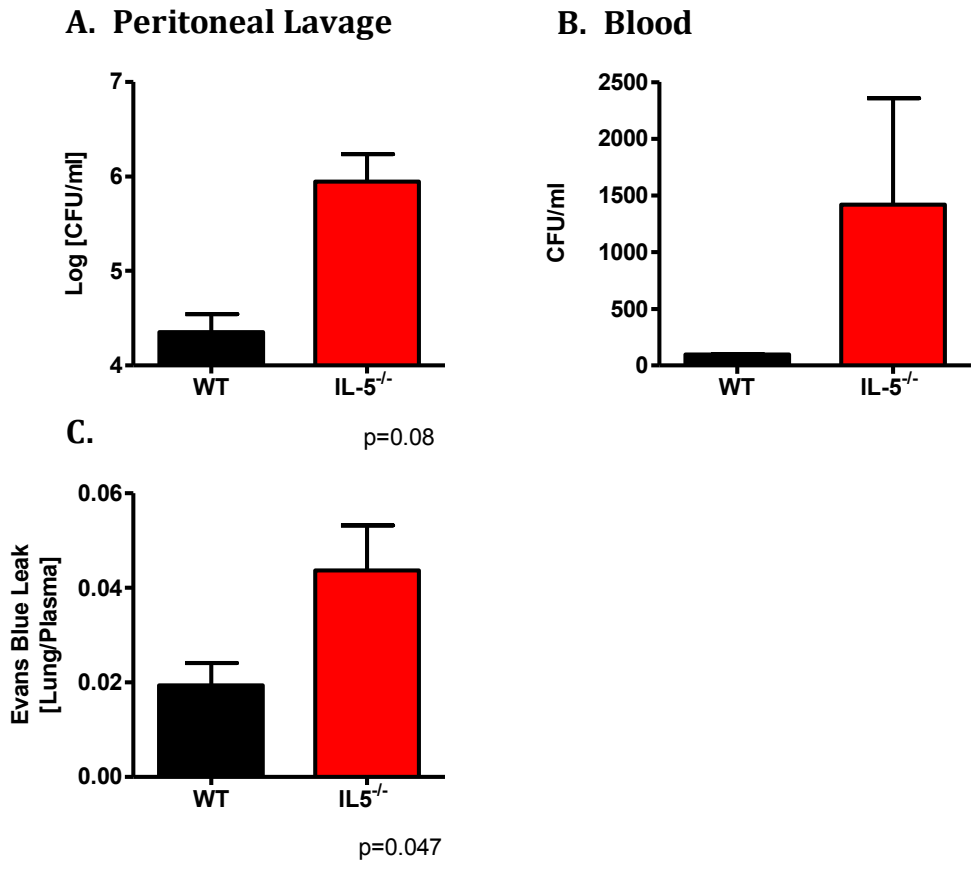


Figure 4.5: IL-5$^{-/-}$ mice (n=5) or C57Bl/6 (n=4) mice were subjected to CLP. Animals were sacrificed 18 hrs post-surgery. Quantitative colony counts of the peritoneal lavage fluid (Panel A) and blood (Panel B) were made by serial dilution. For the Evan's Blue dye assay, animals were injected i.p. with 20μg Evan's Blue dye 18 hrs post-CLP. Animals were sacrificed 1 hr later; plasma and lung homogenates were made to measure the ratio of dye in the plasma vs. the lung. Data were analyzed by student's t-test Bars represent means +/- SEM. Peritoneal lavage fluid P=0.08; Evans Blue dye leak *P=0.047

Figure 4.6: IL-5$^{-/-}$ mice have a trend toward increased IL-6 and IL-10, but no significant difference in IL-12 production in CLP sepsis

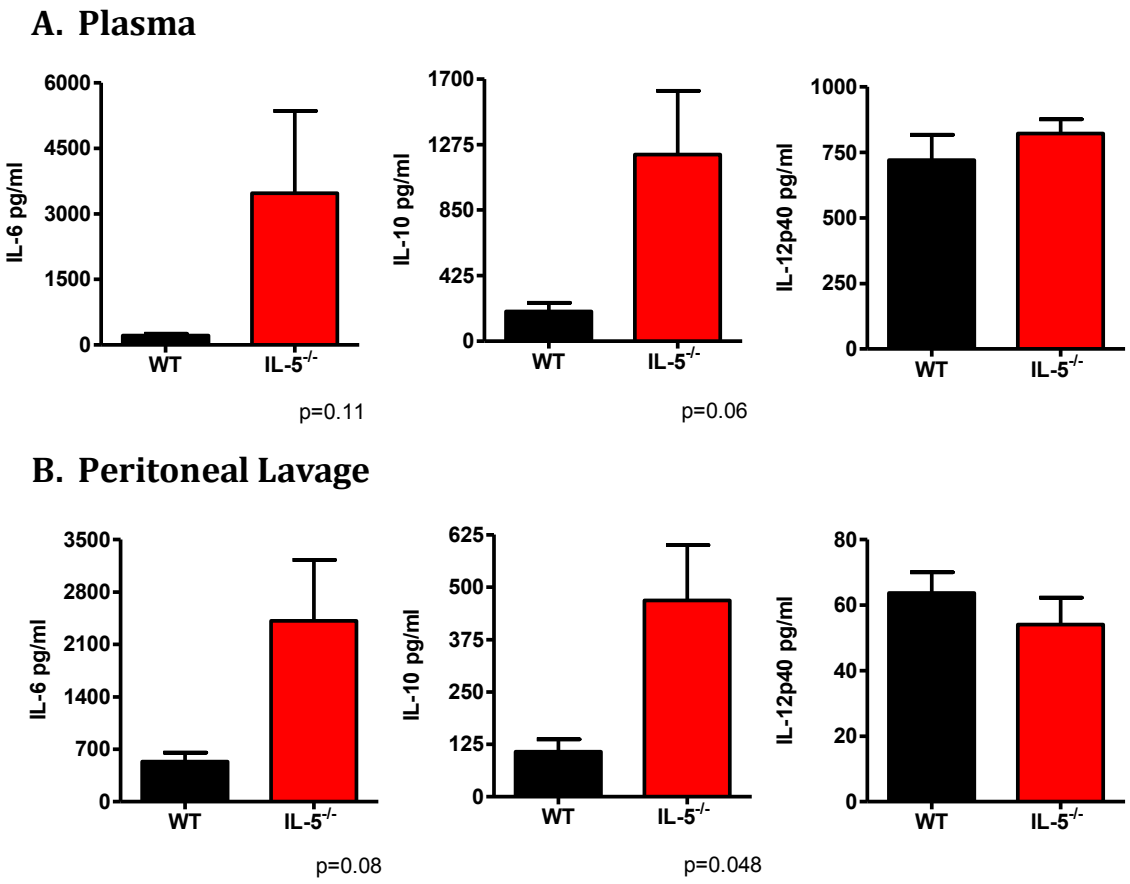
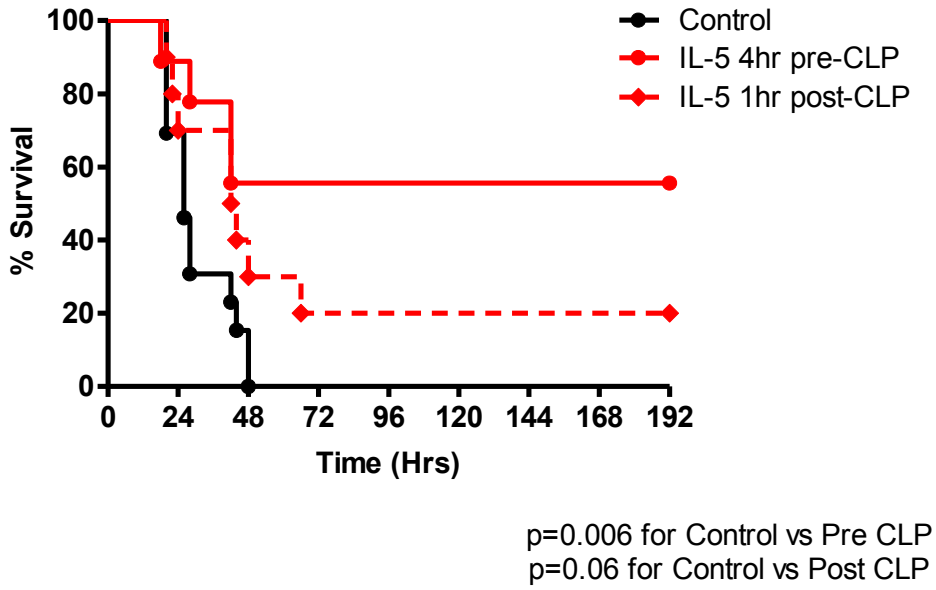


Figure 4.6: IL-5$^{-/-}$ mice (n=5) or C57Bl/6 (n=4) mice were subjected to CLP. Mice were sacrificed 18 hrs following surgery. IL-6, IL-10, and IL-12 were measured in plasma (Panel A) and peritoneal lavage fluid (Panel B) by ELISA. Data were analyzed by student's t-test. Bars represent means +/- SEM. All P values not listed are non-significant (P>0.05). Plasma: IL-6 P=0.11; IL-10 P=0.06. Peritoneal lavage fluid: IL-6 P=0.08; IL-10 *P=0.048

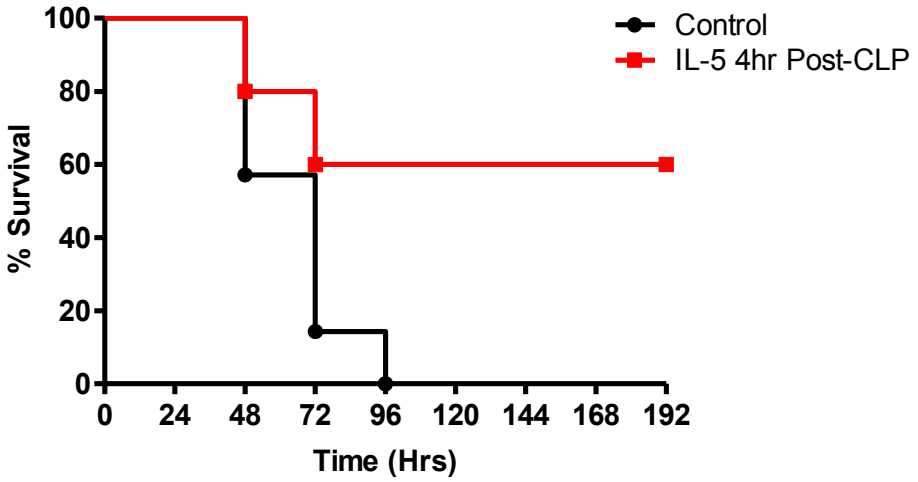
Figure 4.7: Treatment with recombinant IL-5 improves survival in CLP-induced Sepsis

A.



p=0.006 for Control vs Pre CLP
p=0.06 for Control vs Post CLP

B.



p=0.046

Figure 4.7: Treatment with recombinant IL-5 improves survival in CLP-induced Sepsis

Wild type C57Bl/6 mice were treated i.p. with 1 μg recombinant mouse IL-5 (n=10 for both groups) 4 hrs before or 1 hr following CLP using a 19 g needle, or PBS control (n=13) 4 hrs before CLP using a 19 g needle (Panel A). Wild type C57Bl/6 mice were treated with 1 μg recombinant mouse IL-5 (n=5) or PBS control (n=7) 4 hrs following CLP surgery using a 22 g needle (Panel B). Animals were monitored for survival for 7 days. No additional animals died after this time point. Data were analyzed using the Log-rank test. IL-5 4 hrs Pre-CLP ***P=0.006; IL-5 1 hr Post-CLP P=0.06; IL-5 4 hrs Post-CLP *P=0.046

Figure 4.8: Recombinant IL-5 administration induces Neutrophil recruitment into the peritoneal cavity

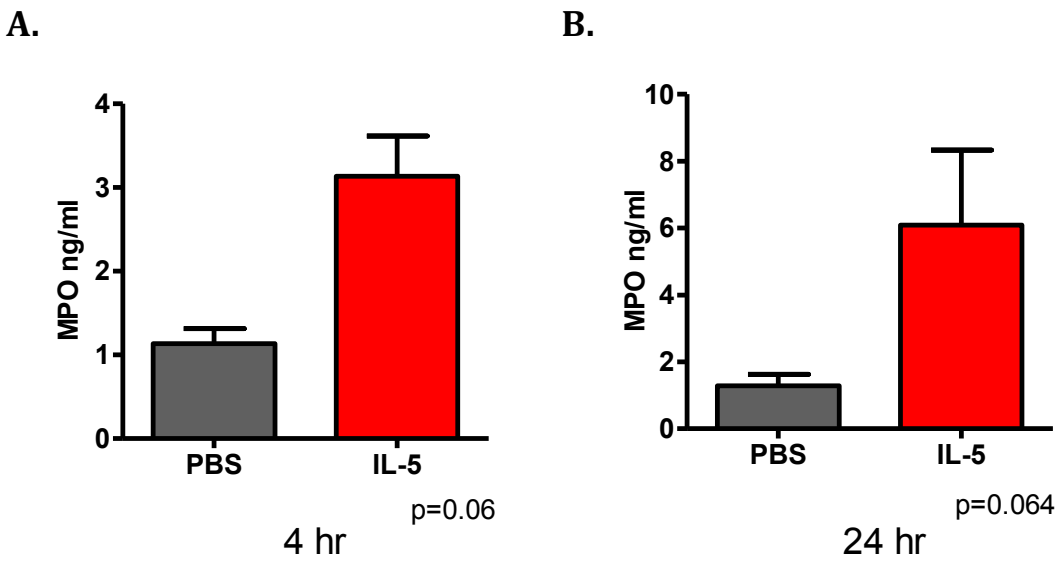


Figure 4.8: Wild type C57Bl/6 mice were administered 1 µg recombinant mouse IL-5 (n=10) or PBS control (n=8) into the peritoneal cavity. Four (Panel A) or 24 hrs (Panel B) later animals were sacrificed. Peritoneal lavage fluid was analyzed for MPO by ELISA. Data were analyzed by student's t-test. Bars represent means +/- SEM. 4 hr P=0.06; 24 hr P=0.064

Figure 4.9: IL-5 levels are elevated in Sepsis and increased IL-5 is associated with improved outcomes

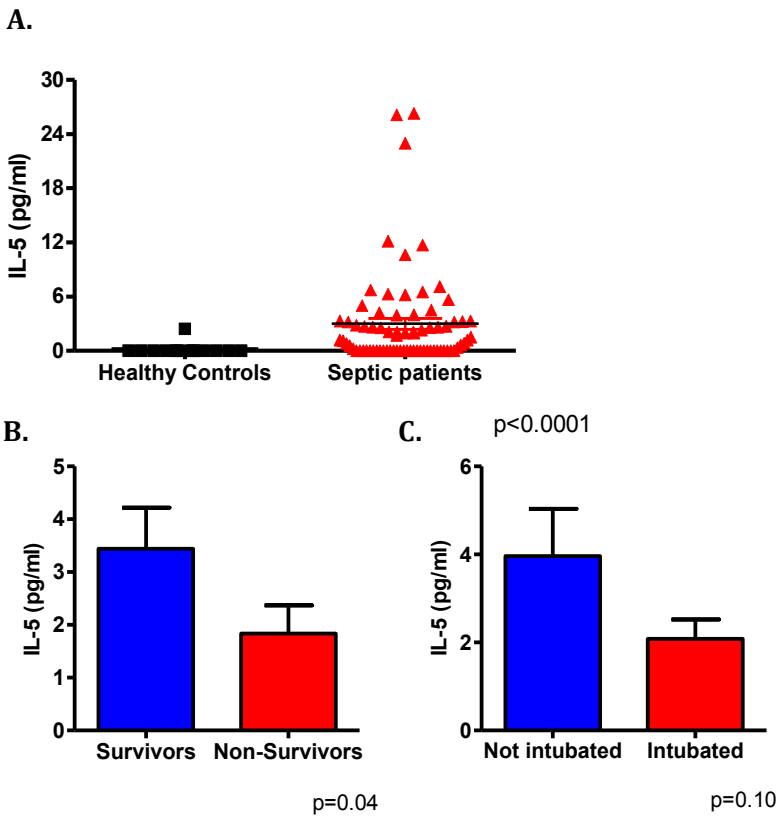


Figure 4.9: Blood was collected from septic patients (n=73) in the ICU at OHSU, or from healthy volunteers (n=13) (Panel A). IL-5 levels were measured by ELISA. Septic patients were further subdivided into either survivors vs. non-survivors (Panel B) or those who required intubation vs. those who did not (Panel C). Data were analyzed by student's t-test. Bars represent means +/- SEM. Panel A: ***P<0.0001; Panel B: *P=0.04; Panel C: P=0.1

Discussion

Data in Chapter 3 indicated that mouse eosinophils had potent yet underappreciated antibacterial activity, both *in vitro* and *in vivo*. In addition, IL-5 overexpressing mice, which have a profound eosinophilia, had improved survival over littermate control mice in CLP sepsis (Yousefi et al., 2008). A study in humans observed that higher levels of IL-5 were found in sepsis survivors compared to non-survivors (Bozza et al., 2007). Since sepsis is associated with a loss of eosinophils, this apparent paradox suggests that the elevation of IL-5 is independently beneficial during sepsis, and is not due to enhancing the function and survival of eosinophils.

In fact, IL-5 does have eosinophil-independent effects. This was demonstrated using the NJ.1638/PHIL mice, which have elevated levels of IL-5 in the absence of eosinophils. Interestingly, these mice had a baseline elevation in the number of neutrophils and monocytes/macrophages in the blood and spleen in the absence of infection or inflammation. This finding is surprising because these cells are not known to respond to IL-5 stimulation or express the receptor for this cytokine, though it was shown that non-eosinophilic myeloid cells express the promoter region for the IL-5Rα (Sun et al., 1995). The presence of increased numbers of neutrophils and monocytes suggests that IL-5 can serve as a growth factor for them. This is contrary to most previous reports regarding the role for IL-5 (Kouro and Takatsu, 2009; Takatsu and Nakajima, 2008). However, there is some precedent for this. A few studies have shown IL-5 induced proliferation of RAW264.7 macrophages and microglia (macrophage-derived cells in the brain) (Liva and de

Vellis, 2001; Ringheim, 1995). The possible effects of IL-5 on neutrophils and monocytes/macrophages need to be further studied, as these cells may play an important role in mediating the protective effects of IL-5.

In addition to expansion of monocytes/macrophages and neutrophils, NJ.1638/PHIL mice had improved survival in the CLP model of sepsis. Bacterial killing was also increased as detected using the Pseudomonas peritonitis model. This was true both locally in the peritoneal lavage fluid, as well as systemically in the blood. It seems probable that increased bacterial killing in NJ.1638/PHIL mice is due to the increased presence of neutrophils and monocytes/macrophages. This confounder makes it difficult to definitively determine whether administration of IL-5 on its own at the onset of sepsis would be beneficial or if improved bacterial clearance and survival is simply mediated by the expansion of innate effector cells due to *congenital* IL-5 overexpression. However, evidence that exogenous administration of IL-5 during sepsis improved survival illustrated that IL-5 is protective when used as a rescue therapy. Further studies are necessary to assess which cells mediated the protective effects of IL-5 in sepsis, but based on these data it seems likely to be neutrophils and macrophages.

Since evidence suggested IL-5 is protective in sepsis, it was expected that loss would be harmful to the host. Indeed, IL-5 knockout mice had decreased survival in CLP sepsis, which was accompanied by decreased bacterial clearance. As mentioned earlier, these mice do not have a defect in T and B cell development, and still maintain a population of eosinophils, though they do not develop eosinophilia

during helminth infection. These mice do have a minor delay in innate B-1 cell development though this defect is significantly diminished in adult mice (Kopf et al., 1996). It remains possible that this minor defect has an impact on survival observed in the CLP model, as one study showed that loss of IgM, which is normally produced by B-1 cells, increases mortality in sepsis (Boes et al., 1998). However, IL-5 knockout mice do not have a defect in IgM production even in response to viral infection, therefore it remains unlikely that this defect is increasing mortality in sepsis (Kopf et al., 1996).

Furthermore, IL-5 knockout mice had increased tissue damage as evidenced by increased Evans Blue dye leak into the lung. This dye remains bound to albumin in the blood, and when found within the tissues indicates loss of membrane integrity and increased vascular permeability. These mice also had a trend toward increased IL-6 and IL-10 with no change in IL-12 compared to wild type mice. Changes in IL-10 were not observed in uninfected IL-5 knockout mice compared to wild type mice (Kopf et al., 1996), and while levels of IL-6 were not determined in that study, it is unlikely that this cytokine is elevated at baseline. These data suggest that the loss of IL-5 allows for increased antiinflammatory cytokine production and mortality, though it does not indicate the mechanism for this. One limitation for these results is that the small sample size used for cytokine analysis and lack of significance makes it difficult to analyze these data, so care should be taken when interpreting this result.

Loss of IL-5 increased tissue damage and permeability in sepsis and induced production of antiinflammatory cytokines IL-6 and IL-10. Interestingly, exogenous administration of IL-10 or elevated levels of endogenous IL-10 are protective in CLP sepsis (Sewnath et al., 2001; van der Poll et al., 1995; Walley et al., 1996; Wang et al., 2001). However, IL-5 knockout animals have increased mortality from sepsis despite elevated levels of IL-10. This may be due to elevated IL-6 production, which has been associated with increased mortality and an overall imbalance in the inflammatory response in sepsis. Maintaining this balance is important for survival and homeostasis during sepsis (Remick et al., 2005; Walley et al., 1996). It is curious that IL-12 levels were not affected in IL-5 knockout mice, since IL-12 is produced along with IL-6 following bacterial or viral recognition by macrophages, and studies indicate that IL-12 is necessary in sepsis to augment phagocytosis and microbicidal activity by neutrophils (Langrish et al., 2004; Moreno et al., 2006; van Till et al., 2007). Moreover, decreased bacterial clearance in sepsis is associated with decreased IL-12 (Murphey et al., 2004). Contrary to these studies, despite no change in IL-12 levels compared to wild type mice, there was impaired bacterial clearance in IL-5 knockout mice. This might suggest that bacterial clearance and killing has less to do with levels of IL-12 specifically and more to do with an overall imbalance and profound immunosuppressive state induced during sepsis. Nevertheless, loss of IL-5 is harmful to the host during sepsis by decreasing bacterial clearance and survival, and increasing antiinflammatory cytokine production and vascular permeability.

It is important to consider that genetic deletion of IL-5 may have unanticipated consequences on cell proliferation, as overexpression does. This limitation can be diminished through the use of small-interfering RNA knockdown of IL-5 before and during sepsis. This would eliminate the possibility that there are confounding effects of genetic deletion of IL-5. Moreover, administration of exogenous IL-5 to IL-5 knockout mice would confirm if this were indeed the case.

The loss of IL-5 increased mortality in CLP sepsis. To test the feasibility of IL-5 administration as a means to improve survival in sepsis, IL-5 was first administered prophylactically as proof of the protective effects of this cytokine in sepsis. Using a highly lethal model of CLP, administration of recombinant IL-5 to mice 4 hours prior to surgery significantly improved survival, confirming the protective role of IL-5 in sepsis. In a separate group, IL-5 was administered 1 hour after CLP surgery to test the ability to treat with IL-5 therapeutically. IL-5 administration after CLP resulted in a trend toward improved survival in mice. However, the rapid onset of disease (death starting around 18 hours post-CLP) made it very difficult to delay the time to treatment. This was accomplished using a smaller needle to perforate the cecum, thereby providing a model of CLP with reduced mortality and delayed onset of disease. Using this model of CLP, therapeutic administration of IL-5 4 hours following surgery significantly improved survival in mice, confirming the protective role of IL-5 in sepsis. While these results provide strong evidence for the therapeutic benefit of IL-5, they do not specifically address the cells mediating these protective effects and will need to be determined before use in septic patients. Furthermore, it

will be necessary to determine the effects of IL-5 on the cytokine milieu in sepsis, as this is an important mediator of survival.

While it was apparent that IL-5 had eosinophil-independent effects during CLP, it remained a possibility that exogenous IL-5 treatment was recruiting eosinophils to the peritoneal cavity, thereby improving survival through eosinophil-mediated functions. IL-5 administration to wild type mice did not affect eosinophil recruitment into the peritoneal cavity, compared to PBS treated control mice. Surprisingly, IL-5 induced neutrophil recruitment following administration, as evidenced by increased release of myeloperoxidase into the peritoneal cavity. These data suggest that one possible reason for the protective effects of IL-5 in sepsis is through enhanced recruitment of neutrophils to the site of infection. It supports earlier data showing expansion of neutrophils in IL-5 overexpressing NJ.1638/PHIL mice. It is curious that the effects of IL-5 on neutrophils have not been previously appreciated. However, this data is contrary to previous knowledge regarding eosinophils and IL-5 and was probably an oversight. There are a few studies indicating that IL-5 is a chemotactic factor for neutrophils (Hakansson and Venge, 1994; Lilly et al., 1996), but it is possible that the connection between IL-5 and neutrophils is unique to sepsis or other critical illnesses. This question remains unanswered.

Finally, it was necessary to determine if the role of IL-5 in sepsis was a species-specific phenomenon only observed in mice. One study demonstrated that increased IL-5 levels were associated with improved outcomes in septic patients (Bozza et al.,

2007). Data presented here confirmed the previous study, and demonstrated that septic patients at OHSU had increased IL-5 compared to healthy volunteers. Moreover, elevated levels of IL-5 were associated with improved outcomes in sepsis. These data give credence to the hypothesis that IL-5 administration remains a viable therapeutic option for patients, and needs to be further explored.

While IL-5 seems to be protective in sepsis, it is well known that excess IL-5 found in asthma and hypereosinophilic diseases can be detrimental to the host by causing excessive eosinophil recruitment and activation, and hence destruction of healthy tissue (Adamko et al., 1999; Foster et al., 1996; Kita et al., 1992; Kopf et al., 1996; Kouro and Takatsu, 2009; Takatsu and Nakajima, 2008). Data presented in this Chapter are contradictory to these studies. However, they can be reconciled through the observed loss of eosinophils in sepsis (Abidi et al., 2008; Bass et al., 1980; Shaaban et al., 2010). The loss of eosinophils in sepsis remains until patients recover. Expression of IL-5 in this state is unlikely to induce the excessive tissue damage caused by these cells, and may actually provide a way to boost the already depressed immune system.

In summary, these data provide evidence *in vivo* for the protective effects of IL-5. In particular, these effects were eosinophil-independent and implicated a role for neutrophils and macrophages. While these data suggest the benefit of IL-5 and IL-5 administration in sepsis, they do not provide a definitive answer for the role of neutrophils and macrophages in mediating this protection. This will be examined in greater detail in Chapters 5 and 6.

Data from this Chapter are published as:

Linch, S. N., Kelly, A. M., Danielson, E. T., Tamakawa, R. and J. A. Gold. The IL-5 receptor is expressed on neutrophils and macrophages and treatment with IL-5 improves survival in polymicrobial sepsis (manuscript in preparation).

CHAPTER 5:

THE IL-5 RECEPTOR IS EXPRESSED ON NOVEL CELL POPULATIONS IN SEPSIS AND IS INDUCIBLE *IN VITRO* USING TOLL-RECEPTOR LIGANDS

Abstract

Sepsis results from dysregulation of the host immune response. Clinical trials aimed at dampening the proinflammatory response have failed. The discovery of immunosuppression in sepsis led to focus on enhancing the inflammatory response as a therapeutic modality. Elevated levels of IL-5 in sepsis survivors and improved survival in septic mice overexpressing IL-5 suggest a novel and protective role for IL-5 in sepsis. Data presented in Chapter 3 demonstrated that eosinophils kill bacteria and were protective *in vivo*. However, data from Chapter 4 indicated the protective role for IL-5 in sepsis was in fact eosinophil-independent. In this Chapter, data demonstrate novel expression of the IL-5Rα on unexpected leukocytes. In particular, neutrophils and monocytes/macrophages expressed the IL-5Rα both in mice and in humans with sepsis. In addition, expression of this receptor was specific to sepsis, i.e. not present in other critically ill patients, and expression of this receptor waned as patient morbidity improved. Interestingly, soluble IL-5Rα was elevated in septic shock, suggesting that this receptor may serve as a cytokine sink to limit IL-5 activity. Finally, IL-5Rα expression was induced *in vitro* by stimulation with bacteria and bacterial-derived products, including LPS and CpG, as well as a mild sterile inflammatory stimulus, thioglycollate. Receptor expression was induced in an NF-κB-dependent manner, as inhibition using a vaccinia-derived peptide prevented induction of the IL-5Rα. Collectively, these data provide novel evidence for expression of the IL-5Rα on non-eosinophilic leukocytes. These data combined with the absence of eosinophils in sepsis suggest that the protective effects of IL-5 are mediated by neutrophils and macrophages.

Introduction

Sepsis is defined as the systemic inflammatory response to infection. The immune response during sepsis consists of an initial proinflammatory response followed by a compensatory immunosuppressive phase. Efforts aimed at reducing the immunosuppressive phase or augmenting innate immune function represent viable therapeutic strategies in sepsis.

Data in Chapter 4 demonstrated that IL-5 was protective in sepsis. In particular, loss of IL-5 resulted in decreased bacterial clearance, increased tissue damage, mortality, and antiinflammatory cytokine production. Therapeutic administration of IL-5 improves mortality in polymicrobial sepsis, providing evidence for the use of IL-5 treatment in septic patients. Even in the absence of eosinophils, IL-5 mediated improved survival in sepsis, suggesting effects on other cell populations. Neutrophils and monocytes/macrophages were implicated for these effects, as IL-5 overexpression in the absence of eosinophils caused an increase in the absolute numbers of these myeloid cells. Moreover, administration of IL-5 into the peritoneal cavity of wild type mice resulted in enhanced neutrophil recruitment while having no effect on eosinophil recruitment. These data collectively suggest an important role for neutrophils and monocytes/macrophages in mediating the protective effects of IL-5 in sepsis.

Traditionally, eosinophils and B cells are the only immune cells known to express the IL-5Rα, which is the unique ligand-binding portion of this receptor. However,

receptor expression has not been examined in humans with sepsis or bacterial infection. There is some evidence that other cell types respond to this cytokine, including airway smooth muscle cells, neutrophils, microglial cells and macrophages (Bober et al., 1995; Dewachi et al., 2006; Liva and de Vellis, 2001; Ringheim, 1995; Saeftel et al., 2003; Wen et al., 2003). Interestingly, only one of these studies examined IL-5R expression. In particular, they found that neutrophils from horses with heaves express the IL-5Rα (Dewachi et al., 2006). This is the only documented study demonstrating IL-5Rα expression on neutrophils; there are no reports documenting this receptor on monocytes. Furthermore, expression of the promoter region for IL-5Rα is eosinophil *and* myeloid cell-specific (Sun et al., 1995). These data suggest that non-eosinophilic leukocytes like neutrophils and macrophages are a potential target of IL-5 in sepsis.

Finally, evidence for IL-5Rα expression on neutrophils and monocytes in humans is evidenced by a single study using anti-IL-5Rα treatment. The use of this therapy in asthma patients resulted in depletion of eosinophils as expected; what was not expected was the concomitant reduction in neutrophils and monocytes, albeit to a lesser extent (Busse et al., 2010). These data provide *indirect* evidence for expression of the IL-5Rα on these leukocytes. However, direct evidence is necessary to confirm that the receptor is present, as anti-IL-5Rα treatment may simply have off-target effects. What remains puzzling is the reason that IL-5Rα expression on neutrophils and monocytes/macrophages has never been observed previously. The circumstances and reasons for IL-5Rα expression on these myeloid populations also

remains unclear. This Chapter examines the hypothesis that the IL-5Rα is in fact present on neutrophils and macrophages in sepsis.

These data demonstrate that IL-5Rα is indeed expressed on neutrophils and monocytes/macrophages in sepsis. This expression is unique to sepsis, and was not found in other critically ill patients. Additionally, IL-5Rα is induced following stimulation with bacteria or bacterial products, including LPS or CpG. This expression was inhibited using an inhibitor of TRAF6, which signals through the NF-κB pathway, providing one possible mechanism for IL-5Rα expression *in vivo*. Collectively, these data indicate that other myeloid cells express the IL-5Rα in sepsis. Data from the previous Chapter combined with this data provide evidence that neutrophils and macrophages mediate the protective effects of IL-5 in sepsis.

Methods

Neutrophil and Macrophage/Monocytes isolation

See General Methods Section IVc and IVd

CLP model of polymicrobial sepsis

See General Methods Section IIa and IId

Soluble IL-5Rα detection by ELISA

See General Methods Section IVb

IL-5Rα analysis by flow cytometry and western blot

See General Methods Section IVa and IVc

Human selection criteria

See General Methods Section Ia and Ic

Results

IL-5Rα is expressed on CD14+ Neutrophils and CD16+ Monocytes from septic patients and is unique to sepsis

Data from Chapter 4 indicate the presence of elevated IL-5 levels in survivors of sepsis suggesting a protective role for this cytokine. Moreover, the reduction of neutrophils and macrophages in anti-IL-5Rα treated humans with asthma suggests the presence of this receptor on these two populations (Busse et al., 2010). However, this has not been definitively established. To determine if neutrophils and monocytes express the IL-5Rα in human sepsis, surface IL-5Rα was assessed using flow cytometry on blood leukocytes from septic patients in the ICU. Interestingly, human $CD14^{+}SSC^{hi}$ neutrophils (Figure 1a) and $CD16^{+}$ SSC^{lo} monocytes (Figure 1b) from septic patients expressed IL-5Rα. In addition, expression of this receptor waned over time on these monocytes in surviving patients, i.e. as patients recovered from sepsis, monocytes lost expression of IL-5Rα and consequently an ability to respond to IL-5 (Figure 5.2b). One possible explanation for the appearance of the IL-5Rα on these cells is that it occurs during general illness or infection. To assess this possibility, IL-5Rα expression was examined on leukocytes from both septic patients and other non-septic, critically ill patients in the ICU. Interestingly, expression of the IL-5Rα was unique to sepsis, as receptor expression was not observed on neutrophils or monocytes from non-septic ICU patients (Figure 5.2a).

It is known that soluble cytokine receptors often act as a cytokine sink, to bind excess cytokine and prevent further inflammation (Bazan, 1990; Nicola, 1994). Moreover, there is a soluble isoform of the IL-5Rα, which can be generated by alternative splicing or cleavage of the membrane bound receptor (Liu et al., 2002a; Liu et al., 2002b; Tavernier et al., 1991; Tavernier et al., 1992). Circulating levels of the soluble receptor were assessed in septic patients by ELISA. Indeed, the soluble IL-5R was found in septic patients, and was elevated in patients with septic shock compared to those without shock (Figure 5.3).

IL-5Rα is expressed on Neutrophils and Monocytes from septic mice

It has not been demonstrated previously that mouse neutrophils or monocytes/macrophages express the IL-5Rα. However, there is some evidence that these myeloid cells express IL-5Rα in certain disease states (i.e. horses with heaves) and can respond to IL-5 via chemotaxis (Dewachi et al., 2006; Hakansson and Venge, 1994). The presence of the IL-5Rα on human neutrophils and monocytes during sepsis provides evidence to suggest the presence of this receptor on mouse neutrophils and monocytes/macrophages.

To determine whether IL-5Rα was present on these two cell types in CLP-induced sepsis, neutrophils were isolated from both the blood and the BAL of septic mice and analyzed by flow cytometry. Indeed, $Ly6g^{+}$ neutrophils from wild type mice expressed IL-5Rα during polymicrobial sepsis, in the blood (Figure 5.4b) and the

lung (Figure 5.4c). This was also true for $CD11b^{+}Ly6g^{-}$ blood monocytes (Figure 5.5a) and $F4/80^{+}$ macrophages from the BAL fluid (Figure 5.5b) and spleen (Figure 5.5c) of septic mice. These data indicate that the IL-5Rα is in fact present on non-eosinophilic myeloid cells in mice, and further suggest a relationship among IL-5, neutrophils, and monocytes/macrophages in sepsis.

IL-5Rα is inducible *in vivo* using thioglycollate stimulation as well as *in vitro* using TLR ligands or Interferon-α

Since IL-5Rα is not expressed on circulating neutrophils or macrophages at baseline in healthy animals and humans, the next step was to determine if receptor expression could be induced on these cells in order to provide a model for this system *in vitro* and allow functional studies of the IL-5Rα on these cells. Indeed, this receptor was induced on peritoneal macrophages following administration of a thioglycollate solution, which is a mild but sterile inflammatory stimulus (Figure 5.6). In addition, IL-5Rα was present on bone marrow neutrophils isolated from healthy mice (Figure 5.7).

To determine whether this receptor was induced *in vitro*, RAW264.7 mouse macrophages were stimulated with LPS and receptor expression was assessed using flow cytometry. At 24 and 48 hours following LPS stimulation, there was potent upregulation of IL-5Rα on macrophages (Figure 5.8). Upregulation of the receptor was also observed on RAW264.7 macrophages following CpG stimulation (Figure

5.9), as well as Interferon-α stimulation of thioglycollate-elicited macrophages (Figure 5.6), though this was to a lesser extent than LPS stimulation. To test the mechanism for IL-5Rα upregulation, CpG stimulated macrophages were treated with a peptide derived from the Vaccinia virus protein A52R, known to inhibit signaling through TRAF6 and the NF-κB pathway (Harte et al., 2003; McCoy et al., 2005). NF-κB inhibition completely abolished IL-5Rα expression on RAW264.7 macrophages, suggesting IL-5Rα expression proceeds through an NF-κB-mediated manner (Figure 5.9). Taken together, these novel data indicate that the IL-5Rα is expressed in septic mice on neutrophils and macrophages and that expression of IL-5Rα observed in septic mice can be modeled *in vitro*. The ability to model this system *in vitro* will allow further assessment of the effects of IL-5 on neutrophils and macrophages in Chapter 6.

Finally, expression of this receptor *in vitro* was not a species-specific phenomenon as IL-5Rα expression was induced using human-derived THP-1 monocytes. THP-1 monocytes were differentiated in macrophages *in vitro* using phorbol myristate acetate (PMA). Stimulation of differentiated THP-1 macrophages *in vitro* with either LPS (Figure 5.10a) or CpG (data not shown) induced upregulation of IL-5Rα expression on the cell surface. Additionally, stimulation of PBMC from healthy volunteers with heat killed *E. coli* also induced IL-5Rα upregulation on the cell surface (Figure 5.10b). These data indicate that bacteria and bacterial products, such as LPS and CpG, induce IL-5Rα expression in macrophages. These data indicate that *in vitro* systems can be used to further study the role of IL-5Rα on non-

eosinophilic leukocytes in the innate immune response to sepsis and specifically assess changes in cellular activation or function following IL-5 stimulation.

Summary

The data presented in this Chapter indicate that the IL-5Rα was present on neutrophils and monocytes during sepsis, in both mice and humans. Furthermore, IL-5Rα expression was induced on these cells *in vitro*, through stimulation with bacteria or bacterial-derived products. This will allow the effects of IL-5 signaling in neutrophils and macrophages to be studied *in vitro*. Functional studies in Chapter 6 will help elucidate the role of IL-5 in sepsis, and the functional effects it has on neutrophils and macrophages.

Figure 5.1: IL-5Rα is expressed on Neutrophils and Monocytes from Septic patients

A. **B.**

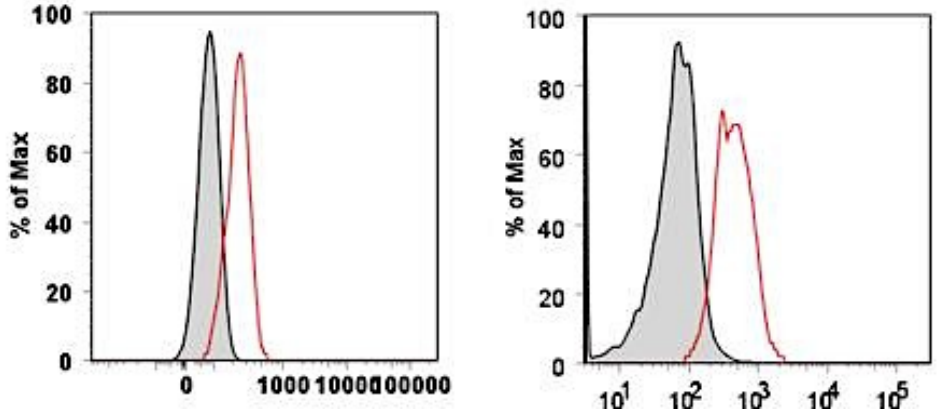


Figure 5.1: Blood was taken from septic patients within 24 hrs of admission to the ICU. Cells were analyzed for IL-5Rα expression by flow cytometry. Panel A represents $CD14^+$ neutrophils; Panel B represents $CD16^+$ monocytes. Black histogram represents unstained control; grey histogram represents isotype control; red histogram represents IL-5Rα expression. X-axis is IL-5Rα expression.

Figure 5.2: IL-5Rα expression on Monocytes is unique to Sepsis and decreases as patient morbidity improves

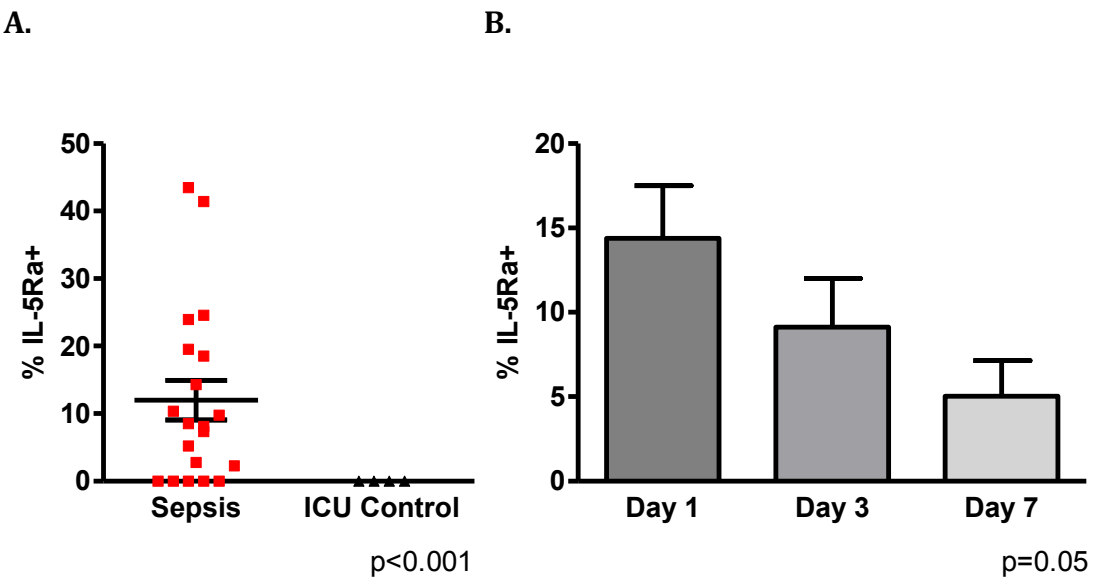


Figure 5.2: Blood was taken from septic patients within 24 hrs of admission to the ICU. Cells were analyzed for receptor expression by flow cytometry. The percentage of $CD16^+$ monocytes expressing IL-5Rα was graphed for each patient. Bars represent means +/- SEM. Data were analyzed by student's t-test (Panel A) or ANOVA (Panel B). Panel A shows IL-5Rα expression in septic patients compared to non-septic, critically ill controls (ICU controls). ***P<0.001. Panel B shows IL-5Rα expression on $CD16^+$ monocytes over time on septic patients. *P=0.05

Figure 5.3: Soluble IL-5Rα is found in Septic patients and is increased during Septic Shock

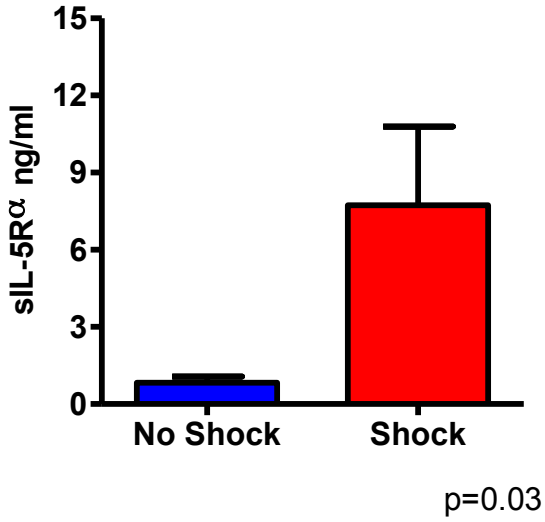
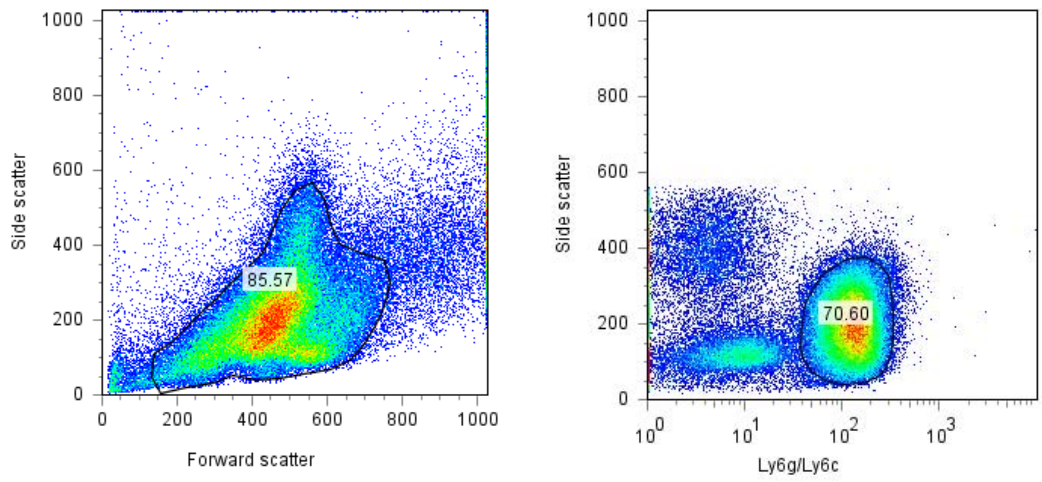


Figure 5.3: Blood samples were collected from septic patients within 24 hrs of admission to the ICU. Soluble IL-5Rα (sIL-5Rα) was assessed by ELISA. Shock was defined as the need for vasopressors. Bars represent means +/- SEM. Data were analyzed by student's t-test. **P=0.03

Figure 5.4: IL-5Rα is expressed on Neutrophils from Septic mice

A.



B. Serum

C. Lung

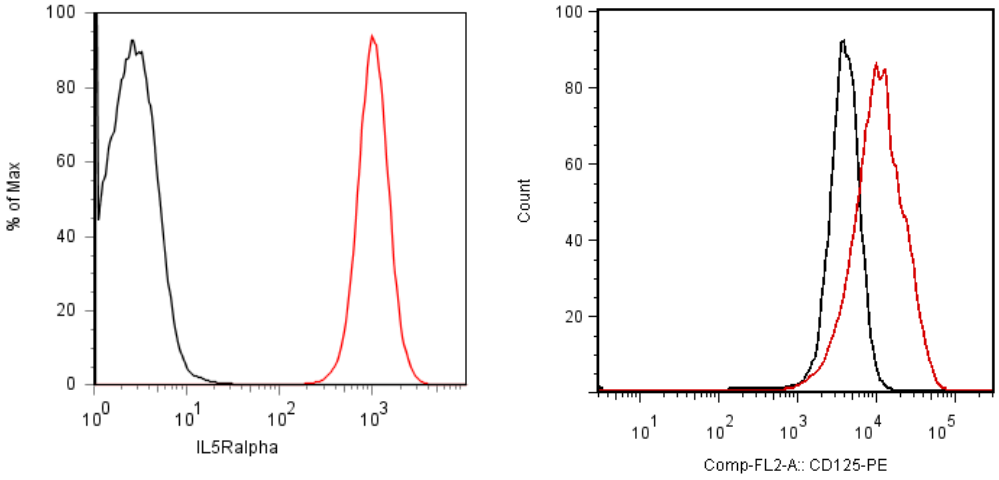
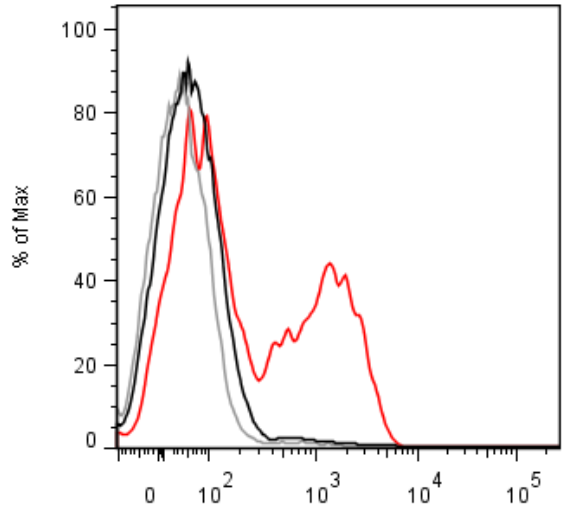


Figure 5.4: IL-5Rα is expressed on Neutrophils from Septic mice

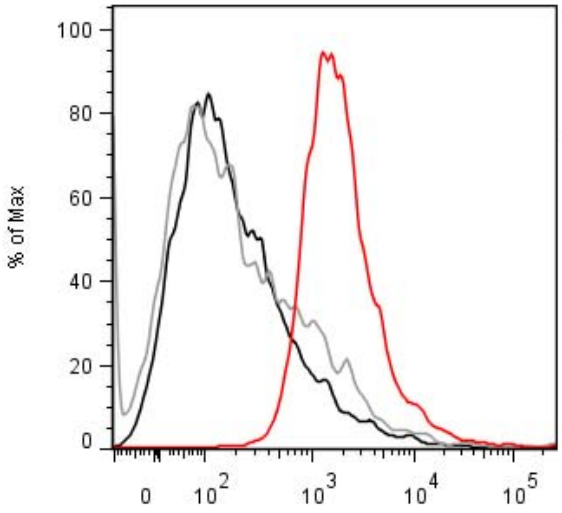
Sepsis was induced via CLP in wild type mice. Eighteen hrs later, animals were sacrificed. Blood and BAL fluid were analyzed by flow cytometry for IL-5Rα expression. Representative plots show total cells (Panel A left) and total cells gated on side scatter and Ly6g expression (Panel A right), indicative of neutrophils. Bottom panels represent IL-5Rα expression from the $Ly6g^{+}$ population in either the blood (Panel B) or the BAL fluid (Panel C). Black histogram represents Isotype control; Red histogram is IL-5Rα.

Figure 5.5: IL-5Rα is expressed on Monocytes/Macrophages from Septic mice

A. Blood Monocytes



B. BAL Macrophages



C. Splenic Macrophages

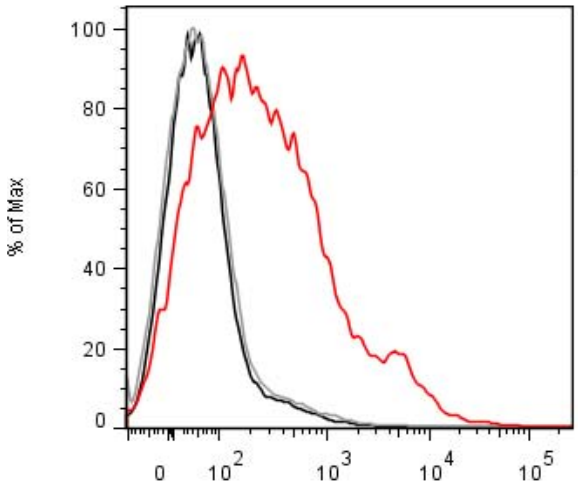


Figure 5.5: IL-5Rα is expressed on Monocytes/Macrophages from Septic mice

Sepsis was induced via CLP in wild type mice. Eighteen hrs later, animals were sacrificed. Blood, spleen homogenates and BAL fluid were analyzed by flow cytometry for IL-5Rα expression. Representative plots showing blood monocytes ($Ly6g^{-}CD11b^{+}$) (panel A), BAL macrophages ($F4/80^{+}$), and splenic macrophages ($F4/80^{+}$) expression of IL-5Rα. Black histogram represents unstained control; grey histogram represents Isotype control; Red histogram is IL-5Rα. X-axis is IL-5Rα expression.

Figure 5.6: IL-5Rα is expressed on Thioglycollate-elicited Macrophages

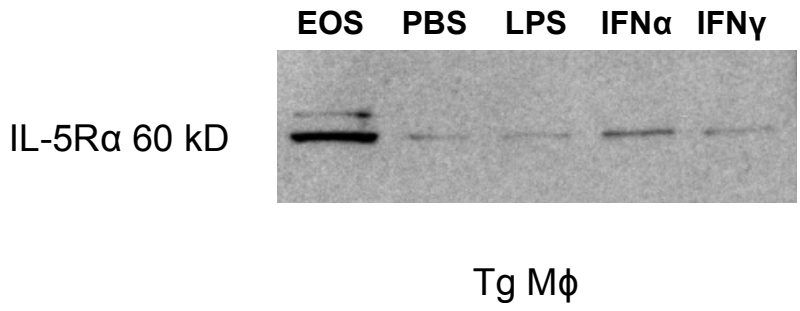
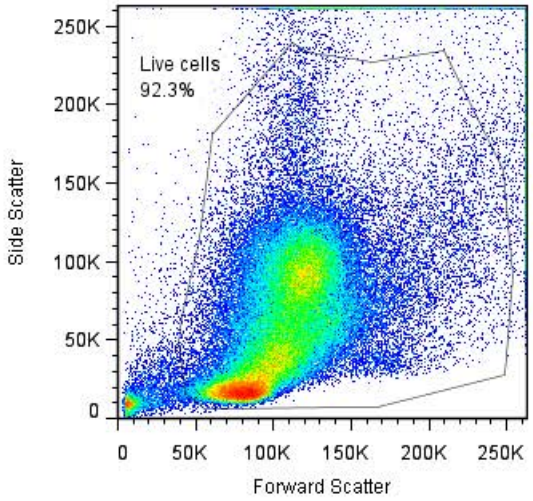


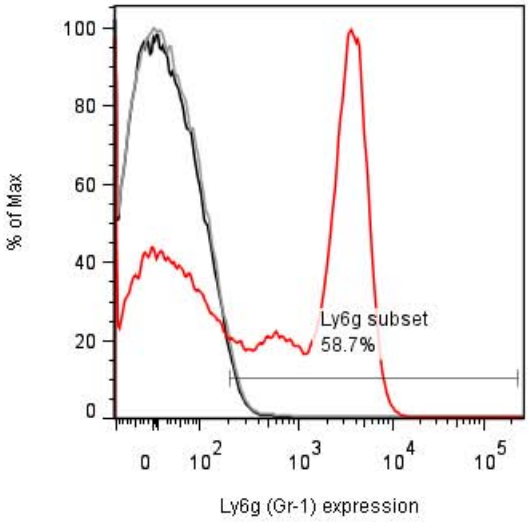
Figure 5.6: Thioglycollate-elicited macrophages were stimulated *in vitro* for 24 hrs with PBS control, LPS, IFNα, or IFNγ. Western blot for IL-5Rα was performed on whole cell extracts. EOS is a positive control for IL-5Rα expression.

Figure 5.7: IL-5Rα is expressed on Bone-Marrow Neutrophils

A.



B.



C.

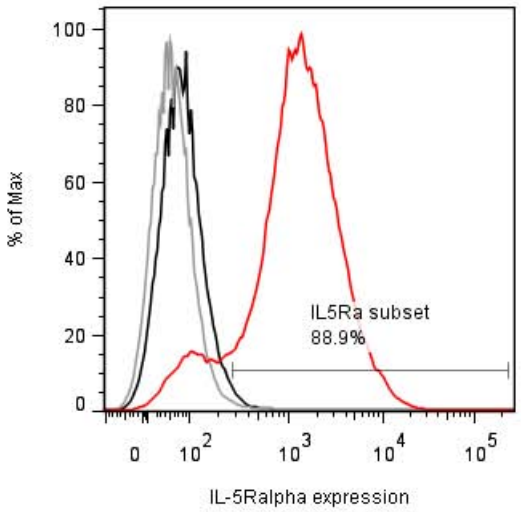
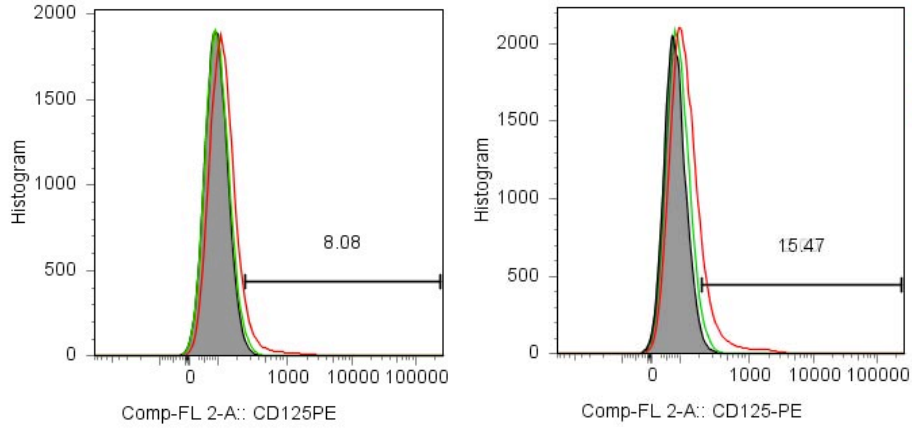


Figure 5.7: IL-5Rα is expressed on Bone-Marrow Neutrophils

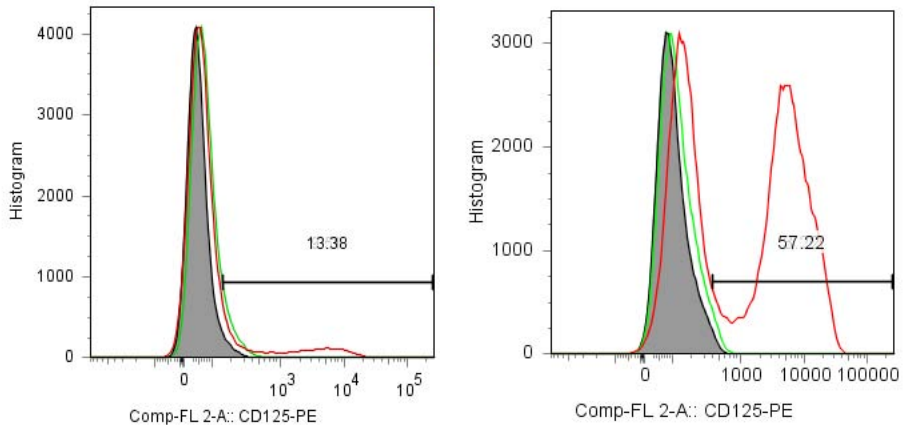
Bone marrow neutrophils were isolated by density gradient centrifugation. Flow cytometry was used to analyze neutrophils for IL-5Rα expression. Total cells (Panel A) were gated stained for the neutrophil marker Ly6g (Panel B). Black histogram is unstained control; grey histogram is isotype control; red histogram represents Ly6g expression. IL-5Rα expression on the $Ly6g^{+}$ subset was assessed (Panel C). Black histogram is unstained control; grey histogram is isotype control; red histogram represents IL-5Rα expression. X-axis is IL-5Rα expression.

Figure 5.8: IL-5Rα expression is induced by LPS stimulation on Macrophages

A. **PBS** **LPS**



B.



C.

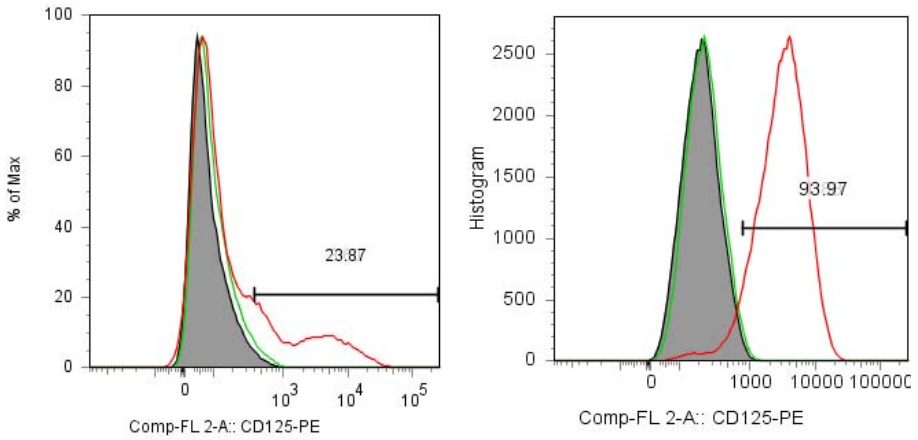


Figure 5.8: IL-5Rα expression is induced by LPS stimulation on Macrophages

RAW264.7 macrophages were stimulated *in vitro* with 10 ng/ml LPS or PBS control and flow cytometry was used to examine surface IL-5Rα expression at several time points following stimulation—3 hrs (Panel A), 24 hrs (Panel B) and 48 hrs (Panel C). Black histogram represents unstained control; shaded histogram is isotype control; red histogram is IL-5Rα expression; green histogram is a negative control antibody not expressed on macrophages. X-axis is IL-5Rα expression.

Figure 5.9: IL-5Rα expression is induced by CpG stimulation on Macrophages and inhibited by an NF-κB inhibitor

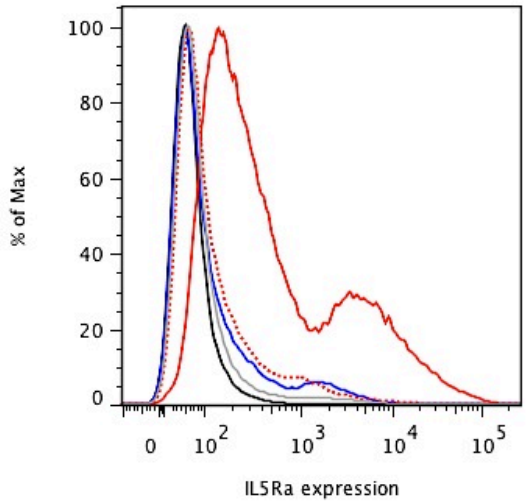


Figure 5.9: RAW264.7 macrophages were stimulated *in vitro* with 1μg/ml CpG or PBS control and flow cytometry was used to examine surface IL-5Rα expression 24 hrs later. Black histogram represents unstained control; grey histogram is isotype control; blue histogram is PBS treated control; red histogram is CpG stimulation; dotted red histogram is cells treated with CpG and an NF-κB inhibitor (Vaccinia virus-derived peptide P13). X-axis is IL-5Rα expression.

Figure 5.10: Stimulation with LPS or heat-killed *E. coli* induces IL-5Rα expression on human Monocytes/Macrophages

A. THP1 Monocytes **B. PBMCs**

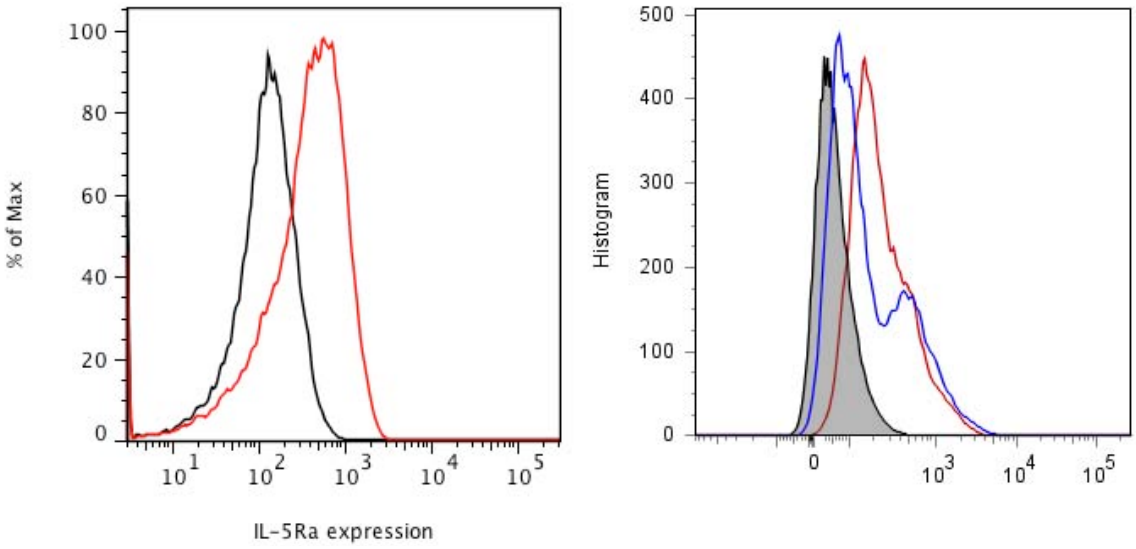


Figure 5.10: Panel A: THP1 monocytes were differentiated *in vitro* using PMA, followed by stimulation with 10 ng/ml LPS for 24 hrs. Panel B: Human PBMCs were isolated and stimulated with heat-killed *E. coli* for 24 hours. Cells were analyzed for receptor expression by flow cytometry. Panel A: Black histogram represents unstained control; Red histogram represents IL-5Rα expression. Panel B: Black histogram represents Isotype control; grey histogram represents isotype control; Blue histogram represents PBS control treated PBMCs; Red histogram represents *E. coli*-stimulated PBMCs. X-axis is IL-5Rα expression.

Discussion

IL-5 is traditionally associated with Th2-related diseases including asthma, allergic diseases, and helminth infection. This is because of its effects on eosinophils and B cells by inducing recruitment and chemotaxis, antibody production, survival, and degranulation. However, there is some evidence to suggest IL-5 affects the function of other leukocytes and cells (Al-Qaoud et al., 2000; Awatsuji et al., 1993; Hakansson and Venge, 1994; Liva and de Vellis, 2001; Ringheim, 1995; Saeftel et al., 2003). IL-5 signaling in these cells would require expression of the IL-5Rα, which has not been found previously. Interestingly, in a human study of anti-IL-5Rα treatment in asthma, there was a notable decrease in both neutrophils and monocytes. Taken together these studies suggest the presence of the IL-5Rα on these myeloid populations.

Here, IL-5Rα expression was documented for the first time on neutrophils and macrophages from septic humans. This receptor was expressed on $CD14^+$ neutrophils and $CD16^+$ monocytes from the blood of septic patients. This novel finding suggests an alternative role for IL-5 given the absence of eosinophils and apoptosis of B cells in sepsis (Abidi et al., 2008; Bass, 1975; Bass et al., 1980; Hotchkiss and Nicholson, 2006). Expression of the IL-5Rα on $CD16^+$ resident monocytes may be important for inflammatory cytokine production, as these cells are the major source of TNFα and IL-1 during infection. During immunosuppression, stimulation of the IL-5Rα on these monocytes may promote release of these cytokines and contribute to the resolution of infection. This hypothesis needs to be

tested by stimulating monocytes from septic patients *in vitro* with IL-5 and measuring inflammatory cytokine production. This may be one possible mechanism for the protective effects of IL-5 observed in sepsis.

It is known that many cytokines have both soluble and membrane bound receptors. While membrane bound receptors induce intracellular signaling cascades, soluble receptors frequently act as either: a cytokine sink to absorb excess amounts of a given cytokine in the circulation; or as cytokine amplifiers by binding, stabilizing cytokines, and prolonging their half-life (Levine, 2004). IL-5Rα is known to have several soluble isoforms, which are generated by cleavage of membrane-bound receptors on the cell surface in addition to alternative splicing of mRNA (Liu et al., 2002a; Tavernier et al., 1992). Data here demonstrate that soluble IL-5Rα levels were increased in patients with septic shock compared to those without shock, suggesting that expression of the soluble form of the receptor is detrimental in sepsis. Previous data indicate that IL-5 is protective in sepsis; therefore, these data suggest that the soluble IL-5Rα is acting as a cytokine sink to absorb excess IL-5 in the blood and prevent further signaling. These data are in accordance with another study indicating that soluble IL-5Rα limits inflammatory effects of IL-5 in the context of asthma and eosinophils (Liu et al., 2002b). However, in the context of sepsis and immunosuppression, limiting the inflammatory effects of IL-5 through soluble IL-5Rα seems to be detrimental to the host immune response.

Since expression of the IL-5Rα has not been observed previously on neutrophils or macrophages, one potential explanation for these observations is that the IL-5Rα is

present on these leukocytes during generalized critical illness and not specific to sepsis. However, data presented here indicate IL-5Rα expression was unique to sepsis, and not found on monocytes from non-septic patients. Furthermore, as patients recovered, expression of the IL-5Rα on $CD16^+$ monocytes waned again suggesting the specificity of IL-5 and IL-5Rα expression during sepsis. IL-5Rα expression may be a protective mechanism for the host in order to maintain the immune response to IL-5 in the absence of eosinophils. An alternative explanation is that IL-5Rα expression on these populations is a consequence from the rapid mobilization of bone marrow leukocytes to assist in combatting infection. This is supported by IL-5Rα expression on bone marrow neutrophils. An important consideration for these data is that IL-5Rα expression on these cells does not imply functionality. This question needs to be tested to determine the functional consequences of IL-5 stimulation in neutrophils and macrophages.

In addition to expression of the IL-5Rα on neutrophils and monocytes in humans, data in this Chapter demonstrated this was not a species-specific phenomenon as mouse neutrophils and monocytes/macrophages also expressed this receptor. IL-5Rα was present on $Ly6g^+$ neutrophils in both the blood and in the lungs. Moreover, IL-5Rα was expressed on $Ly6g^-CD11b^+$ monocytes in the blood, and $F4/80^+$ macrophages in the spleen and in the lung. This receptor was not expressed on neutrophils or monocytes/macrophages in wild type mice, which is likely the reason why this has not previously been observed. Since sepsis is not typically a Th2-associated disease, investigators have not assessed the role for this cytokine or its

receptor in sepsis. The presence of two distinct populations of blood monocytes, one which expresses the IL-5Rα in sepsis and one that does not (Figure 5.5a) is interesting when taking into consideration the presence of different populations of blood monocytes, i.e. inflammatory ($CCR2^+$) and resident monocytes ($CX3CR1^+$). These data do not make the distinction as to which population of monocytes specifically expresses the IL-5Rα in sepsis and should be tested by examining CCR2 and CX3CR1 on the surface of monocytes. Since these two populations have different functional profiles like human monocytes, this may be important in the context of sepsis.

The same is true of macrophage expression of IL-5Rα. There are several functional classifications for macrophages and it will be important to determine the phenotype of those expressing IL-5Rα. Wound healing macrophages are important for tissue repair; classically activated macrophages are important for production of inflammatory cytokines, like IL-1 and TNFα, and clearance of an infection; regulatory macrophages are important for production of antiinflammatory cytokines, like IL-10, and reducing inflammation. It seems unlikely that the population of IL-5Rα expressing macrophages is of the regulatory phenotype since elevated numbers of regulatory macrophages increases host susceptibility to disease (Mosser and Edwards, 2008). This is supported by the fact that IL-5 is protective to the host during sepsis, and that loss of IL-5 resulted in increased IL-10. It may be that IL-5 prevents accumulation of regulatory macrophages, and allows for a continued proinflammatory response. This is especially important during

sepsis given the overt immunosuppression, which occurs in patients. The issue of macrophage phenotype needs to be addressed in the future.

While IL-5Rα expression was observed *in vivo* in septic mice and humans, it was not expressed in healthy animals or non-septic patients. Additionally, sepsis is associated with apoptosis of leukocytes, which makes it difficult to isolate an adequate number of leukocytes that express the IL-5Rα. In order to assess functional changes on these cells following IL-5 stimulation, it was necessary to model IL-5Rα expression on macrophages/monocytes *in vitro*. To accomplish this, thioglycollate-elicited macrophages or RAW264.7 macrophages were stimulated *in vitro* with a number of different inflammatory mediators. LPS-stimulation of RAW264.7 macrophages potently induced IL-5Rα expression at 24 and 48 hours following stimulation as determined by flow cytometry. Moderate upregulation of IL-5Rα was observed 24 hours following CpG or IFNα stimulation providing yet another way to model receptor expression *in vitro*. Again, receptor expression was not species-specific as IL-5Rα was inducible on PMA-differentiated THP1 human macrophages following LPS stimulation. Likewise, IL-5Rα expression was induced on PBMCs stimulated with heat-kill *E. coli*. These data indicate that the receptor is induced on macrophages following stimulation with bacteria or bacteria-derived products. This provides one possible mechanism for receptor expression *in vivo*, though it does not provide definitive proof that this is indeed the cause.

In addition, these data demonstrated that the mechanism for IL-5Rα expression *in vitro* was in an NF-κB-dependent manner. This was determined by inducing

receptor expression on these cells using CpG and using a known TRAF6 inhibitor to examine changes in receptor expression. Expression of this receptor in an NF-κB-dependent manner is not unusual, as this has been described for other cytokine receptors (Siebenlist et al., 1994). One caveat is that IL-5Rα expression was measured at 24 hours following CpG stimulation, and NF-κB-mediated gene expression can occur in a shorter time scale, i.e. several hours. It remains possible that IL-5Rα expression is a secondary effect and NF-κB-mediated gene expression resulted in cytokine or chemokine expression, which in turn resulted in upregulation of this receptor. Doing a more in-depth time-course analysis for IL-5Rα expression following stimulation would assess this possibility. More studies are necessary to determine the precise mechanisms regulating IL-5Rα expression in neutrophils and monocytes/macrophages.

Expression of the IL-5Rα on neutrophils and monocytes during sepsis is especially important when considering the use of anti-IL-5Rα therapy as a means to deplete eosinophils. In a recent study, administration of anti-IL-5Rα treatment for asthma reduced the number of circulating monocytes and neutrophils in patients by 20% and 13%, respectively (Busse et al., 2010). This could have unappreciated consequences in patients and may predispose them to infection. Moreover, depletion of these populations of IL-5Rα expressing myeloid subsets would likely prevent the protective effects observed during sepsis. This population of IL-5Rα expressing myeloid cells is likely found in asthmatic patients; data indicated in this Chapter demonstrate that this population is also present during sepsis. Taken

together, data suggest that this population may be present in other diseases, such as autoimmunity or cancer. Precaution must be taken when administering these therapies to patients as they may have unanticipated consequences like predisposing them to sepsis by reducing neutrophils and monocyte populations or even increasing mortality.

Collectively, these data indicate that IL-5Rα is expressed on atypical, non-eosinophilic leukocytes during sepsis. In the previous Chapter, data indicated that IL-5 was protective in sepsis, and elevated levels are associated with improved outcomes in humans. More importantly, IL-5 treatment was used as a rescue therapy in mice and improved survival. Collectively, these data suggest that the protective role for IL-5 in sepsis was through its effects on neutrophils and macrophages. The primary functions of these innate effector cells is: to alert the host of infection through pathogen recognition; to induce chemokine and cytokine secretion; to participate in pathogen clearance through killing and phagocytosis; and to participate in wound healing. In the next Chapter, the effect of IL-5 on neutrophils and macrophages will be assessed through examination of several primary functions of these myeloid cells.

Data from this Chapter are published as:

Linch, S. N., Kelly, A. M., Danielson, E. T., Tamakawa, R. and J. A. Gold. The IL-5 receptor is expressed on neutrophils and macrophages and treatment with IL-5 improves survival in polymicrobial sepsis (manuscript in preparation).

CHAPTER 6:

IL-5 AUGMENTS MACROPHAGE ACTIVATION, SURVIVAL, PHAGOCYTOSIS, AND BACTERIAL KILLING, WITH NO EFFECT ON NEUTROPHIL BACTERIAL KILLING OR SURVIVAL

Abstract

Sepsis is the leading cause of death in the ICU. Despite numerous advances in knowledge of this disease, clinical trials have failed. Studies documenting elevated levels of IL-5 in sepsis survivors and improved survival in septic mice overexpressing IL-5 suggest a novel and protective role for IL-5 in this disease. Previous data indicate the protective role for IL-5 in sepsis is eosinophil-independent. Moreover, the IL-5Rα is atypically expressed on neutrophils and macrophages in sepsis and receptor expression is inducible. However, the precise function of this receptor on these populations is unknown. In this Chapter, data indicate that IL-5 augments macrophage function, while having minimal impact on neutrophils. Thioglycollate-elicited macrophages stimulated with IL-5 *in vitro* have increased STAT1 nuclear translocation and produce IL-6 and IL-12 in a dose-dependent manner, indicating the receptor is functional on these leukocytes. IL-5 stimulation results in release of intracellular calcium in both myeloid populations. In neutrophils, IL-5 has no effect on bacterial killing or spontaneous cell death. However, in macrophages IL-5 stimulation enhanced phagocytosis and bacterial killing. Moreover, IL-5 prolonged macrophage survival *in vitro*. Finally, *in vivo* evidence indicates that while IL-5 treatment improves survival in septic mice, these protective effects are abolished in macrophage-depleted animals. Taken together, these data indicate that IL-5 mediates protection and improved survival in sepsis through enhancement of macrophage function. Further, these data indicate that IL-5 may represent a viable immunostimulatory in sepsis and depletion may have unwanted side effects on macrophage function.

Introduction

The immune response during sepsis consists of an early proinflammatory response followed by a compensatory immunosuppressive phase. Immunosuppression in sepsis is highlighted by reduced neutrophil-mediated bacterial killing; reduced HLA-DR expression, antigen presentation, and proinflammatory cytokine production by monocytes; and increased apoptosis of leukocytes (Ayala and Chaudry, 1996; Ayala et al., 1996; Guo et al., 2006; Hotchkiss and Nicholson, 2006; Monneret et al., 2004; Pachot et al., 2006; Wang and Deng, 2008; Wolk et al., 2000). Clinical studies have demonstrated a benefit for immunostimulation in patients with overt immune dysfunction (Austin et al., 1995; Levin et al., 2000; Levy and Elsbach, 2001; Meisel et al., 2009; Orozco et al., 2006; Presneill et al., 2002). Data presented in Chapter 4 indicate that exogenous administration of IL-5 was protective in sepsis. Furthermore, these data indicated that the beneficial role for IL-5 was eosinophil-independent, exhibited by IL-5Rα expression on neutrophils and monocytes/ macrophages in sepsis. This receptor was inducible *in vitro* in both mice and human myeloid cells. However, it is not known how IL-5 affects these cells, nor is it known whether these cells are required for the protective effects of IL-5 *in vivo*.

Historically, the functional effects of IL-5 signaling were determined in eosinophils and B cells, as these cells express a functional IL-5Rα. In eosinophils, IL-5 induces signaling through the JAK/STAT pathway, and JAK2 and STAT1 are required for signaling, though STAT3 and STAT5 have also been implicated in eosinophils and B cells (Adachi and Alam, 1998; Martinez-Moczygemba et al., 2007; Takatsu and

Nakajima, 2008; van der Bruggen et al., 1995). In eosinophils, IL-5 inhibits apoptosis (Huang et al., 2000; Ochiai et al., 1997; Stern et al., 1992; Yousefi et al., 1996), upregulates adhesion molecule and MHC class II expression (Guida et al., 1994; Walsh et al., 1990), induces chemotaxis and degranulation (Hakansson and Venge, 1994; Kita et al., 1992; Wang et al., 1989), stimulates cytokine production (Nakajima et al., 1996), and induces growth and differentiation (Clutterbuck et al., 1987; Clutterbuck et al., 1989). In B cells, IL-5 induces production of IgM and IgA, and growth and differentiation of B-1 cells (Hiroi et al., 1999; Huston et al., 1996; Kopf et al., 1996; Moon et al., 2004).

However, the role of IL-5 on neutrophil and monocyte/macrophage function is more limited as it was not previously known that these cells can express IL-5Rα. One study determined that mouse RAW264.7 macrophages proliferate in response to IL-5 (Ringheim, 1995). Another study demonstrated that human neutrophils exhibit chemotaxis to IL-5, though it required much higher doses than required for eosinophil chemotactic responses (Hakansson and Venge, 1994). These data indicate the potential for neutrophils and macrophages to respond to IL-5.

The primary functions of neutrophils during infection are to: localize to sites of infection; recruit and alert additional leukocytes; and kill any microorganisms detected. Likewise, the same is true for monocytes/macrophages with the addition of cytokine secretion to activate the adaptive immune response, and janitorial service, i.e. to clean up dead cellular debris and initiate wound healing. During sepsis, there are defects in the typical response by these two populations. Any

therapy to stimulate these leukocytes or prevent their dysfunction would be of enormous benefit to septic patients. The hypothesis test in this Chapter is that IL-5 is protective in sepsis by augmenting neutrophil and monocyte/macrophage function, improving pathogen control, and promoting host defense.

Data in this Chapter demonstrate the specific functional effects of IL-5 signaling in neutrophils and macrophages. In particular, IL-5 induced calcium signaling in neutrophils, but did not affect bacterial killing or spontaneous cell death. In contrast, IL-5 induced calcium signaling in addition to augmenting phagocytosis, bacterial killing, and cytokine production by macrophages. Furthermore, IL-5 increased survival of macrophages *in vitro*. The importance of macrophages for the protective effects of IL-5 was observed *in vivo* using MaFIA mice depleted of macrophages. IL-5 administration to macrophage-depleted mice no longer rescued mice from the lethality of sepsis, demonstrating that macrophages were necessary for IL-5-mediated protection in sepsis. Collectively, these data demonstrate a novel role for IL-5 in sepsis through augmenting functional effects of macrophages as a means to improve host control of infection.

Methods

Neutrophil and macrophage isolation

See General Methods Section IIc, IIIa, and IIIc-e

Western blot for IL-5Ra and STAT-1

See General Methods Section IVa

Intracellular calcium release using fluorescence microscopy

See General Methods Section IVd

Bacterial killing, Phagocytosis and cytokine analysis

See General Methods Section IIIf, IVb, IVe, and IVf

Cell survival assessed by flow cytometry

See General Methods Section IVg

MaFIA mice and macrophage depletion

See General Methods Section Ib

CLP model of polymicrobial sepsis

See General Methods Section IIa

Results

IL-5Rα induces STAT-1 nuclear translocation and cytokine release by macrophages

In order to demonstrate that the receptor was functional on these cells, it was necessary to demonstrate activation of downstream signaling events. Nuclear translocation of STAT-1 in thioglycollate-elicited macrophages was assessed by western blot, as it is downstream of IL-5 signaling in eosinophils (Pazdrak et al., 1995). IL-5 treatment of macrophages caused increased STAT-1 nuclear translocation compared to vehicle-treated controls (Figure 6.1), indicating functionality of this receptor. Moreover, macrophages secreted IL-6 and IL-12 in a dose-dependent manner following IL-5 stimulation (Figure 6.2). These data establish the IL-5Rα is functional on macrophages, through the induction of downstream signaling events and cytokine production.

IL-5 enhances macrophage phagocytosis and bacterial killing with no effect on neutrophil killing

IL-5 causes recruitment, differentiation, maturation and enhance survival of eosinophils (Clutterbuck et al., 1989; Hakansson and Venge, 1994; Lopez et al., 1988; Rothenberg and Hogan, 2006; Yamaguchi et al., 1988). Using *in vitro* techniques, the specific effects of IL-5 on neutrophils and macrophages were

examined. First, changes in intracellular calcium occur during phagocytosis, as well as chemotaxis and bacterial killing, by both neutrophils and macrophages (Hallett et al., 1999; Marks and Maxfield, 1990; Partida-Sanchez et al., 2001; Smith et al., 1983; Stephens and Snyderman, 1982). To examine cytosolic changes in calcium in these cells following IL-5 stimulation, cells were loaded with a calcium-sensitive fluorescent dye, stimulated with IL-5, and examined by microscopy. Both neutrophils and macrophages responded to IL-5 by releasing stored intracellular calcium as indicated by the increase in fluorescence intensity of this dye following stimulation (Figure 6.3).

Since calcium flux occurs for many different effector functions, the next step was to determine the effects of IL-5 on phagocytosis and bacterial killing. Bacterial killing was assessed using a simple microbicidal assay, where bacteria and neutrophils are co-cultured for one hour to allow killing followed by serial dilution of the lysed supernatant, which allows for quantitation of killing following stimulation with IL-5. Interestingly, mouse neutrophil-mediated killing of two different bacterial species was not affected by IL-5 (Figure 6.4). This was also true of human neutrophils and *E. coli*, though the nearly complete neutrophil-mediated killing of bacteria makes it difficult to detect any potential increase in killing by IL-5 stimulation.

To examine an effect of IL-5 on macrophage phagocytosis, fluorescently labeled *E. coli* bioparticles were added to RAW264.7 macrophages pretreated with IL-5 or buffer control. Using fluorescence microscopy, there was an increase in macrophage phagocytosis of heat-killed *E. coli* bioparticles following IL-5 stimulation (Figure

6.5). Using the same simple assay to assess neutrophil bacterial killing, a moderate increase in bacterial killing was detected from IL-5 stimulated macrophages, though not significant (Figure 6.6). However, it is difficult to determine if this was due to increased phagocytosis by the macrophages. Nevertheless, these data combined with data demonstrating IL-6 and IL-12 production following IL-5 stimulation indicate that IL-5 augments macrophage function.

IL-5 has no effect on spontaneous cell death in neutrophils but prolongs survival of macrophages

IL-5 is well known to enhance the survival of eosinophils and B-1 cells (Adachi and Alam, 1998; Moon et al., 2004; Yamaguchi et al., 1988). To measure this, several methods involving flow cytometry or microscopy were used. Initially, apoptosis of these cells was measured using Annexin V, which binds to PS expressed on the exterior of apoptotic cells. However, the presence of Fc receptors combined with autofluorescence due to size and granularity created difficulty when analyzing the data. In addition, a recent article confirmed the results observed with these experiments, and showed that PI also stained intracellular RNA, which generated up to 40% false positive events (Rieger et al., 2010).

To more accurately assess changes in survival following IL-5 stimulation, cells were stained with the LIVE/DEAD® dye. This dye binds to cell surface and free amines within the cell, allowing distinction between live (dimly stained) and dead (brightly

stained) cells (Perfetto et al., 2006). Changes in spontaneous cell death of neutrophils and macrophages following IL-5 stimulation were determined using LIVE/DEAD® dead cell stain by flow cytometry. Surprisingly, bone marrow neutrophils stimulated with IL-5 had no change in survival at 9, 24, or 48 hours post-treatment (Figure 6.7). However, RAW264.7 mouse macrophages treated with IL-5 *in vitro* did have increased survival over PBS treated controls at 24 and 48 hours post-treatment. This was not spontaneous cell death, but rather cell death induced by LPS stimulation (which is required to induce IL-5Rα expression)

Macrophages are necessary for the protective effects of IL-5 *in vivo*

Functional studies indicated that IL-5 stimulation augmented multiple macrophage functions *in vitro*. To assess whether macrophages were necessary for the protective effects observed following IL-5 treatment in sepsis, MaFIA mice were used. These mice express a Fas receptor transgene under control of the colony stimulating factor receptor-1 (CSFR-1). Using a Fas receptor dimerization compound, animals are reversibly depleted of macrophages by apoptosis, such that 94% of macrophages are eliminated from the peritoneal cavity (Burnett et al., 2004). Macrophage depletion alone had no effect on survival during sepsis. However, the loss of macrophages no longer conferred the protective effects observed following IL-5 administration when given 4 hours prior to CLP sepsis (Figure 6.8). These novel

data indicate that macrophages were necessary for the protective effects of IL-5 *in vivo* during sepsis.

Summary

In sum, these data indicate the IL-5Rα was functional on neutrophils and macrophages. Specifically, IL-5 signaling in macrophages resulted in STAT-1 nuclear translocation, activation through release of intracellular calcium, cytokine secretion, enhanced phagocytosis and bacterial killing, and increased survival. In neutrophils, IL-5 induced activation through release of intracellular calcium, with no change in bacterial killing or spontaneous cell death. Finally, macrophages were necessary *in vivo* for the protective effects of IL-5 in sepsis.

Figure 6.1: IL-5 causes increased STAT-1 nuclear translocation in Macrophages following LPS-stimulation

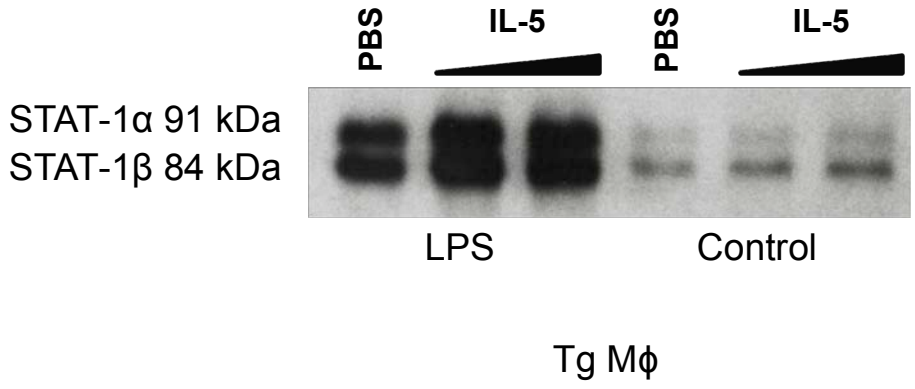


Figure 6.1: IL-5 causes increased STAT-1 nuclear translocation in Macrophages following LPS-stimulation

Thioglycollte-elicited macrophages were stimulated *in vitro* with 10 ng/ml LPS or PBS control for 24 hrs. Cells were stimulated with IL-5 at increasing doses (100 ng/ml and 1μg/ml) or PBS for 4 hrs. Western blot for STAT-1 was performed on nuclear extracts.

Figure 6.2: IL-5 induces a dose-dependent production of IL-6 and IL-12 by Macrophages

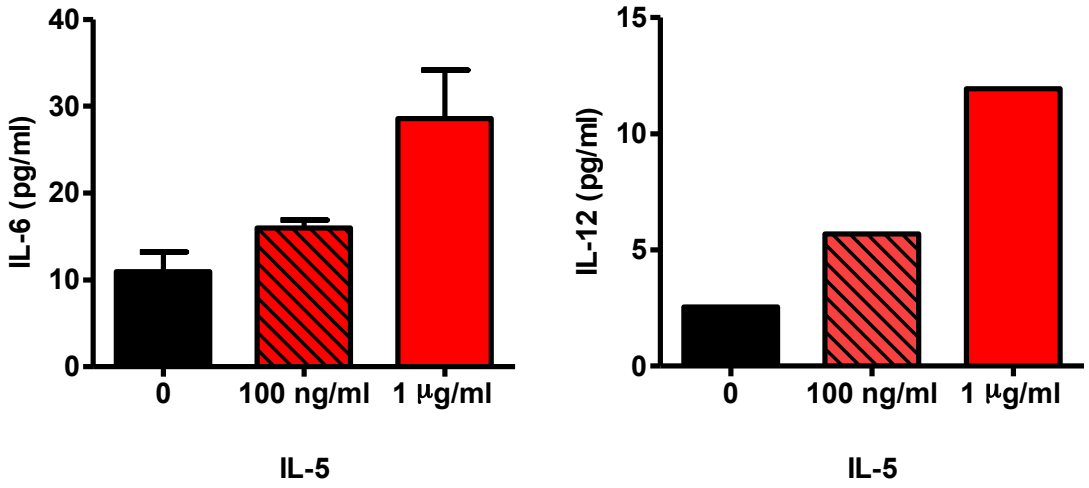
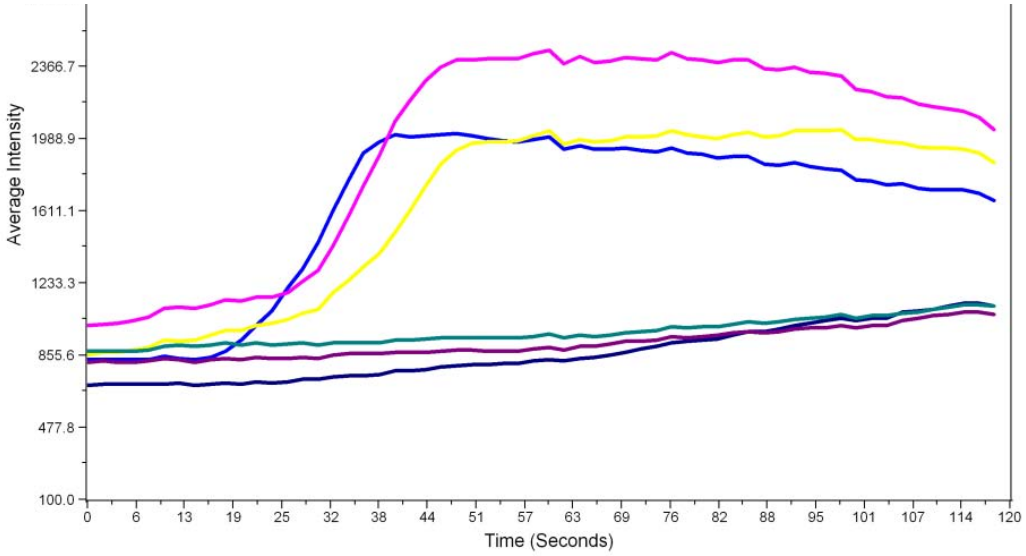
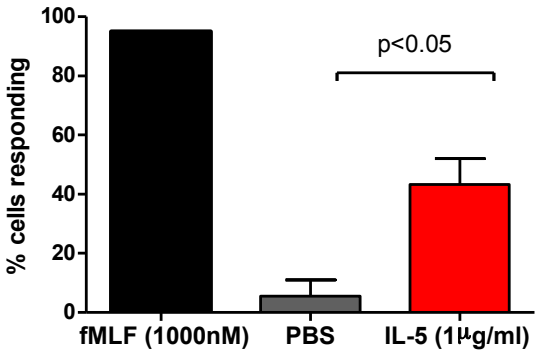


Figure 6.2: Thioglycollate-elicited macrophages were stimulated *in vitro* with IL-5 at varying doses for 24 hrs. IL-6 and IL-12 levels were measured in the supernatants by ELISA. Bars represent means +/- SEM.

Figure 6.3: IL-5 induces activation of Neutrophils and Macrophages by causing intracellular calcium flux



A. Macrophages



B. Neutrophils

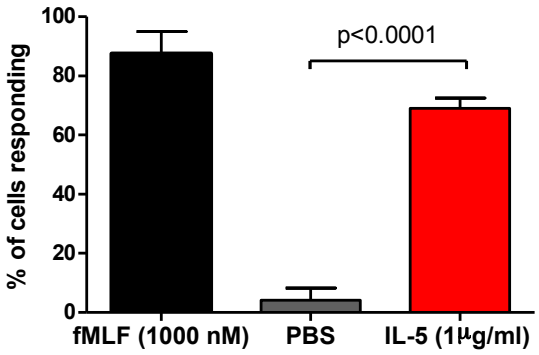
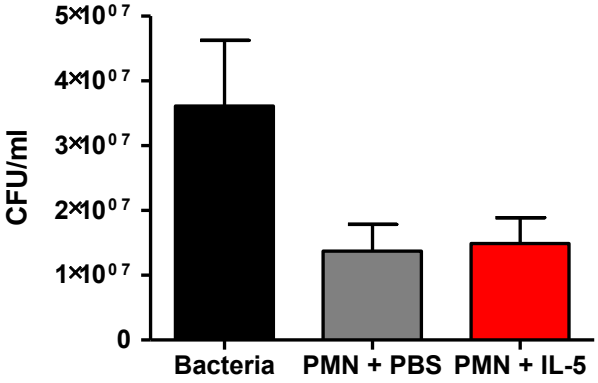


Figure 6.3: IL-5 induces activation of Neutrophils and Macrophages by causing intracellular calcium flux

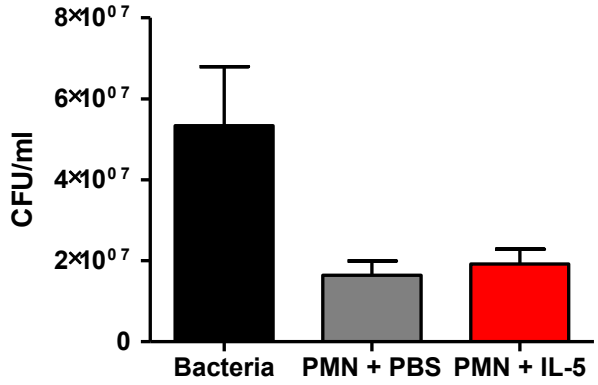
Thioglycollate-elicited macrophages or neutrophils were allowed to adhere to microscope slides, then loaded with Fluo-4 calcium indicator, and stimulated with 1 μg/ml IL-5 or PBS control. Time-lapse images were taken and the graph on the top is a representative image of this data. The blue, magenta, and yellow lines are 3 individual cells that are considered responding to the stimulus; the 3 darker, flatter lines are non-responding cells. fMLF is used as a positive control. Images were taken of >75 cells per experiment for a total of 3 experiments. The percentage of responding cells was calculated for each experiment. Bars represent means +/- SEM. Data were analyzed by student's t-test. Macrophages treated with IL-5: *$P<0.05$; Neutrophils treated with IL-5: ***$P<0.0001$

Figure 6.4: IL-5 does not affect bacterial killing by Neutrophils

A. Mouse Neutrophils with *P. aeruginosa*



B. Mouse Neutrophils with *E. coli*



C. Human Neutrophils with *E. coli*

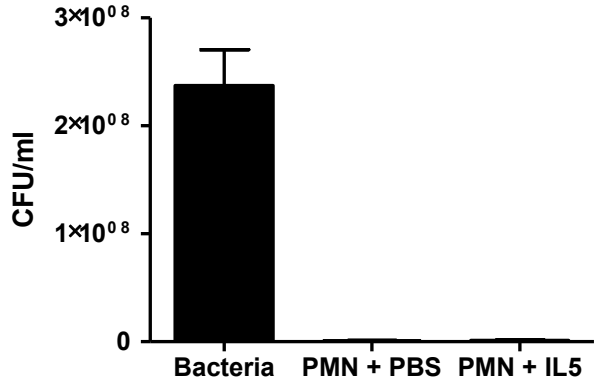


Figure 6.4: IL-5 does not affect bacterial killing by Neutrophils

Thioglycollate-elicited mouse (Panel A and B) neutrophils or human neutrophils (PMN) (Panel C) were incubated with either *P. aeruginosa* (Panel A) or *E. coli* (Panel B and C) at an MOI of 10 for 1 hr with either 1 μg/ml IL-5 or PBS. Cells were lysed and viable bacteria were plated by serial dilution. Bars represent means +/- SEM. Data were analyzed by student's t-test. All P-values are non-significant (P>0.05)

Figure 6.5: IL-5 enhances Macrophage phagocytosis

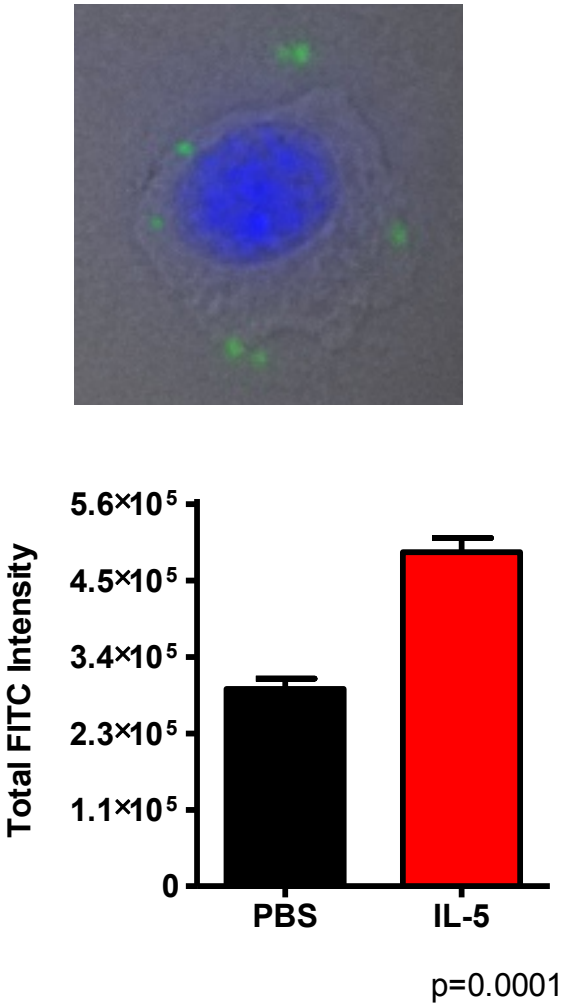


Figure 6.5: Thioglycollate-elicited macrophages were pretreated for 1 hr with 1 μg/ml IL-5 or PBS followed by incubation with FITC-labeled *E. coli* bioparticles for 20 min to allow phagocytosis to occur. Extracellular bacteria were rinsed, and images were taken of >75 cells/experiment for a total of 3 experiments. Images were quantitated by subtracting out background intensity for each image and plotting total fluorescence intensity for each cell. Bars represent means +/- SEM. Data were analyzed by student's t-test. ***P=0.0001

Figure 6.6: IL-5 enhances Macrophage bacterial killing

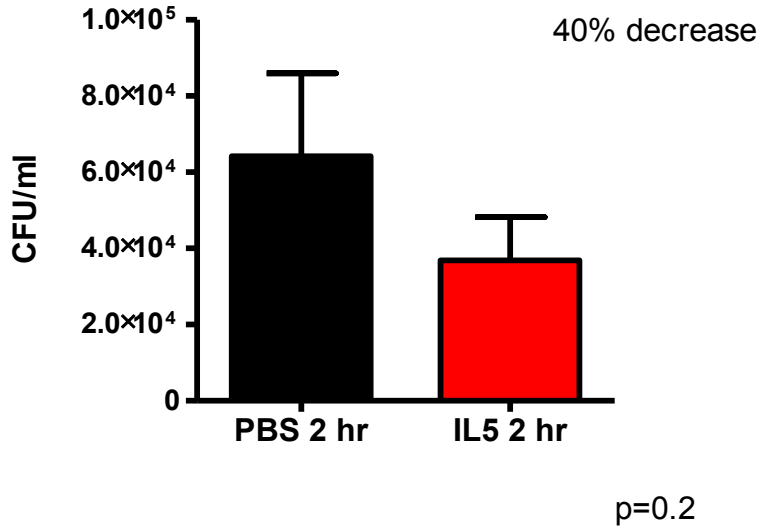


Figure 6.6: Thioglycollate-elicited mouse macrophages were incubated with either 1 μg/ml IL-5 or PBS, and *E. coli* at an MOI of 10 for 30 min to allow phagocytosis to occur. Extracellular bacteria were killed with gentamicin. Cells were lysed 1.5 hrs later and viable bacteria were plated by serial dilution. Bars represent means +/- SEM. Data were analyzed by student's t-test. P=0.2

Figure 6.7: IL-5 does not affect survival in Neutrophils

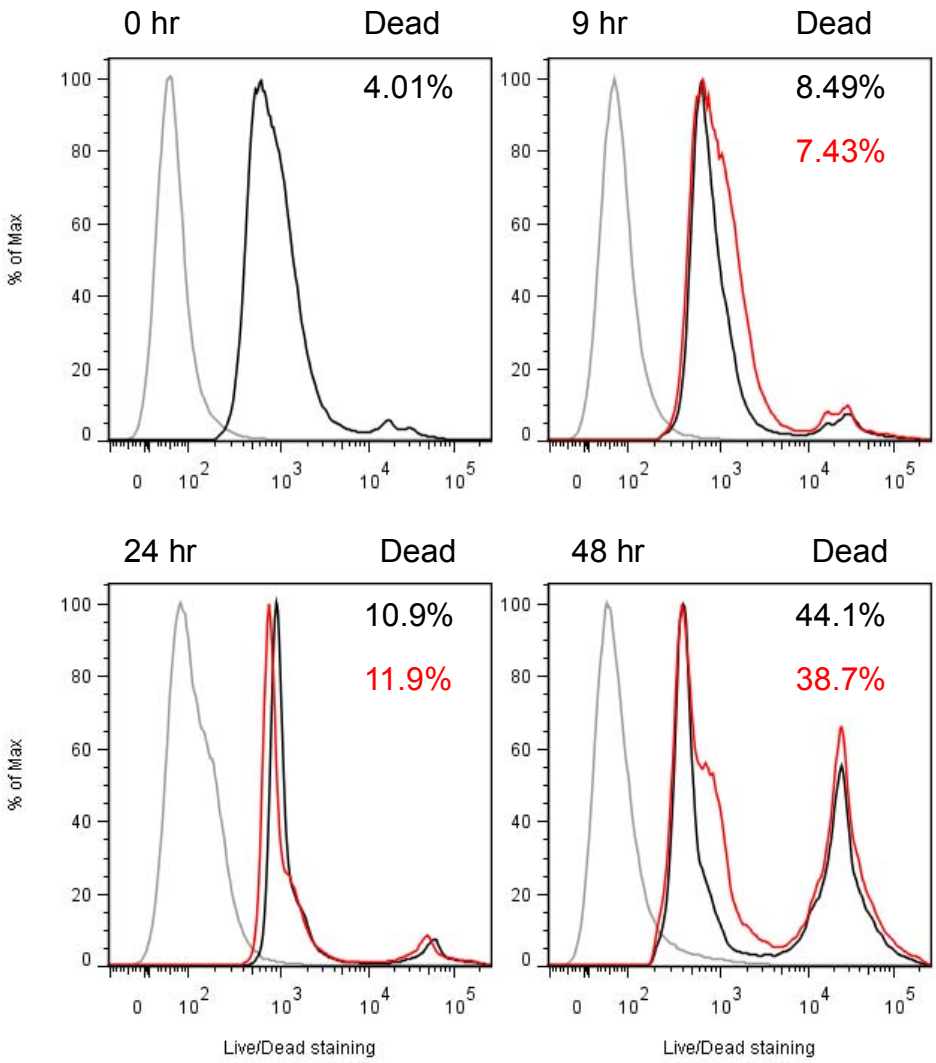


Figure 6.7: Bone marrow neutrophils were isolated and treated with 1µg/ml IL-5. Neutrophils were stained using the LIVE/DEAD® dye. Cells were stained at 0, 9, 24, and 48 hrs following IL-5 stimulation. Grey histogram represents unstained control; black histogram represents PBS control; Red histogram represents IL-5 treated. The percentages on the graphs are the percentage of dead cells at that time point, with the black representing PBS control, and red representing IL-5 treated samples.

Figure 6.8: IL-5 increases Macrophage survival *in vitro*

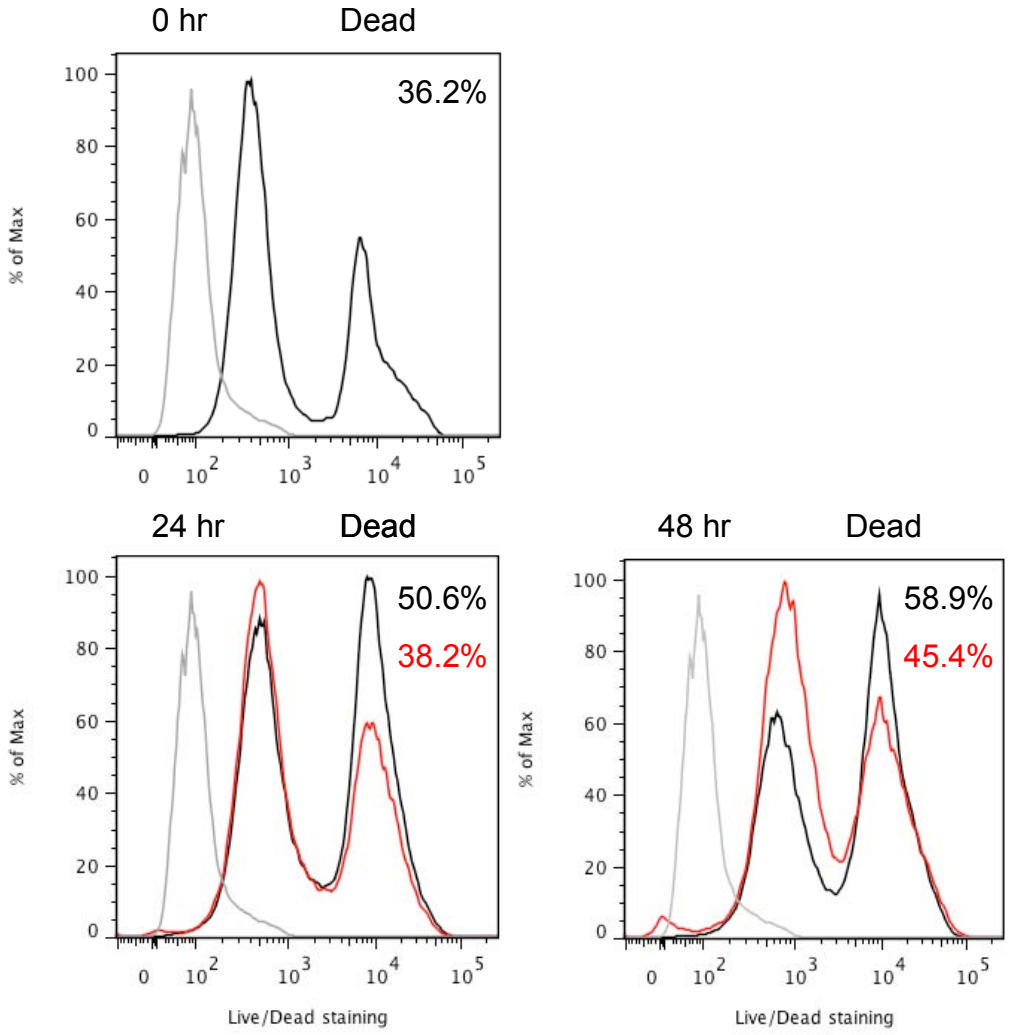


Figure 6.8: RAW 264.7 macrophages were incubated with 10 ng/ml LPS for 24 hrs to upregulate IL-5Rα. Cells were then stimulated with either PBS or 1µg/ml IL-5. Macrophages were stained using the LIVE/DEAD® dye. Cells were stained at 0, 24, and 48 hrs following IL-5 stimulation. Grey histogram represents unstained control; black histogram represents PBS control; Red histogram represents IL-5 treated. The percentages on the graphs are the percentage of dead cells at that time point, with the black representing PBS control, and red representing IL-5 treated samples.

Figure 6.9: Macrophage depletion eliminates the protective effects of IL-5 in CLP-induced Sepsis

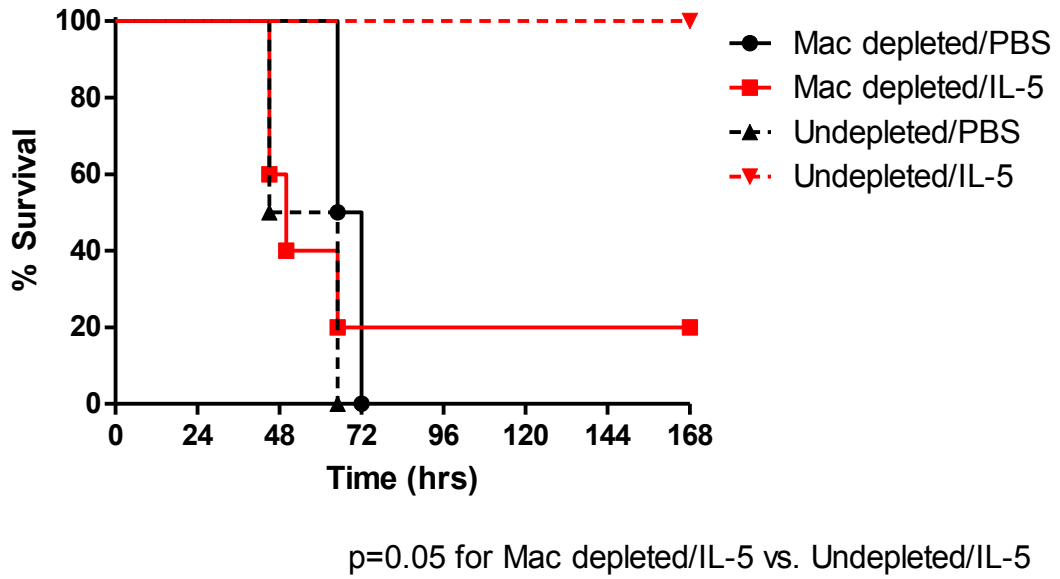


Figure 6.9: MaFIA mice were administered AP20187 (Mac depleted) or vehicle control (undepleted) for 5 days to assess the effect of macrophage depletion on survival in CLP. Three days later, mice were administered 1µg of IL-5 (n=5) or PBS (n=5) i.p. 4 hrs prior to CLP. Mice were monitored for survival for 7 days. No additional animals died after this time point. Data were analyzed using the Log-rank test. *P=0.05 for Macrophage depleted/IL-5 treated vs. Undepleted/IL-5 treated mice. P=0.48 for Macrophage depleted/IL-5 treated vs. Macrophage depleted/PBS treated.

Discussion

Sepsis represents a major financial and health burden in the US. Despite recent advances in knowledge of this disease and adequate health care, morality remains excessively high. Data presented in Chapter 4 demonstrate that therapeutic administration of IL-5 improved survival in polymicrobial sepsis, providing strong evidence that IL-5 is a viable treatment option for septic patients in the ICU. Moreover, these data determined that loss of IL-5 impaired bacterial clearance, increased tissue damage, and mortality. Elevation of IL-5 in the absence of eosinophils resulted in increased numbers of neutrophils and monocytes and administration of IL-5 into the peritoneal cavity of mice induced neutrophil recruitment, indicating that these populations may mediate the protective effects of IL-5 in sepsis. This was confirmed by documentation of the IL-5Rα on neutrophils and macrophages/monocytes in sepsis both in mice and in humans. However, the possibility still remains that the membrane-bound IL-5Rα on neutrophils and macrophages is a non-functional decoy receptor to absorb excess IL-5 and prevent intracellular signaling events.

Evidence for a functional IL-5Rα on macrophages comes from increased STAT-1 nuclear translocation, which is required for IL-5 signaling in eosinophils (Adachi and Alam, 1998; van der Bruggen et al., 1995). While this suggests that many of the same signaling pathways and adapter molecules used for IL-5 signaling in eosinophils are also used in macrophages and neutrophils, this hypothesis will need to be further tested. Since this is the first time expression of the IL-5Rα has been

shown definitively on neutrophils and macrophages/monocytes from mice and humans, the next question to resolve was the effect of IL-5 signaling on these cells, i.e. what functions does expression of this receptor during sepsis provide and how is IL-5 beneficial.

Previously, data in this thesis demonstrated that exogenous administration of IL-5 therapeutically improved survival in polymicrobial sepsis, suggesting the usefulness of IL-5 treatment for septic patients. However, the failure of numerous clinical trials in sepsis was in part due to poor animal models and a lack of knowledge regarding how a treatment affects the host immune response. Therefore, it was important to understand the specific effects of IL-5 administration on the immune response. In order to assess the potential effects of IL-5 administration *in vivo*, the response of neutrophils and macrophages to IL-5 was determined *in vitro* by assessing major and important functions of these cells, including cellular activation, cytokine production, bacterial killing, phagocytosis, and cell death.

Eosinophils are known to migrate toward a concentration of IL-5 from 10-500 fold-lower (in the picomolar range) than that required for neutrophil migration or macrophage proliferation (Hakansson and Venge, 1994; Ringheim, 1995). Thioglycollate-elicited macrophages were stimulated *in vitro* with 0, 100, or 1000 ng/ml of IL-5. Interestingly, IL-5 stimulation induced a dose-dependent production of IL-6 and IL-12 by macrophages. Since only two doses were tested, it is impossible to know at what point this effect would reach saturation, but it does suggest the possibility for a range of macrophage activation following IL-5 stimulation. This

would be especially important when administering IL-5 to septic patients, as dosing might be modulated to increase or decrease the amount of stimulation and macrophage activation required for each patient and allow for more individualized treatment.

Calcium signaling precedes a number of different functions in neutrophils and macrophages, including chemotaxis, degranulation and superoxide production (Hallett et al., 1999; Marks and Maxfield, 1990; Partida-Sanchez et al., 2001; Smith et al., 1983; Stephens and Snyderman, 1982). In both neutrophils and macrophages, IL-5 stimulation induced intracellular calcium release. Since there is no extracellular calcium in the media, the increase in fluorescence intensity of the dye induced by calcium binding must be occurring due to release of intracellular calcium. However, while calcium release occurs before each of these functions, it does not address the specific results of IL-5 signaling.

To more specifically assess the effects of IL-5 on the effector functions of these leukocytes, bacterial killing by neutrophils was measured. Because of previous studies indicating neutrophil chemotaxis to IL-5 (Hakansson and Venge, 1994) and calcium flux data, it was expected that IL-5 would enhance neutrophil-mediated bacterial killing. Surprisingly, this was in fact not true. Mouse neutrophil-mediated killing of either *E. coli* or *P. aeruginosa* was not altered by IL-5 stimulation. This was also true for human neutrophil-mediated killing of *E. coli*. Even so, there are several limitations to this study. First, this was initially performed with thioglycollate-elicited neutrophils, which are already fully activated upon isolation (Itou et al.,

2006). Compared to bone marrow neutrophils, thioglycollate-elicited neutrophils have increased expression of adhesion molecules, spontaneous release of ROS, and increased chemotaxis. More importantly, production of ROS and degranulation was not increased by thioglycollate-elicited neutrophils following TNFα treatment, whereas in bone marrow neutrophils it was increased by roughly 3-fold (Itou et al., 2006). The inability to augment bacterial killing in thioglycollate-elicited neutrophils following IL-5 stimulation is in accordance with this study. The relationship of neutrophil activation state to bacterial killing is also reflected in the variability observed in these assays. Combined, there is no difference between IL-5 treated or untreated neutrophils. However, in those assays where neutrophils appeared to be less active, i.e. control-treated neutrophils had an intermediate level of killing, IL-5 treatment did augment bacterial killing. This is especially true of the assays conducted using human neutrophils. Since human neutrophils so effectively killed bacteria (>90% killing), it was nearly impossible to detect an increase in killing as a result of IL-5 stimulation. One way to test this would be to titrate the number of neutrophils required to achieve between 40-60% killing. Then, any increase or decrease in killing mediated by IL-5 stimulation would be detected. However, it would be more important to assess IL-5 stimulation of neutrophils that are already defective in bacterial killing, as observed in septic patients, to determine if IL-5 has any effect on reducing neutrophil dysfunction. This possibility will be explored in future studies and will more definitively establish the importance of IL-5 on neutrophil activity in sepsis.

To make one final comment on these data, it was intriguing that in some experiments when either bone marrow or thioglycollate-elicited neutrophils were used to assess bacterial killing, there was an increase in bacterial growth regardless of treatment with IL-5 or PBS control. This was determined by comparison with samples only containing bacteria. This is surprising, because the media used was normal cell culture media (RPMI supplemented with FBS) and in some cases PBS, which do not promote bacterial cell growth. In addition, the time point used to allow neutrophil killing (1 hour) and the rate of bacterial doubling time (roughly 20 min for *E. coli*) was insufficient to account for the increase in bacteria present. These data suggest that neutrophils or a substance secreted by these cells was causing accelerated bacterial growth. This was not true for all experiments making it difficult to decisively determine the cause for this, but it was frequent enough to necessitate mention.

The next step was to measure phagocytosis by macrophages, as this is one of their primary functions during an immune response. Macrophages treated with IL-5 had significantly greater levels of phagocytosis compared to vehicle treated controls. This was determined using heat-killed bacteria, so it remains possible that IL-5 does not augment phagocytosis of apoptotic cells or particles. This would be especially important during sepsis as there is a massive increase in apoptosis of neutrophils, T cells, B cells, and non-immune cells (Hotchkiss and Nicholson, 2006). If IL-5 stimulation augmented macrophage uptake of apoptotic cells it would decrease the number of cells that progressed to secondary necrosis, which is a highly

inflammatory process and causes damage to bystander cells and tissues. This would be an important consequence of IL-5 stimulation, and needs to be assessed in the future. Nevertheless, IL-5 does augment macrophage bacterial phagocytosis, and hence host control of infection. This would be an important consequence of IL-5 treatment in septic patients, given the overt immunosuppression that often occurs.

Interestingly, there was not a statistically significant increase in bacterial killing by macrophages treated with IL-5, though IL-5 treatment did augment killing by 40%. One possibility is that the increase in phagocytosis mediated by IL-5 resulted in more bacteria ingested per macrophage, which made it difficult to detect a significant increase in bacterial killing. Another possibility is that the level of bacterial killing by thioglycollate-elicited macrophages depends on the age of the mice, as aged mice were frequently used and it is known that macrophages are immunosenescent and less proinflammatory in aged animals (Stout and Suttles, 2005). Finally, there may be an inherent difference between RAW264.7 macrophage and thioglycollate-elicited macrophages with regard to phagocytosis and bacterial killing. Whatever the reason for these differences, whether intrinsic or extrinsic, it is more important to assess changes in these functions in monocytes/macrophages isolated directly from septic patients. This will definitively assess the role for IL-5 on these cells during sepsis and determine whether IL-5 treatment will restore their function.

Finally, as mentioned previously sepsis is associated with massive apoptosis of both immune and non-immune cells (Hotchkiss and Nicholson, 2006; Wesche et al.,

2005). Likewise, inhibition of apoptosis through caspase-12 deletion or upregulation of Bcl-2 in mice improved survival in CLP-induced sepsis (Hotchkiss et al., 1999b; Saleh et al., 2006). However, inhibition of neutrophil apoptosis could prove detrimental, as the increased presence of neutrophils in sepsis caused renal dysfunction and tissue damage (Hoesel et al., 2005; Jimenez et al., 1997). Data here indicate there was no effect on spontaneous neutrophil death following IL-5 stimulation. One caveat to these studies is that spontaneous cell death is not the same as neutrophil apoptosis. Due to technical difficulties and concerns with false-positives using Annexin V and PI staining, a simple LIVE/DEAD stain was used to distinguish between these two populations. Looking at cell death alone does not determine whether the cell died by apoptosis, which is an antiinflammatory process, or necrosis, which is a proinflammatory process. The effects of IL-5 on these processes would be an important distinction in sepsis, and needs to be assessed.

Another caveat to these experiments is the use of bone marrow neutrophils. Typically, neutrophils in the blood have a short half-life of 6-12 hours, while bone marrow neutrophils can survive much longer (Boxio et al., 2004). These cells are not activated following isolation like thioglycollate neutrophils are, which may be one reason for the low percentage of dead cells by 24 hours. Activated neutrophils are more apoptotic than resting ones and this may be important *in vivo* for regulating the balance between control of infection and excessive tissue damage. IL-5 may be more effective at inhibiting apoptosis in already apoptotic cells, and these studies do not use activated or apoptotic cells. However, the use of thioglycollate-elicited (or

activated) neutrophils is difficult because of the variability observed between isolations. Further studies are needed to fully qualify the effects of IL-5 on neutrophil apoptosis.

In contrast to studies with neutrophils, macrophage survival was prolonged *in vitro* by IL-5 stimulation. This may be important during sepsis, as macrophages are vital to clear apoptotic cells. Since IL-5 increased both macrophage phagocytosis and survival, this may prevent apoptotic cells from progressing on to necrosis because they can be rapidly cleared *in vivo*. Since macrophages do not release highly cytotoxic granule proteins like neutrophils, prolonging their survival is unlikely to increase tissue damage.

Because IL-5 stimulation augmented macrophage function, the effect of macrophage depletion *in vivo* was tested in the context of IL-5 stimulation. MaFIA mice were depleted of macrophages by administration of AP20187, which causes Fas receptor dimerization on monocytes/macrophages. Macrophage depletion had no effect on survival in the CLP model of sepsis. However, while IL-5 administration 4 hours prior to CLP in wild type mice significantly improved survival, macrophage-depleted mice were no longer rescued by IL-5 treatment. These data indicate that macrophages are *necessary* for the protective effects of IL-5 during sepsis *in vivo*. One limitation to this mouse model, is that dendritic cells are also depleted by AP20187 (Burnett et al., 2004), and several studies have shown that dendritic cells are required for survival in sepsis (Ding et al., 2004; Scumpia et al., 2005). However, these studies used CD11c as a marker for dendritic cells despite the fact that

numerous different populations of macrophages, including alveolar and interstitial macrophages express CD11c (Hume, 2011). The expression of IL-5Rα and role for IL-5 on dendritic cells has not been determined and remains a possible explanation for the protective effects of this cytokine in sepsis. However, the association of human sepsis with massive dendritic cell death might suggest that this is not the cell type through which IL-5 is mediating protection (Hotchkiss et al., 2002). Nevertheless, these data provide clear evidence for the role of IL-5 in augmenting macrophage function. Future studies to determine the role for IL-5 regulating dendritic cell function are necessary.

In sum, data in this Chapter provide evidence for the role of IL-5 and IL-5Rα expression in sepsis. In neutrophils, IL-5 stimulation mediated chemotaxis *in vivo* (Chapter 4), and activation through release of intracellular calcium. It did not affect survival or bacterial killing. For macrophages, IL-5 stimulation resulted in activation through release of intracellular calcium, increased phagocytosis and bacterial clearance, and prolonged survival. Moreover, macrophages were required *in vivo* for the protective effects of IL-5.

Data from this Chapter are published as:

Linch, S. N., Kelly, A. M., Danielson, E. T., Tamakawa, R. and J. A. Gold. The IL-5 receptor is expressed on neutrophils and macrophages and treatment with IL-5 improves survival in polymicrobial sepsis (manuscript in preparation).

CHAPTER 7:

SUMMARY AND CONCLUSIONS

Summary and Conclusions

Sepsis is characterized by an initial robust proinflammatory response followed by a compensatory immunosuppressive, or antiinflammatory response (Hotchkiss and Karl, 2003; Monneret et al., 2004; Wang and Deng, 2008). The use of inappropriate antibiotics further increases already elevated patient mortality (Harbarth et al., 2003; Ibrahim et al., 2000; Kumar et al., 2009). The need for appropriate treatment options for septic patients is dire, though the failure of countless clinical trials does not provide much hope for effective treatments. Recently, investigators have focused on trying to reduce the severity of immunosuppression, or restore the inflammatory response in patients, and these trials have had early success (Austin et al., 1995; Bilgin et al., 2001; Gennari et al., 1994; Levin et al., 2000; Levy and Elsbach, 2001; Meisel et al., 2009; Orozco et al., 2006; Presneill et al., 2002; Schneider et al., 2004; Weiss et al., 1995).

Interestingly, severe sepsis and other acute infections are associated with a loss of eosinophils and as patients recover, this population of leukocytes returns (Abidi et al., 2008; Bass, 1975; Bass et al., 1980; Shaaban et al., 2010). The significance of this observation is not understood. One possible explanation for this is that eosinophils are participating in the host response to infection, by migrating into tissues, secreting cytokines, and releasing their highly cationic, antimicrobial proteins through cytolytic degranulation. There is evidence to support this possibility (Jong et al., 1980; Lehrer et al., 1989; Rothenberg and Hogan, 2006; Shi, 2004; Watanabe et al., 1995; Wong et al., 2007; Yazdanbakhsh et al., 1986), though it is difficult to

detect a cell that has degranulated. Early autopsy studies in the late 1800's by Paul Ehrlich documented the absence of these cells even in the tissues, but the use of MBP immunohistochemistry was not available as a means to detect degranulated eosinophils.

To definitively determine whether eosinophils participate in the immune response *in vivo*, a bacterial peritonitis model with *P. aeruginosa* was employed. First, these data demonstrated that mouse eosinophils kill *P. aeruginosa in vitro*. Purified mouse eosinophils killed *P. aeruginosa in vitro*. This killing ability was confirmed for the first time *in vivo* through the use of adoptive transfer of eosinophils to wild type mice and PHIL mice, which have a congenital ablation of eosinophils. These data document for the first time that mouse eosinophils participate in bacterial clearance *in vivo*. Moreover, data in Chapter 3 showed that eosinophil granule proteins enhance bacterial clearance *in vitro* and *in vivo*. These data support the idea that eosinophils participate in bacterial infection *in vivo*, in part through degranulation, and provide an explanation for the lack of visual evidence in very early clinical studies of sepsis and acute infection. This could be definitively assessed using immunohistochemistry for the presence of MBP in tissue, which would detect eosinophil degranulation. While it is difficult to induce mouse eosinophils to degranulate (communication with J. J. Lee), we did observe release of MBP in supernatants from eosinophil killing assays *in vitro* (data not shown) further supporting a role for eosinophils and their granule proteins in bacterial infection.

There are several reports that indicate human eosinophils and eosinophil granule proteins, including MBP and EPO, possess antimicrobial properties, which suggests a role for these granulocytes during infection (Calafat et al., 1998; Gleich et al., 1979; Inoue et al., 2005; Ishihara et al., 2003; Lehrer et al., 1989; Persson et al., 2001; Rosenberg and Domachowske, 2001; Svensson and Wenneras, 2005). Conversely, the loss of eosinophils during acute infection or sepsis might also suggest that these cells are not necessary for the immune response to bacterial infection. Their increased presence in asthma and other allergic diseases is in fact harmful to the host response, by increasing airway hyperreactivity and tissue destruction (Adamko et al., 1999; Foster et al., 1996; Garofalo et al., 1992; Gundel et al., 1991; Hogan et al., 2008; Humbles et al., 2004). Loss of eosinophils during sepsis may be a protective mechanism for the host to limit tissue destruction. However, while neutrophils are necessary to clear an infection, an increase in neutrophils during bacterial infection also causes tissue destruction and in excess, can contribute to organ failure in sepsis (Brown et al., 2006; Hoesel et al., 2005; Jimenez et al., 1997; Olcay et al., 2008; Ottonello et al., 1995). Eosinophils, like neutrophils, represent a double-edged sword during infection; their presence is associated with increased tissue damage, but also more rapid clearance of virus (Adamko et al., 1999; Phipps et al., 2007).

The precise function of eosinophils during infection remains an enigma. It is clear that they can kill bacteria, and that this can occur *in vivo*, but it is not clear why the host response to bacterial infection is largely neutrophilic and eosinopenic. What is even more interesting is that humans with hypereosinophilic syndromes rarely

present with bacterial infection (communication with G. Gleich), again raising the question of the role for these cells in bacterial infection. Within the last decade the canonical role for eosinophils during parasitic infection has been questioned, and numerous data indicate that eosinophils are dispensable for the killing of most helminths, while neutrophils are in fact important during some helminth infections for worm encapsulation and killing (Afshar et al., 2007; Al-Qaoud et al., 2000; Saeftel et al., 2003). Furthermore, data presented in this thesis demonstrate a role for eosinophils and eosinophil granule proteins during bacterial infection *in vivo*. While these studies may be confusing and difficult to reconcile, they suggest more overlap in eosinophil and neutrophil function than was previously appreciated. It seems unlikely that their only job is to promote tissue destruction as they are genetically conserved in numerous mammalian species. Future studies aimed at delineating these functions will help elucidate the role for the eosinophil in disease and infection.

In addition to identifying a novel role for eosinophils *in vivo* during bacterial infection, the data presented in this thesis also demonstrate an important and protective role for IL-5 in sepsis. It was previously shown that IL-5 overexpressing mice had improved survival and bacterial clearance in sepsis (Yousefi et al., 2008). Because of the role established in Chapter 3 for eosinophils *in vivo*, and the profound eosinophilia observed in these mice, it seemed probable that this activity would be attributed to the eosinophil rather than overexpression of IL-5. However, a recent study suggested that elevated levels of IL-5 were protective in septic patients (Bozza

et al., 2007), necessitating a careful evaluation of the individual roles for IL-5 and eosinophils in sepsis.

Indeed, there was a unique role for IL-5 in sepsis as determined using IL-5 overexpressing mice, in the absence of eosinophils (NJ.1638/PHIL mice). These mice were protected from the lethality of sepsis, had increased bacterial clearance, and an intriguing expansion of neutrophils and monocytes at baseline in healthy animals. This is the first time IL-5 has been described as having protective effects in sepsis *in vivo*, and in the absence of eosinophils. This was confirmed by assessing the effect of IL-5 deletion in CLP model of sepsis. IL-5 knockout mice have normal numbers of tissue eosinophils and B-1 cells at the time of surgery (8-12 weeks) (Kopf et al., 1996). These mice had decreased survival accompanied by decreased bacterial clearance and increased tissue damage. The trend toward increased IL-6 and IL-10 suggests that IL-5 may be important in regulating their production during sepsis, though a compensatory upregulation of these cytokines cannot be ruled out. The loss of IL-5 was clearly detrimental to the host immune response in sepsis, and exogenous administration of IL-5 prophylactically and therapeutically improved survival in sepsis. These data suggest a therapeutic benefit for IL-5 administration in sepsis.

One interesting idea that cannot be excluded is the role for B cells in the protective effects induced by IL-5 in sepsis. In one clinical study, G-CSF infusion increased neutrophil function and mobilization as expected, but it also mobilized B cells from the bone marrow and increased levels of IL-4 (Valente et al., 2002). IL-5 is known to

induce B cell survival and antibody production. It is possible that IL-5 administration is increasing B cell antibody production, which in the context of disease would promote increased opsonization of pathogens, thereby allowing increased recognition and phagocytosis of microbes by phagocytes. While IL-5 stimulation of macrophages did increase phagocytosis in the absence of B cells *in vitro*, there may be an additive or synergistic role for these two cell types in response to IL-5 in sepsis and needs to be assessed in future studies.

The beneficial role for IL-5 in sepsis is mediated through expression of the IL-5Rα on neutrophils and monocytes/macrophages in mice and humans, which is demonstrated here for the first time. IL-5 signaling in mouse neutrophils induced chemotaxis *in vivo*, and *in vitro* resulted in intracellular calcium flux with no effect on spontaneous cell death or bacterial clearance. IL-5 signaling in mouse macrophages induced intracellular calcium flux, augmented phagocytosis and bacterial killing, as well as prolonged survival. These data are not the first to document the response of these myeloid cells to IL-5 (Al-Qaoud et al., 2000; Bober et al., 1995; Hakansson and Venge, 1994; Ringheim, 1995; Saeftel et al., 2003). However, these are the first studies to document the response of these cells to IL-5 in the context of sepsis and polymicrobial infection. More importantly, these findings indicate that IL-5 has pleiotropic effects on neutrophils and macrophages, and that these effects may only be apparent in specific diseases. One key question that remains is the extent of these effects on human neutrophils and macrophages from septic patients. Patients who are immunosuppressed have significant defects

in neutrophil-mediated bacterial killing and monocyte/macrophage antigen presentation and cytokine production. It remains to be determined whether IL-5 can still augment macrophage function in immunosuppressed patients. Moreover, IL-5 may enhance neutrophil bacterial killing in defective neutrophils from septic patients. These same functions need to be assessed in numerous septic patients in order to fully assess the effects of IL-5 administration in sepsis.

In vitro data demonstrate that the IL-5Rα receptor is induced on macrophages by TLR stimulation, using a number of different TLR ligands including LPS, CpG, or heat-killed bacteria. The presence of infection may be one possible way to induce expression of this receptor *in vivo*. This has not specifically been examined in this thesis though it would be possible to determine levels of IL-5Rα expression based on the presence versus the absence of an infectious organism in each patient. If the IL-5Rα is still expressed in patients in the absence of a demonstrable infection, then that would suggest additional mechanisms for receptor expression in patients. More patient samples are needed to determine this possibility.

The expression of IL-5Rα on neutrophils from mouse bone marrow might suggest that this receptor is expressed on more immature neutrophils, and that during infection the rapid mobilization of neutrophils from the bone marrow occurs before this receptor can be down modulated. It is known that IL-5Rα expression is a critical step in eosinophil development (Kouro and Takatsu, 2009). However, neutrophils isolated from the bone marrow here express high levels of this receptor and Ly6g (a neutrophil lineage marker), and were morphologically identified as neutrophils. In

one study of bone marrow purified neutrophils, these cells were analyzed visually and biochemically, and were identified as 94.4% neutrophils (they released large amounts of lactoferrin upon stimulation) (Boxio et al., 2004). Even if the remaining 5.6% of these cells were eosinophils, they cannot account for the entire population of IL-5Rα expressing cells. Perhaps IL-5Rα expression is not a step toward terminal differentiation of eosinophils. It has been shown by numerous groups that differentiation of hematopoietic stem cells is a somewhat plastic process, and various phenotypes in neutrophils and macrophages can be induced in the same cell using different stimulation (De Santo et al., 2010; Edwards et al., 2006; Kreider et al., 2007; Lumeng et al., 2007; Mosser and Edwards, 2008; Tsuda et al., 2004). This hypothesis will need to be tested but it is an interesting possibility given the evidence to suggest a dual role (both harmful and beneficial) for these cell types in disease. Nevertheless, IL-5Rα expression on neutrophils and monocytes in sepsis is unique, and mechanisms for its expression need to be further explored.

One distinct possibility that was not directly tested is that IL-5 may induce neutrophil and monocyte/macrophage chemotaxis. It is apparent that neutrophils exhibit delayed and reduced chemotaxis in sepsis (Reddy and Standiford, 2010; Torres-Duenas et al., 2006). Indirect evidence *in vivo* suggests that IL-5 administration induces neutrophil chemotaxis, but this was not definitively determined *in vitro*. Further evidence for this role comes from a study showing CXCR1 and CXCR2, the receptors for IL-8 and other chemokines, were induced on monocytes by Th2 cytokines, including IL-4 and IL-13 (Bonecchi et al., 2000).

Likewise, another recent paper demonstrated that IL-33, which is known to be a potent inducer of Th2 related cytokines IL-4, IL-5, and IL-13 (Arend et al., 2008), rescues animals from sepsis by inducing neutrophil recruitment to the site of infection and preventing downregulation of CXCR2 (Alves-Filho et al., 2010; Rios-Santos et al., 2007). It is possible that induction of IL-33 in sepsis results in elevated IL-5, which prevents downregulation of CXCR2 in sepsis. This cannot be confirmed by the study by Alves-Filho because while IL-4 and IL-13 were measured following IL-33 administration, IL-5 levels were not determined. One more interesting point about this study was that IL-10 and IL-6 were reduced following IL-33 administration (Alves-Filho et al., 2010). This is in agreement with data from IL-5 knockout mice showing elevated levels of IL-10 and IL-6 in sepsis, and may further support a role for IL-5 in the regulation of these antiinflammatory cytokines. Nonetheless, the role for IL-5 in neutrophil chemotaxis needs to be definitively determined *in vivo*, and *in vitro* using neutrophils from septic patients.

A final question that remains is the cellular source of IL-5 in sepsis. Numerous cell populations produce IL-5, including T cells, NK cells, NKT cells, mast cells, epithelial cells, and bone marrow stromal cells (Hogan et al., 2000; Kurowska-Stolarska et al., 2008; Martinez-Moczygemba and Huston, 2003; Sakuishi et al., 2007; Salvi et al., 1999; Warren et al., 1995). The massive loss of T cells observed in sepsis suggests that this may not be the source of IL-5 in sepsis. Furthermore, T cell and B cell deficient $Rag1^{-/-}$ mice have no difference in mortality or levels of IL-5 following CLP, establishing that T cells are unlikely the source of IL-5 (Bosmann et al., 2011). To

determine the source of IL-5, the intracellular cytokine staining of whole blood from septic patients should be examined. While this is the simplest way to begin, it is possible that the cellular source of IL-5 is localized to infected tissues or lymph nodes. This would make it more difficult to definitively assess the source. However, obtaining tissue sections from people who died from sepsis or from septic animals would still allow for IL-5 detection by this method.

The model (Figure 7.1) suggested by these data is that during sepsis, the IL-5Rα is induced *in vivo* on neutrophils and macrophages, possibly following LPS, CpG or IFNa stimulation. Moreover, there is release of IL-5 by some population(s) of cells. IL-5 signals through IL-5Rα expressed on neutrophils and macrophages in sepsis, and induces a variety of effector functions. IL-5 may recruit cells to the site of infection. There, IL-5 causes increased phagocytosis, survival, and release of inflammatory cytokines by macrophages. Data do not suggest that IL-5 increased neutrophil function in sepsis; however, IL-5R expression may provide another way to recruit neutrophils to the site(s) of infection. Ultimately, IL-5 is a viable therapeutic candidate for septic patients as a means to improve pathogen control and ultimately survival.

These data are very interesting when taking anti-IL-5 and anti-IL-5Rα therapies into consideration. Treatments to inhibit IL-5 and IL-5 signaling are being tested for use in asthmatic patients, as a means of reducing eosinophil recruitment into the lungs and the resulting airway hyperreactivity. These therapies have been modestly effective at reducing eosinophilia in patients, but may pose a threat to already

immunocompromised or suppressed patients. Since IL-5 is protective in sepsis, then the possibility remains that this cytokine is also beneficial in other diseases where its presence may have been overlooked. Indeed, the presence of IL-5 in other diseases needs to be addressed. This is highlighted by a recent study with anti-IL-5Rα treatment, which resulted in an unexpected reduction in neutrophils and monocytes (Busse et al., 2010). Data presented in this thesis demonstrate that these cells express this receptor in sepsis. While the anti-IL-5Rα study does not definitively establish the presence of this receptor on neutrophils and macrophages in asthma, it does suggest it. The functional consequences for this in asthma are not understood, and further *in vitro* human studies should be performed before additional clinical trials.

The detrimental role for IL-5 in eosinophilic disorders and asthma is apparent; however, patients typically have very heterogeneous backgrounds and various comorbidities. While inhibiting eosinophilia with these therapies might be beneficial, inhibition of IL-5 may negatively affect the ability of the host to control infection. The presence of the IL-5Rα on neutrophils and macrophages in sepsis indicates that receptor expression is not as specific as previously believed. As our knowledge of the immune system and disease advances, there is more overlap observed between leukocyte functions and the more complex the overall picture becomes.

It will be very interesting to see where the field of innate immunology goes in the next few decades. It seems that in the past, scientists have tried to draw discrete

boxes around cell types, and these boxes are associated with a specific phenotype and function. As research advances, we draw more boxes within boxes to represent several subsets of one cell type, again based on different but specific phenotypes and functions. Data presented in this thesis, in addition to numerous additional studies, suggest that this is not necessarily the best way to approach the field. Very often this results in inappropriate classification of cell populations as having very specific functions, as with eosinophils and helminth infection, or with various classifications of monocytes and macrophages. Moreover, observations like those made in this thesis will often be missed due to preconceptions of a cell or receptor's specific function. It seems the best way to approach research in this area would be to accept that there is more likely a range of phenotypes within a cell subset, and that phenotype can change based on cellular location, environmental cues, as well as the presence of a specific disease. While it makes research more complex, it ultimately improves our understanding of the immune response and allows for a better translation into humans of discoveries made in animals.

In summary, this thesis has established an important role for eosinophils and eosinophil granule proteins *in vivo* during bacterial infection. Data demonstrated that this effect is not through any alteration in the inflammatory milieu, but due to direct killing of bacteria. Finally, while there is a loss of eosinophils during sepsis, elevated IL-5 is protective through IL-5Rα expression on neutrophils and macrophages. Elevated IL-5 is protective in animals and humans with sepsis, and this is likely trhough augmenting macrophage function and enhancing neutrophil

recruitment. Importantly, depletion of macrophages *in vivo* eliminated the protective effects observed following IL-5 administration, indicating that macrophages are necessary for the protective effects of IL-5 *in vivo*. These data support the use of two novel immunomodulatory candidates: eosinophil granule protein administration in patients with bacterial infections as an adjuvant to antibiotic therapy; and IL-5 administration in septic patients to boost the innate immune response and improve host control of infection.

Figure 7.1: Model of IL-5 effects in Sepsis on Neutrophils and Macrophages

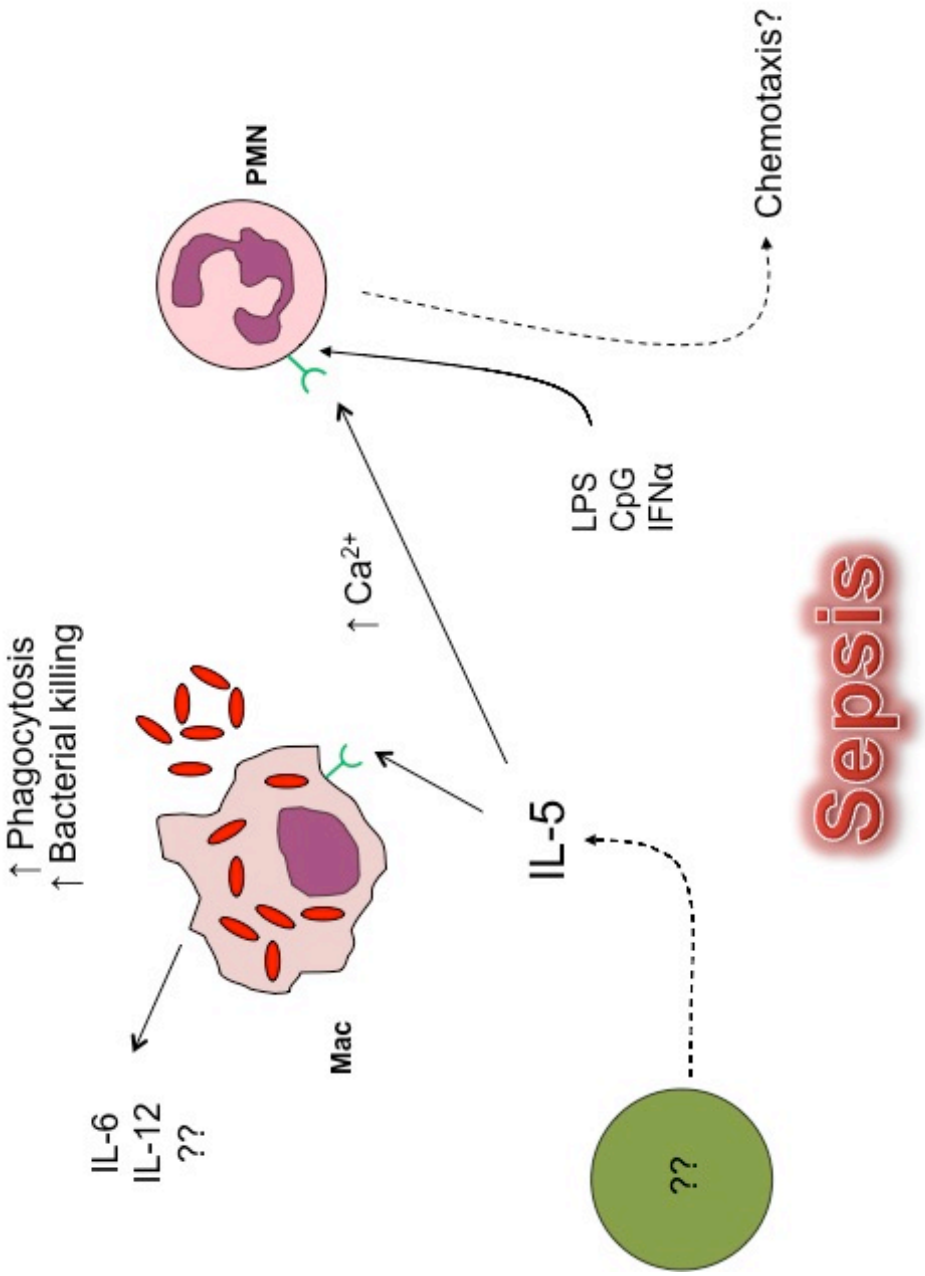


Figure 7.1: Model of IL-5 effects in Sepsis on Neutrophils and Macrophages

During sepsis, there is a release of IL-5 by some cell type, including T cells, epithelial cells, NK cells, or macrophages. IL-5 then signals through IL-5Rα expressed on neutrophils and macrophages in sepsis, by potentially recruiting cells to the site of infection, and causing increased phagocytosis and release of inflammatory cytokines by macrophages. It is not known specifically how the receptor is induced *in vivo*, but *in vitro* this can occur following LPS, CpG, or IFNα stimulation and signaling through the NF-B pathway. It doesn't appear that IL-5 increased neutrophil function in sepsis, however, IL-5R expression may simply be providing another way to recruit cells to the site of infection. At the very least, not augmenting neutrophil function ensures that IL-5 is not increasing tissue damage caused by these cells, which would be beneficial during sepsis. IL-5 treatment represents a good therapeutic candidate for septic patients as a means to improve pathogen control and ultimately survival.

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