Regulation of Sepsis and Endotoxic shock by Regulatory T cells

BY

EMEKA B. OKEKE

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Immunology

Faculty of Medicine

University of Manitoba

Winnipeg, Manitoba, Canada

Abstract

One of the major challenges facing clinicians in modern medicine is how to effectively manage excessive host immune response to pathogenic insults such as sepsis. This is demonstrated by the fact that despite over half-century research efforts, sepsis and its spectrum of diseases (severe sepsis and septic shock) are still associated with poor clinical outcome. Currently, sepsis is a leading cause of death in intensive care units.

The immune system protects the host against pathogens and is therefore armed with an arsenal of deadly ammunitions (including chemicals, cells and proteins) necessary for the elimination of microbes. It is therefore paramount that the immune system must develop mechanisms necessary to prevent destruction of the host it is designed to protect. A good example of such a mechanism is found in the subset of lymphocytes known as regulatory T cells (Tregs). There is unequivocal experimental evidence of the role of Tregs in the maintenance of immune homeostasis and self tolerance and aberrant Treg function has been linked with several inflammatory diseases. Since sepsis is a disease marked by a hyper-inflammatory state, I hypothesized that Tregs play a crucial role in dampening sepsis-induced excessive inflammation.

Using a murine model of lipopolysaccharide (LPS) infusion and bacterial infection, I show that Tregs are essential for survival during sepsis because their depletion leads to acute death to an otherwise non-lethal dose of LPS. This enhanced susceptibility to LPS following Treg depletion was also observed using live *E. coli* infection. Hence, using two models of sepsis, I unequivocally demonstrate that Tregs are important for survival during sepsis.

Next, I probed the mechanism by which Tregs protect against LPS challenge and bacterial infection. I found that defective Treg function leads to exaggerated activity of two immune cells

– $CD4^+$ effector T cells and neutrophils in response to LPS, leading to severe inflammatory response. Hence, this work successfully illustrates the critical role of Tregs in regulating other immune cells and the catastrophic consequences of defective Treg function during an immune response.

Tregs have long been known to regulate effector T cells but their regulatory effect on cells of the innate immune system is severely underexplored. Hence, the finding that Tregs regulate neutrophil activity and function is highly significant and illustrates an interesting mechanism of regulation of innate immune cells by cells of the adaptive immune system.

Overall, this work highlights the significant role of Tregs in the regulation of bacteria associated inflammatory processes. The findings hold implications for the successful management of sepsis and have potential for use in development of adequate therapeutic intervention for sepsis.

ACKNOWLEDGEMENTS

Getting to this point would have been twice difficult if not impossible save for the support I got along the way. First, I thank the Almighty who has brought me thus far.

I am grateful for the support, encouragement and supervision of my PhD supervisor, Dr. Jude Uzonna. He gave me his unflinching support and believed in me even when I didn't believe in myself. Under his supervision, I have grown to be an independent researcher and together we have made big contributions to Immunology.

A big thank you to my advisory committee members: Drs Abdelilah Soussi Gounni, Neeloffer Mookherjee and Prashen Chelikani. I sincerely appreciate your support, inspiration and mentorship the last few years and humbly count it as one of my many blessings. I also appreciate the mentorship of Dr. Redwan Moqbel as part of my advisory committee.

I wish to thank the members of Uzonna Lab both past and present, especially Ping Jia, Dong Liu, Ifeoma Okwor, Shiby Kuriakose, Forough Khadem, Nonso Onyilagha, Rani Singh and Olivia Ihedioha. You all provided a friendly environment suitable for the pursuit of academic and research excellence. We were like a family and our friendship and warm interactions will never be forgotten. I am also grateful for the support of all those who work at the fourth floor of the Apotex building. Indeed, it is an excellent work environment. In particular, I would love to thank the members of Gounni Lab especially Lianyu Shan for their support. I will also like to thank my gossip buddy Bill – it was always nice sharing the news together. I sincerely appreciate the efforts of the department office staff, Karen Morrow and Susan Ness. There was not a single time you people were not willing to help all these years.

I wish to acknowledge the agencies that funded my research. In particular, Children's Hospital Research Institute of Manitoba and Research Manitoba for supporting me all these years. Thank you to all the faculty members of the department of Immunology especially those who taught me. You all inspired me in one way or the other.

My deepest appreciation for my friends particularly those in Manitoba who made this journey less cumbersome, especially, Chibuike Udenigwe, Chukwudi Okoye, Ugochukwu Obodo, and Obinna Nwangwu. Your friendship and support over the years have made a significant positive contribution to my academic success.

I thank my family members for their love and prayers. I deeply appreciate the love and support of my wife Nkeiru. Thank you for journeying with me along the way. I want to express my thanks to members of the Okeke family for their support, my parents Boniface and Virginia, my siblings, Ifeoma, Ogochukwu, Okwudili, Chinyere, Chukwuma. Thank you for your love and prayers. I also wish to thank my aunts Josephine Obianika and Julie Anigbo for supporting me to reach this height of academic success. It is impossible to write the name of everyone who has been instrumental in the completion of this doctoral degree. Sincerely speaking, in whatever capacity you contributed, your help is deeply appreciated.

DEDICATION

I dedicate this accomplishment to the one who made the biggest sacrifice for my success – my mother

Virginia Obiageli Okeke

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENT OF COPYRIGHT FOR WHICH PERMISSION WAS OBTAINED

1. Chapter 3, texts and figures, Okeke et al, Shock July 2013; 40(1) 65-73. DOI: 10.1097/SHK.0b013e318296e65b. © 2013 by Wolters Kluwer Health Lippincott Williams & Wilkins

2. Chapter 4, texts and figures, Okeke et al, J Immunol. July 2014; 193(2) 655-662. DOI: 10.4049/jimmunol.1303064. © 2014 by American Association of Immunologists

LIST OF ABBREVIATIONS

ACCP	American college of chest physicians
APACHE	Acute Physiology and Chronic Health Evaluation II
APCs	Antigen presenting cells
BMDM	Bone marrow-derived macrophages
BMDN	Bone marrow-derived neutrophils
CCL	CC chemokine ligand
CD	Cluster of differentiation
CFSE	5- (6-) carboxyfluorescein diacetate succinimidyl ester
CHS	Contact hypersensitivity
CLP	Cecal ligation and puncture
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CVD	Cardiovascular disease
CXCL	CXC chemokine ligand
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagles Medium
ECs	Endothelial cells
ELISA	Enzyme linked immunosorbant assay
FOXP3	Forkhead box P3
GITR	Glucocortocoid-induced tumor necrosis factor receptor-family related genes
GM-CSF	Granulocyte macrophage colony-stimulating factor
HIV	Human immnodeficiency virus
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
i.p	Intraperitoneal
ICAM 1	Intercellular adhesion molecule 1
ICU	Intensive care unit
IFN-γ	Interferon gama
IκB	Inhibitor of κ light chain gene enhancer in B cells
IKK	IκB kinase
IRAK-4	IL-1 receptor-associated kinase-4
IRF3	Interferon regulatory factor 3
LAG-3	lymphocyte activating gene-3
LBP	LPS binding protein
LFA 1	Lymphocyte function-associated antigen 1
mAb	Monoclonal antibody
Mal	MyD88-adapter-like
MAMPs	Microbial-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidase
MHC	Major histocompatibility complex

MyD88	myeloid differentiation primary response gene 88
NETs	Neutrophil extracellular traps
NF-kB	Nuclear factor-κB
NK cells	Natural killer cells
NOD-LRR	Nucleotide-oligomerization domain leucine-rich repeat
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3 Kinase
PMA	Phorbol myristate acetate
PMNs	Polymorphonuclear neutrophils
PRRs	Pattern recognition receptors
RAG1	Recombination activating gene 1
RIP1	Receptor-interacting protein 1
RPMI	Rosewell Park Memorial Institute
SARM	sterile α and HEAT-Armadillo motifs-containing protein
SCID	Severe combined immunodeficiency
SIRS	Systemic inflammatory response syndrome
TAK1	Transforming growth factor-β-activated kinase 1
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK binding kinase 1
TCR	T cell receptor
Th	Helper T cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β
UBC 13	Ubiquitin-conjugating enzyme 13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1 isoform A
VCAM 1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WT	Wild type

1.0 CHAPTER ONE

INTRODUCTION

1.1 Sepsis, severe sepsis and septic shock

The word sepsis appeared in literature as early as 8th Century B.C and literally means decay of organic matter in the presence of bacteria (1). Since then, the interpretation of the word has not really changed. In 1991, the American College of Chest Physicians (ACCP) laid out terms for the definition of sepsis (2). Sepsis was subsequently defined as a systemic response to infection. If this response occurs in the absence of infection, the condition was described as systemic inflammatory response syndrome (SIRS). Sepsis resulting in organ dysfunction is termed severe sepsis while septic shock is sepsis with acute circulatory failure and hypotension. Over the years, increase in knowledge of the pathophysiology of sepsis and the heterogeneity of the clinical presentation of the syndrome have highlighted these definitions as broad and unspecific. In 2001, the International Sepsis Definition Conference (ISDC) was convened with the intention of changing the definition of sepsis and basing it on biomarkers. However, participants at the conference agreed to let the definition of sepsis by ACCP stand and couldn't agree on a reliable biomarker for sepsis. However, for the ease of sepsis diagnosis, they provided a staging system for sepsis called **PIRO** that stratified patients on the basis of their **P**redisposing conditions, the nature and extent of the **I**nsult (**I**nfection, in the case of sepsis), the nature and magnitude of the host **R**esponse, and the degree of accompanying **O**rgan dysfunction (3). This staging system for sepsis is very basic and relies heavily on the judgment of the physician.

1.2 Incidence of sepsis

It is difficult to estimate the global burden of sepsis as data is nearly non-existent from developing countries but it is estimated that there are 19 million cases of sepsis worldwide annually (4). In Canada, there were a total of 30,587 sepsis hospitalizations (excluding the province of Quebec) between 2008-2009 (5). This represents a 14 percent increase from 2004-2005. In the United States, between 1979 and 2000, the number of reported cases of sepsis increased from about 164,000 cases per year to about 660,000 cases per year, representing an annual increase of nearly 9% (6). A more recent study indicates approximately 13% national increase in the incidence of severe sepsis in the United States (7). Also severe sepsis is responsible for about 2% of hospital admissions and 10 percent of admissions in the intensive care unit (8). Similar data have been reported in other developed countries (9). Currently, sepsis is a leading cause of death in intensive care units (ICU) (8). Between 2008 and 2009, sepsis accounted for 10% of all hospital deaths in Canada (excluding Quebec) (5). However, there is evidence of the success of the surviving sepsis campaign and mortality from sepsis has decreased in recent years. A retrospective study of patients with severe sepsis in Australia and New Zealand revealed that between 2000 and 2012, mortality decreased from 35% to 18.4% representing nearly 17% decrease in mortality (10). Besides mortality, sepsis has a significant socio-economic burden since survivors of sepsis have been shown to have a lower quality of life compared to the general population and the disease has a huge financial cost estimated at nearly $17 billion annually (8, 11). Studies have shown greater incidence of sepsis in males compared to females and in non-Caucasians compared to Caucasians (8, 12).

1.3 Causes of sepsis

Sepsis usually results from bacterial infections although the cause can be due to viral or fungal infections. Lung infections and pneumonia are the most common causes of severe sepsis followed by intra-abdominal and urinary tract infections (8). Recent studies have shown that Gram-negative bacteria are the major cause of sepsis (13). This is in contrast to previous studies which identified Gram-positive infections as the most common cause of severe sepsis (6). The increase in Gram-negative infections may be due to increase in antibiotic resistance. Gram-negative bacteria unlike Gram-positive organisms have an outer membrane which can further inhibit the entry of antibiotics and microbicides thereby promoting antibiotic resistance. Frequently identified Gram-negative organisms include *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* species (14). For Gram-positive species, *Staphylococcus aureus* and *Streptococcus pneumoniae* are the most common isolates (14).

Certain factors are known to increase the risk for development of sepsis; for example, the presence of chronic illness such as HIV infection or cancer (8). Recently, there is interest on the role of genetic factors towards susceptibility to infection which will be of significant contribution towards effective diagnosis and treatment of sepsis (15). For example, Garred et al showed that allelic variants of mannose binding lectin (MBL) a major innate defense molecule is associated with susceptibility to sepsis (16). Also, Wang et al identified single nucleotide polymorphisms of TLR4 and CD14 that were associated with greater risk of sepsis in Chinese population (17). The identification of genetic susceptibility factors in sepsis is highly desirable as this will assist in the delivery of appropriate therapy that is tailored to individual need of patients with sepsis.

1.4 Diagnosis of sepsis

The clinical signs of sepsis vary and depend on several factors like the type of inciting pathogen, the site of infection and patient predisposition. The ACCP has guidelines for sepsis diagnosis (3). However, the use of factors such as body temperature, white blood cell count and hypotension is largely unspecific for sepsis and many clinicians have found the ACCP guidelines remotely helpful (18). Early diagnosis of sepsis has been shown to be important for survival (19). The use of a biomarker for accurate diagnosis of sepsis will be a landmark achievement in sepsis therapy. Preliminary studies highlight procalcitonin (PCT) as a potential biomarker for sepsis. Procalcitonin is a precursor peptide of calcitonin composed of 116 amino acids and is produced by the C cells of the thyroid gland. Proteolytic cleavage of PCT gives rise to calcitonin. PCT is a more desirable biomarker compared to calcitonin because PCT has a longer half-life. Serum concentration of PCT has been shown to correlate with the severity of infection and PCT has been demonstrated to be a potential reliable biomarker for sepsis (20-21). However, in their meta-analysis review of literature, Tang et al showed a low diagnostic performance for PCT and further demonstrated that PCT measurement could not accurately differentiate sepsis from systemic inflammatory response syndrome (22). Advances in technology hold promise for the possibility of a reliable biomarker for sepsis. A biomarker able to establish the progression of sepsis to severe sepsis and septic shock will be ideal. The use of multiple parameters may improve accuracy and reliability compared to a single biomarker as shown by the work of Gibot and co-workers (23).

1.5 Treatment options for sepsis

The international consortium of critical care physicians recently released revised guidelines for treatment and management of sepsis (24). Concisely, the guideline consists of two main parts: an early management therapy encompassing the first six hours of patient arrival and guideline for management of patient in ICU. These guidelines have been shown to be effective in reducing sepsis mortality (25). Containing infection is critical in sepsis therapy and rapid implementation of antibiotic therapy has been shown to reduce sepsis mortality (26). It is recommended that blood cultures be done prior to the administration of antibiotics. Normalization of respiration and blood pressure is essential in the early management therapy. This usually encompasses the use of vasopressors, intravenous fluid administration, and the use of mechanical ventilation if necessary. In the ICU, prevention of organ dysfunction and avoidance of other complications is the major goal. It is also essential to monitor the progress of the patient and note when care such as antibiotic treatment should be withdrawn. This ensures the safety of the patient and prevents the development of antibiotic resistance.

One of the earliest therapies for sepsis treatment was the administration of corticosteroids (27). However, the beneficial effects of corticosteroid therapy for patients with sepsis are an ongoing debate. The administration of high or low dose of corticosteroids has been shown to be beneficial in animal models of septic shock (28-29). Several reports have also shown the beneficial effects of corticosteroid therapy for septic shock patients (30-31). In a recent review, Annane et al reported a significant improvement in survival of septic shock patients given low doses of corticosteroids (32). The use of hydrocortisone is recommended for treatment of patients with septic shock (24).

The discovery of new therapies for sepsis based on the abundant knowledge of the pathophysiology of the disease has proven to be an arduous task. More than 40 clinical trials of anti-inflammatory therapies were not successful to be approved as standard of care. Systemic inflammatory response to microbes involves the activation of inflammatory mediators. The inflammatory response leads to tissue injury and subsequent activation of the coagulation pathway in addition to down-regulation of anticoagulant effects.

In November 2001, Drotrecogin alfa was approved for the treatment of patients with severe sepsis at a high risk of death (Acute Physiology and Chronic Health Evaluation II, APACHE ≥ 25). Drotrecogin alfa was the first biological agent to be approved for the treatment of severe sepsis in the United States and this was seen as a breakthrough in critical care medicine. Drotrecogin alfa (Xigris, Eli Lilly and Company) is recombinant human activated protein C (APC). APC is an anticoagulant protein that has also been shown to have anti-inflammatory and anti-apoptotic properties (33-34). Drotrecogin alfa (activated) was initially shown to result in 19.4% decrease in the relative risk of death and 6.1% decrease in the absolute risk of death although this was associated with a greater risk of serious bleeding (35). However, controversies trailed the administration of the drug and its approval by the FDA. A major concern was the serious bleeding complication associated with the drug. Also, prescription of Drotrecogin alfa was dependent on APACHE scoring system, which varies among clinicians. In addition, the drug was shown to be of no benefit to patients at a low risk of death (36). Ten years later, following extensive review of several clinical outcomes, Drotrecogin alfa was determined to be of no benefit to patients with severe sepsis and clinicians were advised not to recommend its use (37). In October 2011, Eli Lilly announced the withdrawal of the drug from the market. The outcome

of treatment with APC buttresses the fact that clinical trials involving patients with sepsis are difficult to interpret and reproduce.

Statins are a class of drugs that inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), an essential enzyme in the mevalonate pathway for the synthesis of cholesterol. Since increased levels of LDL-cholesterol are associated with increased risks of cardiovascular diseases (CVD) (38), which are leading causes of mortality and morbidity, statins were discovered in a bid to interfere with cholesterol synthesis (39). Hence, statins were first marketed as drugs for the prevention and treatment of CVD. Two decades after its discovery, statins have turned out to be a 'wonder pill' grossing the most revenue in pharmaceutical history and having reported benefits for several diseases like sepsis (40), diabetes (41) and cancer (42). Several experimental and clinical studies have shown some beneficial effects of statins in sepsis. Merx et al showed that administration of statin either before or after the onset of sepsis improves survival in a murine model of CLP (43). In the clinical setting, the use of statins in critical care is rapidly increasing and has been recently reviewed (44). In a retrospective cohort study, Dobesh et al showed that patients that received statin therapy had a 35% relative reduction in mortality compared with those that didn't (45). Other authors have also reported the protective effect of statins in sepsis (46-47). Conversely, in a recently concluded multicenter randomized clinical trial, Kruger et al showed that there was no difference in IL-6 concentrations in plasma of patients given statins or placebo group (48). They noted that individuals in the group identified as prior statin users had lower baseline IL-6 levels and discontinuation of statin led to greater mortality in this sub-group while continuation of statin use led to improved survival. The authors concluded that the use of statin did not lead to better outcome except for the sub-group of prior statin users. Interestingly, the same group had earlier reported that there was no beneficial role of

continuing preexisting statin therapy on sepsis and inflammatory parameters (49). In another study, Fernandez et al showed that in patients at high risk of ICU-acquired infections, statin therapy was associated with worse outcome (50). These conflicting reports highlight the necessity of properly defining the criteria for clinical trials in sepsis. In the meantime, continued investigation on the role of statins in sepsis is essential.

Since sepsis is a heterogenous and dynamic syndrome, modulation of the immune system may proffer better therapy for patients with sepsis and this is a major consideration of this work.

1.6 Pathophysiology of sepsis

Following invasion of a host by a pathogen, the host immune defense mechanisms are activated in order to protect it from injury associated with the pathogenic insult. The immune response involves activation of immune cells, recruitment of leukocytes, and vascular changes including increased permeability of the endothelium and vasodilation (51-52). Simultaneously, there are regulatory mechanisms that help to prevent excessive immune responses and ensure that the immune system returns to homeostasis following pathogen containment. Failure of these immune regulatory mechanisms will result in excessive immune activation leading to an extended pro-inflammatory immune state, which will ultimately result in tissue injury and organ dysfunction (53). This failure in immune regulatory mechanisms can also result in extended anti-inflammatory state leading to anergy and immunosuppression (54).

Microorganisms such as bacteria, fungi, viruses and parasites have unique molecular components which, when recognized by the host, trigger host immune responses. These molecular components have been aptly described as pathogen-associated molecular patterns (PAMPs) or more appropriately microbial-associated molecular patterns (MAMPs) because they are also

present in non-pathogenic microorganisms (55). The ligation of PAMPs by germ-line encoded pattern recognition receptors (PRRs) leads to the initiation of a series of host immune responses aimed at eliminating the pathogen. There are four classes of PRRs: 1) toll-like receptors (TLRs); 2) nucleotide-oligomerization domain leucine-rich repeat proteins (NOD-LRR); 3) cytoplasmic caspase activation and recruiting domain helicases such as retinoic acid-inducible gene I-like helicases and 4) C-type lectin receptors expressed on dendritic and myeloid cells (56-57). TLRs are the most studied PRRs and are expressed by different innate immune cells including endothelial cells, macrophages, dendritic cells and monocytes. TLR4 is the ligand for lipopolysaccharide (LPS), an endotoxin found on the cell wall of Gram-negative bacteria. Following recognition of LPS by TLR4, several intracellular signal transduction pathways are activated resulting in hemodynamic changes and the recruitment of immune cells such as neutrophils and macrophages to the site of infection. Additionally, there is systemic release of cytokine mediators such as tumor necrosis factor (TNF), Interleukin (IL)-1, IL-6 and IL-8 (58-59). TNF and IL-1 aid in activation of the complement system and also facilitate vasodilation and the release of secondary mediators such as nitric oxide (NO), platelet activation factor (PAF), prostaglandins and leukotrienes (60-61). In a situation of sustained pro-inflammatory immune state, these mediators cause endothelial damage and capillary leakage. The inflammatory response also leads to the activation of the coagulation pathway, resulting in capillary microthrombi, tissue hypoperfusion and end-organ ischemia (62-63). The extended release of these pro-inflammatory mediators also results in multiple organ dysfunction syndrome (MODS) as seen in severe sepsis and eventually leads to hypotension and septic shock.

To ensure that the immune system returns to homeostasis, anti-inflammatory mediators such as IL-4, IL-10 and transforming growth factor β (TGF-β), are also released by immune cells such as

neutrophils and macrophages in response to infection. Studies have shown that these anti-inflammatory mediators contribute to pathology during sepsis and there is evidence that septic shock patients show signs of immunosuppression (64-65). This is because homeostasis can only be achieved if these anti-inflammatory mediators are released in optimum amounts. The excessive release of anti-inflammatory mediators will inevitably weaken the host immune responses and lead to the inability of the host to fight the infection. The contribution of anti-inflammatory response to the pathogenesis of sepsis has been described as compensatory anti-inflammatory response syndrome (CARS) (64). IL-4 is known to promote Th2 cell responses, inhibit Th1 cell activity and suppress macrophage activity (66-67). IL-10 has been shown to have potent anti-inflammatory activity (67-68). TGF-β has been shown to suppress T and B cell proliferation and differentiation (69-70), and prevents the production of TNF and IL-1-induced NO production (71). In line with this, TGF-β knock-out mice have fatal uncontrolled inflammatory reactions (72). Overall, a high anti-inflammatory response leads to a weakened immune state, inability to clear the infection and enhanced susceptibility to nosocomial or secondary infections.

As mentioned earlier, knowledge of the pathophysiology of sepsis has failed to provide effective therapeutic strategy for sepsis. However, research is ongoing in this regard to translate these discoveries to novel therapies on the bedside. It is expected that increase in knowledge and advances in technology will lead to effective and efficient treatment of sepsis syndrome.

1.7 Immune response to sepsis

The development of the germ theory was a landmark achievement in the understanding of the etiology of several diseases. The understanding of the role of bacteria in the initiation of sepsis led to decades of research efforts in the development of antibiotic therapies for the treatment of sepsis. In the later part of the 20th century, as a result of a better understanding of the nature of the immune response, there began to appear a shift in paradigm implicating the immune response as the mediator of sepsis. Indeed, considering the vast majority of microbes on the planet and the diverse species of microorganisms that maintain a symbiotic association with a healthy individual, the presence of the microbe alone is not sufficient to account for the pathogenesis of sepsis and it is now generally accepted that the immune response plays a crucial role (73-75). In his seminal paper, Lewis Thomas highlighted the significance of the immune response to the outcome of infection (73). According to Thomas, "the micro-organisms that seem to have it in for us in the worst way — the ones that really appear to wish us ill — turn out on close examination to be rather more like bystanders, strays, strangers in from the cold. They will invade and replicate if given the chance, and some of them will get into our deepest tissues and set forth in the blood, but it is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are in more danger from them than from the invaders. We live in the midst of explosive devices; we are mined". Although the immune response is intended to protect the host from the detected 'danger', Thomas describes it as a 'panic response' leading to the release of several molecules that are potentially lethal to the host. The factors that constitute a 'good' or 'bad' immune response still remain the subject of several ongoing research projects around the world and are also the thrust of this PhD thesis. Below, the role of immune cells in the pathogenesis of sepsis is described in greater detail.

1.8 The role of innate immunity

The innate immunity is the 'gatekeeper' of the host immune response and plays an important role in the pathogenesis of sepsis. The significance of the innate immune system for anti-bacterial host defense is further buttressed by the fact that organisms lacking adaptive immunity still maintain strong anti-bacterial immunity (76). The detection of PAMPs by the PRRs of the innate immune system triggers the initial host inflammatory response to infection. The most studied PRRs are the TLRs. The Toll receptor was initially described as a type I transmembrane receptor important for embryogenesis in Drosophila (77). The identification of homologues of the Drosophila Toll in mammals was a landmark achievement in the understanding of the immune response to microbial components. To date, more than 10 human TLRs have been identified (78). Like the Drosophila Toll, all members of the Toll family are transmembrane proteins and have extracellular domains with characteristic leucine-rich repeats (79). However, there are distinct differences in the sequences of different members, hence, their ability to recognize different molecules. The intracellular cytoplasmic domain – the Toll/IL-1 receptor (TIR) domain, which contains about 200 amino acids is evolutionarily conserved (79). Gram-negative bacteria accounts for half the cases of sepsis and the bacterial endotoxin LPS is one of the most potent activators of the immune system (80).

LPS is important for the structural integrity of Gram-negative bacteria and consists of three components – lipid A, a core oligosaccharide, and an O side chain (81). TLR4 is the PRR for LPS (82). The recognition of LPS through TLR4 involves the interaction of several proteins including LPS binding protein (LBP), CD14 and MD-2 (83-84). MD-2 is a protein that is non-covalently associated with TLR4 forming the TLR4/MD-2 receptor complex. There is evidence that MD-2 can directly interact with LPS in the absence of TLR4 (85). LBP binds directly to LPS

leading to the recruitment of CD14 and subsequent activation of the TLR4/MD-2 receptor complex. Following LPS recognition, TLR4 oligomerizes and recruits downstream adaptor proteins through its TIR domain. Five TIR domain-containing adaptor proteins have been identified: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as Mal, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and HEAT-Armadillo motifs-containing protein) (86). Genetic manipulations of these adaptor proteins in animal models have resulted in understanding of their individual roles in TLR signaling.

MyD88 is an important adaptor protein for IL-1 receptor (IL-1R) signaling pathway (87). Studies demonstrated that MyD88-deficient mice were resistant to LPS-induced septic shock and MyD88-deficient macrophages were defective in proinflammatory cytokine production following LPS stimulation (88). However, MyD88-deficient macrophages were able to activate nuclear factor-κB (NF-κB) and induce the expression of Type I interferons genes indicating an alternative pathway to MyD88/TLR signaling (89). TLR signaling is now known to proceed via a MyD88-dependent pathway or MyD88-independent pathway (84).

MyD88 adaptor protein has a death domain (DD), and following LPS stimulation, activates a death domain-containing kinase, IL-1 receptor-associated kinase-4 (IRAK-4), which contains both a death domain and a kinase domain. IRAK-4 deficient macrophages are also resistant to LPS-induced septic shock and are defective in proinflammatory cytokine production following LPS stimulation (90). Downstream of IRAK-4, is the adaptor protein TRAF6 (TNF receptor-associated factor 6), which forms a complex with UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1 isoform A), leading to the activation of

TAK1 (transforming growth factor-β-activated kinase 1) (91). The activation of TAK1 results in the activation of IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways (91). Downstream activation of MAPK pathway leads to the induction of the transcription factor Ap-1 (92). IKKα, IKKβ and IKKγ form a complex and phosphorylate IκB (inhibitor of κ light chain gene enhancer in B cells) proteins. The phosphorylation of IκB results in the degradation of IκB proteins and the translocation of the transcription factor NF-κB. NF-κB is a prominent transcription factor which controls the gene expression of several immune-mediating proteins and proinflammatory cytokines (84).

There is evidence suggesting that under certain conditions, LPS/TLR4 signaling can occur independent of MyD88 signaling. Macrophages deficient in MyD88 are able to induce NF-κB and MAPK activation although they do not produce proinflammatory cytokines (88). The adaptor protein TRIF plays an important role in MyD88-independent signaling through the activation of interferon regulatory factor 3 (IRF3), NF-κB and MAPK. TRIF has a C-terminal region through which it interacts with receptor-interacting protein 1 (RIP1). RIP1 is important for NF-κB activation and absence of RIP1 impairs NF-κB activation (93). Studies have shown that TRIF activates IRF3 through the recruitment of TRAF3. In line with this, TRAF3 deficient cells have defective induction of Type I interferons (94). TRAF3 signals downstream through TRAF family member-associated NF-κB activator (TANK), TANK binding kinase 1 (TBK1) and IKKi (95). The activation of IRF3 and NF-κB leads to the induction of type I interferons, which are essential for anti-bacterial responses (96). The binding of LPS to TLR4 and its downstream signaling effects triggers the effector functions of innate cells discussed below in greater detail.

1.8.1 The role of neutrophils

Polymorphonuclear neutrophils (PMNs) are one of the first responder cells of the innate immune system to bacterial infection and inflammation and constitute a hallmark of innate immunity (97-99). PMNs are the most abundant type of leukocytes in humans and express all known TLRs except TLR3. The ligation of TLR4 on neutrophils by LPS leads to the activation of signal transduction pathways that lead to increased cell survival, chemokine and cytokine production, phagocytosis and the production of enzymes like myeloperoxidase (MPO), which aid in potent bactericidal activity. Hence, inadequate neutrophil function is associated with poor outcome in sepsis (100-101). Paradoxically, neutrophil effector functions can be injurious to host cells and exaggerated neutrophil activity is detrimental in sepsis (102-103). Therefore, the challenge is to maintain optimum neutrophil function during sepsis.

Neutrophil migration to infection foci involves distinct phases: mobilization, margination, adherence and transmigration through the walls of the blood vessel (101). The bone marrow is a reservoir for both immature and mature neutrophils and neutrophils in the bone marrow are rapidly mobilized for release into the circulation during the early phase of sepsis (104). Margination involves interaction between the neutrophil and endothelial cells resulting in the movement of the neutrophil from the central circulation stream to the vessel periphery. The interaction of neutrophils with endothelial cells is mediated by a class of surface adhesion molecules called selectins. Neutrophils constitutively express L-selectin while endothelial cells express E- and P- selectin upon activation. Neutrophil activation also leads to the expression of β integrins, which facilitates the adhesion of neutrophils to the vascular endothelium. The last stage of neutrophil migration is transmigration and it involves the movement of neutrophils through endothelial tight junctions into infected tissue (105). Neutrophil transmigration requires

a difference in concentration gradient of chemokines and inflammatory mediators across the endothelial barrier leading to extravasation of neutrophils to infected foci.

PMNs are one of the first responder cells of the innate immune system and neutrophil effector functions are critical for the resolution of bacterial infection. Studies have shown that PMNs are a source of several cytokines and chemokines including TNF (106), IL-1β (107), IL-8 (108), MIP-1α (109), GM-CSF (110) and many other cytokines (111). Hence, neutrophils play a major role in shaping the host immune response. For example, the production of TNF and MIP-1α, which are potent macrophage activators, implies that neutrophils facilitate macrophage activation. Also, the production of the chemoattractant IL-8 by neutrophils at the infection foci will lead to the recruitment of more leukocytes to the site of infection. Recently, the immunoregulatory functions of neutrophils are being investigated. Interestingly, neutrophils have been shown to produce the immunoregulatory cytokines IL-10 (112) and TGF-beta (113), two cytokines that are associated with CD4+CD25+ regulatory T cells (Tregs). Hence, neutrophil/Treg interaction may be a major pathway of immune regulation and this is one of the major focuses of this thesis. Since sepsis is a disease marked by disequilibrium in inflammatory and anti-inflammatory mediators and neutrophils are major initiators of the inflammation milieu, neutrophil dysfunction following bacterial infection will contribute to the pathogenesis of sepsis.

A prominent mechanism by which neutrophils eliminate bacteria is a process called phagocytosis. Phagocytosis literally means ‘cell eating’ and is a process by which phagocytes engulf bacteria forming a vesicle called phagosome. The phagosome fuses with the lysosome to form a phagolysosome in which the bacteria is degraded (114). The formation of phagolysosome triggers a process called respiratory burst, which involves the production of reactive oxygen species for antibiotic activity. During respiratory burst, oxygen is converted to superoxide (O^{2-})

and hydrogen peroxide (115). Myeloperoxidase (MPO), an enzyme abundant in neutrophils, catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ion. Hypochlorous acid is a microbicide and has potent antibacterial activity. Phagocytosis is critical for the resolution of infection and defects in oxidative metabolism of neutrophils causes chronic granulomatous disease (116). Chronic granulomatous disease is characterized by the formation of granulomas due to an apparent failure to resolve inflammation. Patients with chronic granulomatous disease are susceptible to bacterial and fungal infections.

Recently, another important method of neutrophil antibacterial activity was discovered. Neutrophils form extracellular fibers called neutrophil extracellular traps (NETs) for binding and killing pathogens (117). NETs contain proteins from azurophilic granules like neutrophil elastase, cathepsin G, MPO and histones. Neutrophils trap the pathogen and then mediate killing through the release of antimicrobial proteins. Meng et al demonstrated that inhibition of NETs formation enhances susceptibility to sepsis in mice (118). Paradoxically, NETs formation also leads to tissue injury due to the release of cytotoxic proteins that can be injurious to bystander cells (119). Overall, neutrophil response to bacteria is aimed at eliminating the bacteria but can unavoidably lead to tissue injury and host damage.

1.8.2 The role of monocytes/macrophages

Monocytes are derived from myeloid progenitor cell lineage. They are mononuclear phagocytic cells circulating in the blood stream. Monocytes migrate to tissues and differentiate into macrophages. They can also differentiate into dendritic cells and osteoclasts. Monocytes are also one of the first responder cells of the innate immune system. They migrate to the point of infection through mechanisms similar to those of neutrophils described above. The chemokine

(C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 9 (CXCL9) are important for the recruitment of monocytes during inflammation (120). Tissue resident macrophages can acquire specific characteristics and last for several years. Examples include Kupffer cells in the liver and alveolar macrophages. Interestingly, their function and phenotype is also influenced by the stimuli and microenvironment. The activation of macrophages by the cytokine IFN-γ is described as classical activation and such macrophages are called M1 macrophages. The activation of macrophages by the cytokines IL-4, IL-10 or IL-13 is described as alternative activation and such macrophages are called M2 macrophages (121). M1 macrophages are usually associated with effective immune response against pathogen invasion. In contrast, M2 macrophages are associated with an anti-inflammatory phenotype. For example, tumor associated macrophages and macrophages involved in wound healing are M2 type macrophages.

Similar to neutrophils, macrophages are major players in the immune response to infection. They secrete a lot of cytokines like TNF, IL-1β, and IL-6 (122). They are also able to engulf bacteria by phagocytosis. Additionally, macrophages help in maintaining immune homeostasis through the phagocytosis of dead or apoptotic cells. Also, they are antigen presenting cells and match antigenic peptides to their major histocompatibility complex class II molecules for the activation of antigen specific T cells (123). Hence, macrophages help in the shaping of the adaptive immune response and serve as a link between innate and adaptive immunity.

As a major component of innate immunity, macrophages are important in the pathogenesis of sepsis. Studies have shown that the bacterial endotoxin LPS regulates more than 1000 genes in mouse macrophages (124). Also in human monocytes, LPS stimulation leads to upregulation of several chemokine and cytokine genes (125). Hence, dysfunction in macrophage activity will be of significant consequence in sepsis. Studies have shown that prior exposure of macrophages to

LPS results in downregulation of proinflammatory cytokine production upon subsequent exposure to the endotoxin (126). This phenomenon termed endotoxin tolerance is a teleological approach towards prevention of self-toxicity by overwhelming proinflammatory response to LPS. Endotoxin tolerance may also be of consequence during sepsis. Monocytes from patients with sepsis have been shown to express decreased levels of HLA-DR (127). This decreased monocyte HLA-DR expression is a hallmark of the anti-inflammatory response in sepsis and is believed to lead to immune paralysis and lethality (128). Hence, macrophages are relevant in the pathogenesis of sepsis as they can contribute to the proinflammatory and anti-inflammatory stages of sepsis.

1.8.3 The role of endothelial cells

The endothelium is a significant player in the pathogenesis of sepsis and endothelial dysfunction is a major contributor to sepsis-induced organ dysfunction (129). Endothelial cells express TLR4 and are therefore activated by LPS resulting in downstream activation of NF-κB as described above (130). The activation of endothelial cells by microbial components leads to their secretion of pro-inflammatory cytokines which contribute to the inflammation milieu (131). Additionally, cytokine production by immune cells also leads to activation of endothelial cells (129). Therefore microbial components and immune cell cytokine secretion both constitute dual mechanisms of endothelial cell activation which is an integral mechanism of the immune response to sepsis.

Endothelial cells (ECs) are important for the recruitment of leukocytes to the sites of infection. They express two important adhesion molecules: intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) which are key ligands that interact with integrins on leukocytes and thereby mediate leukocyte migration. ICAM 1 binds to LFA-1

(lymphocyte function-associated antigen 1) on leukocytes while VCAM-1 binds to VLA-4 (very late antigen 4) also expressed on leukocytes (132). Additionally, ECs express other adhesion molecules such as E-selectin which are important in leukocyte migration (133). The expression of E-selectin by ECs is markedly increased upon activation by LPS or cytokines such as TNF and enables leukocyte rolling to infection site (134). Therefore, the regulation of the expression of adhesion molecules by ECs is critical to facilitate optimum immune response to infection via leukocyte recruitment and migration.

In addition to mediating leukocyte migration, ECs help in the maintenance of vascular integrity and sepsis is associated with increase in vascular permeability. ECs provide a permeable barrier for the exchange of fluids, nutrients and molecules between the vascular system and tissues. LPS and cytokines like TNF increase vascular permeability of the endothelium which can result in excessive fluid accumulation in tissues (edema) (135). The increase in vascular permeability of ECs is thought to be mediated by NO produced by ECs (136).

Under normal conditions the endothelium is in an anti-coagulatory state. During sepsis, the endothelium is shifted towards a pro-coagulatory state and disseminated intravascular coagulation contributes to sepsis mortality (63). LPS is known to increase coagulation factors in the endothelium. For example, LPS injection has been shown to result in increased fibrin deposition in various organs (137). Since, the endothelium plays an important role in the pathogenesis of sepsis, preservation of its integrity is critical for survival of sepsis and endothelial dysfunction is a major contributor to sepsis mortality.

1.9 The role of adaptive immunity

The receptors for microbial products such as LPS have been traditionally associated with cells of the innate immune system. Although the injection of LPS in mice or humans leads to rapid mobilization of cells of the innate immune system, the adaptive immune system is also involved in the immune response to sepsis. To begin with, the cytokines secreted by innate immune cells modulate the functions of cells of the adaptive immune system (138). Also, innate immune cells like macrophages present antigen for the activation of T cells. Furthermore, there is emerging evidence of expression of PRRs on cells of the adaptive immune system. Several reports have shown that T cells express different TLRs. The expression of TLRs by T cells is of great significance because it implies they may also function as early responders to bacterial infection and is a major part of this thesis research. Additionally, there are some adaptive immune cells that are at the intersection of innate and adaptive immunity. Good examples include the γδ T cells, which are able to recognize antigen directly and the natural killer T (NKT) cells.

As has been discussed above, the innate immune system provides the first line of defense and is essential for host protection from microbial invasion. However, an acquired immune response is essential for the absolute elimination of pathogen. The adaptive immune response develops over several days following antigen presentation by antigen presenting cells to T cells via major histocompatibility (MHC) complex molecules. Antigen presentation and recognition occurs in lymphoid organs and following antigen recognition, naïve T cells differentiate and proliferate as effector cells and modulate immune response via secretion of cytokines and other effector functions. The role of T cells in the immune response to sepsis is discussed below.

1.9.1 The role of T cells

$CD4^+$ T cells are called helper T cells because of their prominent role in aiding the optimum performance of other cells through cytokine secretion or by direct interaction. For example, $CD4^+$ T cells secrete IFN-γ, which is a major activator of macrophages. Also, activated $CD4^+$ T cells express CD40 ligand (CD40L) and interact with macrophages through CD40/CD40L interaction.

Two distinct subsets of helper T (Th) cells have been identified in both humans and mice – Th1 and Th2 cells (139). Th1 and Th2 cells are distinguished by their cytokine profiles and have been shown to have different functions. Th1 cells produce IFN-g and IL-2 while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (140). Th1/Th2 polarization is determined by several factors including cytokines, type of antigen, dose of antigen and other factors like host genetic composition. Studies have shown that $CD4^+$ T cells stimulated in the presence of IFN-γ and IL-12 develop into Th1 cells (141) while the stimulation of $CD4^+$ T cells in the presence of IL-4 yields Th2 cells (142). Th1 responses have been shown to be essential for the successful elimination of infectious agents whereas Th2 responses are essential for immunity against some parasitic infections but are generally ineffective for the immune control of several infectious agents. In sepsis, Th1 responses are associated with pathogen clearance and improved survival (143-144) whereas Th2 responses are known to contribute to immune suppression and poor outcome (145).

$CD4^+$ T cells are generally known to contribute to host defense during sepsis. RAG1 KO mice which lack T and B cells have increased bacterial burden and increased mortality following CLP compared to WT mice (146). Reconstitution of RAG1 KO mice with T and B cells restores

resistance to infection (147). Mice deficient in $CD4^+$ T cells also have increased mortality following CLP (148). Additionally, lymphocyte apoptosis leading to decreased $CD4^+$ T cell numbers is a major contributor to sepsis-induced immune suppression and prevention of lymphocyte apoptosis has been shown to improve survival in a murine model of sepsis (146). Paradoxically, $CD4^+$ T cells have also been shown to be pathologic during sepsis (149). The pathologic role of $CD4^+$ T cells is a major part of this thesis and will be discussed in detail in the coming chapters.

A major problem of bacterial infections is the possibility of the production of exotoxins that belong to the class of superantigens. Superantigens are one of the most potent immune activators and cause non-specific polyclonal T cell activation (150). They bind directly to MHC class II molecules outside the peptide binding groove. In this way, they cross-link with some T cell receptor (TCR) of the Vβ families. This leads to the activation of 30-50% of the T cell population. This exaggerated T cell activation leads to the production of huge amounts of cytokines especially IFN-γ. The large amount of IFN-γ in turn leads to exaggerated macrophage activation resulting in excessive production of cytokines such as IL-6 and TNF. The end result of such an overwhelming immune response is organ damage and shock. Hence, polyclonal activation of T cells by superantigens results in toxic shock syndrome (151).

$CD8^+$ T cells recognize antigen presented through MHC class I molecules. They are known as cytotoxic T lymphocytes (CTLs) due to their cytolytic action on infected cells. CTLs recognize microbial epitopes presented through MHC class I and eliminate infected cells through the release of perforin or granzyme or through Fas/FasL. CTLs can also produce cytokines such as IFN-γ which aid in pathogen clearance. Several studies have shown that $CD8^+$ T cells contribute to sepsis mortality. Sherwood et al showed that mice lacking $CD8^+$ T cells have improved

survival following CLP compared to WT mice (152). Wesche-Soldato et al showed that $CD8^+$ T cells promote inflammation in the liver after sepsis (153). von Knethen et al showed that CTLs from septic mice have greater cytotoxicity towards allogenic targets compared to CTLs from controls (154). Hence, the overwhelming evidence suggests that $CD8^+$ T cells contribute negatively to sepsis pathology.

1.9.2 The role of regulatory T cells

The immune system protects the host against pathogen invasion and is therefore armed with an arsenal of deadly ammunition (cells and proteins) necessary for the elimination of microbes. It is therefore paramount that the immune system will develop mechanisms necessary to prevent the destruction of self. Hence, T cells and B cells that are reactive to self ligands are eliminated during the process of negative selection in the thymus and bone marrow, respectively. However, it is well established that there are fail-safe mechanisms by which the immune system takes care of self-reactive lymphocytes that were not eliminated during the process of negative selection. One such mechanism that has been proposed for decades is the ability of a subtype of lymphocytes to suppress the function of another lymphocyte. Hence, a subtype of lymphocytes identified as regulatory T cells (Tregs) have been proposed as an important executor of peripheral tolerance and the role and function of Tregs in the maintenance of peripheral tolerance and immune homeostasis constitutes a major thrust of this work.

Evidence for the existence of suppressor T cells was reported as early as the 1970s. In their landmark paper, Gershon and Kondo made the observation that thymocytes were able to induce lymphocyte suppression (155). This gave birth to the theory of T cell – mediated immune suppression. This was followed by the work of Gershon et al which demonstrated that when two

different populations of thymocytes were mixed together, one population was able to suppress the response of the other (156). Baker et al also showed that in the absence of T cells, certain antigens induce exaggerated antibody production by B cells implying the ability of T cells to suppress B cell function (157). These observations led to the acceptance by many immunologists of the existence of a subtype of T cells whose function was to suppress lymphocyte response to antigen. Suppressor T cells were mostly identified by the expression of Lyt-2 (CD8) although in certain instances like hypersensitivity, Lyt-$1^{+}2^{-}$ ($CD4^{+}$) suppressor T cells were identified (158). In retrospect, this is surprising considering that Tregs which are a synonym for suppressor T cells are characterized by the expression of CD4. The theory of T cell suppression was mostly abandoned in the late 1980s because of several factors. Firstly, there were no reliable markers able to distinguish suppressor T cells from other T cells. Also, there was no definitive explanation of the molecular mechanisms that result in suppressive T cell function. The concept of suppressor T cell effectively died when analysis of the murine MHC molecule originally thought to harbor the I-J locus, which was thought to be responsible for suppressor T cell functions, failed to identify the I-J region on the MHC (159).

The theory of T cell suppression was reborn when Sakaguchi et al demonstrated that T cells with suppressive phenotype are characterized by the expression of IL-2 receptor-α chain (CD25) (160). The finding of Sakaguchi et al was significant for many reasons. First, it showed that a specific subset of T cells that can be identified by a reliable marker was involved in maintenance of immune tolerance. Secondly, it showed that ablation of this specific $CD4^{+}CD25^{+}$ T cell population resulted in the development of autoimmune disease in mice. Lastly, the transfer of $CD4^{+}CD25^{+}$ T cell population could rescue these autoimmune prone animals. This marked a new era in the theory of T cell suppression and as if to breed optimism to skeptics, the term

suppressor T cells was discarded and $CD4^+CD25^+$ T cells were labeled regulatory T cells (Tregs) (161).

How do Tregs arise? Naturally occurring Tregs have been shown to arise from the thymus as an essential mechanism of maintenance of peripheral tolerance through 'altered negative selection' (162). In this model, T cells that recognize self-antigens with 'intermediate' affinity are rendered anergic and suppressive and develop to become Tregs (163). Tregs are also induced in the periphery from naïve $CD4^+$ T cells (adaptive Tregs). The development of Tregs from naïve T cells is dependent on certain factors such as maturation state of the antigen-presenting cell (APC), cytokine environment and antigen load (164-167). In this regard, T cell interaction with DC plays an important role in induction of Tregs (166, 168).

Tregs were initially identified by the expression of CD4 and CD25 molecules. The identification of the forkhead box protein 3 (FOXP3) as the transcription factor necessary for the development of Tregs was another milestone in Treg biology (169-170). Interestingly, ectopic expression of FOXP3 can convert $CD4^+CD25^-$ effector T cells into functional Tregs suggesting the essential role of FOXP3 in Treg function (169-170). Additionally, mutations in FOXP3 gene have been linked to autoimmune diseases in both mice and humans. In mice, mutation in the FOXP3 gene leads to a lethal wasting disease characterized by exaggerated $CD4^+$ T cell activity (171). In humans, a similar diseased condition due to mutation in FOXP3 gene is known as immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome (172). These observations highlight the importance of FOXP3 in immune regulation.

In addition to CD4, CD25 and FOXP3, Tregs have been associated with many more markers. Tregs have been shown to constitutively upregulate cytotoxic T lymphocyte-associated antigen –

4 (CTLA-4) (173-174) and glucocortocoid-induced tumor necrosis factor receptor-family related genes (GITR) (175). Differential expression of lymphocyte activating gene-3 (LAG-3) and CD127 has also been reported between Tregs and $CD4^{+}CD25^{-}$ effector T cells with Tregs having higher expression of LAG-3 and lower expression of CD127 (176-177).

Critics readily point out that none of these markers are specific to Tregs. As a matter of fact, all the notable Treg markers are readily expressed by effector T cells upon activation. For example, IL-2 is essential for the proliferation of T cells and all T cells express CD25, which is the α-chain of the IL-2 receptor, upon activation (178). In a similar vein, the upregulation of CTLA-4, GITR and LAG-3 has been reported in activated effector T cells with a corresponding downregulation of CD127 (179-182). This complicated scenario has resurrected the skepticism associated with the existence of a distinct lineage of T cells dedicated to immune suppression.

How do Tregs mediate suppression? After more than two decades of the study of Tregs, the mechanism by which Tregs mediate their suppressive function remain unresolved. Several studies have attempted to explain the suppressive role of Tregs in vitro. However, the mechanism of Treg suppression in vivo is unclear. For example, it is not clear which signals initiate the expansion and homing of Tregs in vivo. It is also not clear where and how Tregs locate and suppress effector T cell function. At what stage of activation are effector T cells subject to Treg suppression? Does Treg suppression of immune cells take place at lymphoid organs or at the site of inflammation? Studies have shown that Tregs mediate their suppressive function through contact dependent and independent mechanisms. For example, it was reported that Tregs mediate suppression by inhibiting the production of IL-2 in responder effector T cells in a contact dependent manner (183). Pandiyan et al showed that Tregs effectively consume IL-2 at a faster rate than effector T cells and thereby induce apoptosis of effector T cells (184). In the contact

dependent activity of Tregs, molecules expressed by Tregs like CTLA-4 and LAG-3 have been implicated. CTLA-4 is differentially expressed by Tregs compared to non-Tregs and has been shown to be important in Treg activity (173) and Treg suppressive function is abrogated in the absence of CTLA-4 (185). It has been postulated that CTLA-4 mediates Treg suppressive ability by interacting with co-stimulatory molecules on APCs CD80 and CD86 to exert suppression (186) or directly by triggering the production of the enzyme indoleamine 2,3-dioxygenase (IDO) whose activity leads to suppression of the immune response (187).

LAG-3 is an adhesion molecule that binds to MHC class II molecules and is markedly expressed by Tregs upon activation (176). Inhibition of LAG-3 activity impairs Treg function and ectopic expression of LAG-3 on $CD4^{+}CD25^{-}$ T cells confers on them suppressive ability (176). It is possible that LAG-3 on Tregs interacts with MHC class II molecules on different lymphocytes and this may be the reason why Tregs are able to suppress different cell types (186). However, it is worthy of note that LAG-3 deficient mice unlike CTLA-4 deficient mice do not develop autoimmune disease.

Tregs have also been shown to mediate their suppressive functions through cytokines, notably IL-10 and TGF-β. Membrane bound TGF-β was reported in human and murine Tregs suggesting a role for TGF-β in Treg function (188-189). However, studies using anti-TGF-β antibody were not able to abrogate Treg function (190). The expression of membrane TGF-β on Tregs has been recently challenged and it has been demonstrated that neither latent nor active TGF-β is expressed on resting human or murine Tregs but both human and murine Tregs have high expression of latent TGF-β upon activation (191-192). Andersson et al showed that the functional relevance of Treg membrane bound TGF-β is to generate de novo Tregs from naive

precursors in a cell contact-dependent manner (192). Also, there is compelling evidence of the importance of TGF-β in Treg function. Mice deficient in TGF-β have reduced numbers of Tregs (193) and develop fatal lymphoproliferative disease (194). Moreover, naïve T cells in the presence of TGF-β acquire a suppressive phenotype (165). However, the exact role of TGF-β in Treg function is unclear since Tregs isolated from TGF-β null mice have been shown to have normal suppressive function in vitro (190).

IL-10 is another cytokine that has been shown to be important in Treg function. Indeed, in the presence of IL-10, clones of $CD4^+$ T cells which secrete IL-10 and TGF-β and suppress antigen specific immune responses are obtained. These suppressive $CD4^+$ T cell clones are called type 1 regulatory T cells (Tr1) (195). Other studies have also demonstrated the importance of IL-10 in Treg function and identified IL-10 as the major cytokine pioneering Treg activity (196-198). The importance of IL-10 in Treg function is buttressed by the fact that Tregs isolated from IL-10 deficient mice have defective function (199) and inhibition of IL-10 activity abrogates Treg function (200).

Another important mechanism by which Tregs mediate their function is the induction of apoptosis in target cells. Grossman et al showed that adaptive Tregs predominantly express Granzyme B and kill allogeneic tumor cells in a perforin dependent manner (201). They also showed that natural Tregs predominantly express Granzyme A and can mediate cytotoxicity against a variety of cell types including $CD4^+$ T cells, $CD8^+$ T cells, monocytes and DCs in a perforin dependent manner (202). Tregs were also shown to kill B cells through the upregulation of Granzymes and perforin (203). In contrast, it has been reported that Treg-mediated cytotoxicity although dependent on Granzyme B is perforin independent (204). Tregs have also

been shown to induce apoptosis through Fas/FasL interaction (205-206). The induction of apoptosis in immune cells by Tregs is of major consequence in immune regulation and is investigated in this doctoral thesis.

Which cell type(s) do(es) Tregs regulate/suppress? The possibility of thymocytes suppressing other immune cells (in this case B cells) was reported a long time ago (157). In the beginning Tregs gained prominence as a critical immune regulatory cell able to suppress autoreactive $CD4^+$T cells (207). Recently, Tregs have metamorphosed into a master regulatory cell able to extend its immunoregulatory dominance across a variety of lymphocytes and APCs including $CD8^+$ T cells (205), B cells (208), NK cells (189), NKT cells (209), monocytes (210), DCs (211), mast cells (212) and neutrophils (213).

From the above, it is evident that several immunoregulatory functions have been attributed to Tregs. Indeed, the function of Tregs in the regulation of host immunity seems to be steadily expanding. Initially described as essential for the prevention of autoimmunity (160), Tregs have been shown to be important in suppression of allergen-induced immune responses (214), oral tolerance (215-216), fetal-maternal tolerance (217), suppression of pathogen-induced immunopathologies (218) and many other functions (219). There is unequivocal experimental evidence about the paramount immunoregulatory function of Tregs in maintenance of host immune homeostasis and peripheral tolerance. However, the use of artificial systems has made the interpretation of the exact function of Tregs in host immunity rather ambiguous.

In addition to naturally occurring $CD4^+CD25^+FOXP3^+$ Tregs, studies have shown that Tregs can arise extrathymically in the periphery. Peripheral Tregs (pTregs) are induced in secondary lymphoid organs in response to homeostatic signals such as antigen and cytokines. Apostolou et

al showed that pTregs arise *de novo* from naïve $CD4^+CD25^-$ T cells by suboptimal antigen activation of dendritic cells that were exposed to very low doses of antigen (167, 220). Two types of induced Tregs have been identified: type 1 regulatory T cells (Tr1) cells arise in the presence of IL-10 and they mediate their suppressive function via secretion of high levels of IL-10 (221). Type 3 regulatory T cells arise in the presence of TGF-beta and have been shown to be important in the maintenance of oral tolerance (222).

Although the existence of $CD8^+$ Tregs was described many years ago, their function was largely ignored and much attention was paid to $CD4^+CD25^+FOXP3^+$ Tregs. Recently, $CD8^+$ Tregs have been shown to play a complementary role to $CD4^+$ Tregs in immune regulation, maintenance of immune homeostasis and prevention of autoimmunity (223-224). Naturally occurring $CD8^+CD122^+$ Tregs recognize activated T cells through the interaction of conventional MHC class I-alphabetaTCR and mediate their suppressive function by secretion of IL-10 (225).

In addition to $CD4^+$ Tregs and $CD8^+$ Tregs, a subset of $alphabetaTCR^+CD3^+CD4^-CD8^-NK1.1^-$ (double-negative; DN) Tregs have been identified and shown to be particularly important in transplantation tolerance (226-227). DN Tregs develop extrathymically and are also essential in immune regulation during infection and prevention of autoimmune diseases (228-229).

The maintenance of immune homeostatis is of significant interest to the wellbeing of the host and it is therefore not surprising that there are multiple mechanisms in immune regulation which may have overlapping and redundant functions. Overall, the most widely characterized regulatory T cell population is $CD4^+CD25^+FOXP3^+$ Tregs and this population is the major focus of this project.

1.9.3 Tregs and Sepsis

This work investigates the role of Tregs in sepsis and bacterial induced immune responses. Studies have shown that Tregs increase in both experimental and clinical sepsis (230-232). However, the exact function of Tregs in sepsis – whether they play a protective role by inhibiting excessive inflammatory response or contribute to the CARS during sepsis by suppressing effector T cells remains a subject of debate (233) and constitutes a major thrux of this work. One study reported that immunological or genetic inhibition of Treg activity has no effect on sepsis mortality (234). Another study showed that depletion of Tregs using monoclonal antibody had no effect on acute lung infection caused by *Pseudomonas aeruginosa* (235). Other studies have actually shown that Tregs contribute to immune suppression during sepsis and advocate the inhibition of Treg activity for survival during sepsis (232, 236-238).

On the other hand, Tregs have also been shown to be protective in experimental models of sepsis. Heuer et al using a murine model of CLP showed that adoptive transfer of *in vitro* stimulated Tregs increased bacterial clearance and improved survival, thereby demonstrating a beneficial role of Tregs in a clinically relevant model of sepsis (239). Another study done by Cambos et al demonstrated that Tregs help to inhibit excessive inflammation in lethal *plasmodium chabaudi adami* infection in which mortality is associated with systemic inflammatory response (240). As stated earlier, a major goal of this work was to investigate the role of Tregs in sepsis. The interesting findings about the role of Tregs in sepsis will be discussed in the following chapters.

1.10 Phosphoinositide pathway in sepsis

The family of lipid kinases able to phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides are termed Phosphatidylinositol 3-kinases (PI3Ks). The enzymatic activity of PI3Ks leads to the activation of signaling pathways involved in several cell functions such as growth, differentiation, proliferation, motility, metabolism, trafficking and survival (241-243).

PI3Ks are divided into 3 classes (classes I-III) based on their substrate activity and sequence homology. Class I PI3Ks have been intensely studied and are further divided into two groups: Class IA and Class IB. Class IA PI3K exists as a heterodimer made up of a p85 regulatory subunit and either a p110α, p110β or p110δ catalytic subunit. The p110α and p110β isoforms are widely expressed in all cells while the p110δ isoform is primarily expressed in leukocytes. Class IB PI3K is also a heterodimer made up of a p101 regulatory subunit and a p110γ catalytic subunit (243-244). The PI3K signaling pathway is involved in inflammation and sepsis. It has been reported that inhibition of PI3K signaling increases mortality in experimental sepsis thereby implying a protective role for PI3K in sepsis (245-248). However, many of these studies used pharmacological agents for the global inhibition of PI3K signaling. The use of specific inhibitors of PI3K isoforms will be more insightful in elucidating the individual roles of the PI3K isoforms in the pathogenesis of sepsis. One study demonstrated that absence of p110γ isoform of PI3K leads to increased infiltration of polymorphonuclear leukocytes in the lungs during sepsis, which greatly enhances sepsis-induced organ damage (249). On the other hand, another study reported that inhibition of p110γ activity improves survival in sepsis (250). Also, Aksoy et al reported that the p110δ isoform of PI3K controls the topology of TLR4 signaling complexes and thereby protects from endotoxic shock (251).

A major part of this work is looking at the p110δ signaling pathway of PI3K. This is because this pathway has been implicated in Treg function and activity. For example, mice with genetic mutation in the p110δ gene (p110δ KI mice) have been shown to have reduced Treg number and function (252). Hence, p110δ KI mice are very suitable for the investigation of the function of Tregs in sepsis and form a major part of this work.

1.11 Animal models of sepsis

There are three widely used methods for experimental sepsis: 1) Injection of an endotoxin; 2) Alteration of endogenous protective barrier e.g. cecal ligation and puncture (CLP); and 3) Injection of exogenous bacteria (253).

Injection of an endotoxin:

The endotoxin LPS has been widely used in the study of experimental sepsis. LPS is the component of the cell wall of Gram-negative bacteria and is a PAMP that is bound to TLR4. As stated earlier, LPS is one of the most potent activators of immune cells and LPS-TLR4 coupling sets a signaling cascade which ultimately leads to the production of pro-inflammatory cytokines by immune cells. In this way, LPS infusion mimics the early stages of clinical sepsis. Also, since LPS infusion leads to activation of several immune cells, it also leads to neutrophil infiltration and organ damage. It has been argued that LPS administration leads to a much elevated level of pro-inflammatory cytokines compared to levels seen in humans. Moreover, the LPS model of sepsis lacks infection, which is also a major feature of clinical sepsis. Nevertheless, LPS infusion still remains a useful approach to understand the role of cytokines in sepsis.

Alteration of endogenous protective barrier e.g cecal ligation and puncture (CLP):

CLP is currently the most widely used method for inducing experimental sepsis. It is a surgical procedure which involves ligation distal to the ileocecal valve followed by puncture with a needle (254). Since the cecum is an endogenous source of bacteria, its puncture leads to peritoneal polymicrobial sepsis. The intensity of the procedure can be manipulated by varying size and number of puncture. CLP is generally accepted as a clinically relevant model of sepsis as it closely mimics features of clinical sepsis including levels of pro-inflammatory cytokines and also sepsis-induced immunosuppression.

However, CLP although regarded as the gold standard in sepsis research still has its limitations. For example, studies have shown that it does not cause lung injury as seen in clinical sepsis (255). Some reports have suggested the injection of cecal slurry as an alternative approach to CLP (256).

Injection of exogenous bacteria:

The injection of live bacteria is another way of inducing experimental sepsis. This approach is particularly useful in studying the ability of the host to clear infection caused by a single pathogen and can be used to reproduce the clinical features of an infection caused by a known organism. It is also a veritable way of investigating antibiotic activity in sepsis.

The above listed animal methods are still currently used for sepsis research worldwide. However, controversies have trailed animal models of sepsis and they have been blamed for the failure of several clinical trials. To begin with, most studies use healthy, young mice for experimental sepsis and supportive care is usually minimal or absent in experimental sepsis. In contrast, patients with sepsis are usually elderly and have existing co-morbidities, which increase the risk

of mortality. Moreover, mortality is recorded in clinical sepsis despite the existence of ultramodern intensive care. Additionally, the endpoint in experimental sepsis is usually days whereas in humans, it is usually weeks and months. Furthermore, there are genomic differences in inflammatory responses across species. In their landmark paper, Seok et al showed that genomic responses in humans to inflammatory stimuli differ greatly from that of mice (257). They showed that genomic disturbances in mice were recovered within 4 days whereas in humans, it took 1-6months. They also showed that there was little or no correlation between relevant signaling pathways in humans and mice during the inflammatory response (257).

Nevertheless, animal models of sepsis have been quite useful in understanding of the pathogenesis of sepsis. As our knowledge of the condition improves, modifications to experimental procedure are essential. For example, in an attempt to mimic the existence of co-morbidity in clinical sepsis, Leelahavanichkul and collegues induced kidney disease in mice before CLP and showed that this worsened survival (258). These modifications to experimental sepsis are essential to achieve clinical translatable result.

1.12 Study rationale

Knowledge of the mechanisms involved in the pathogenesis of sepsis has rapidly increased over the years. However, there still exists a gap in knowledge regarding critical regulatory mechanisms of the immune system during sepsis. Initial emphasis was placed on the inflammatory response in sepsis but it is now known that the anti-inflammatory response is also involved in sepsis pathology. One thing is certain, the maintenance of optimum immune response is crucial for survival of sepsis and elimination of infection.

Since their discovery, the activity of Tregs has proven to be an essential immune regulatory mechanism for the maintenance of immune homeostasis and prevention of exaggerated immune response and defects in Treg function are frequently associated with immune pathology (207). Despite their importance in immune regulation, the suppressive nature of Tregs has also been shown to be detrimental and contribute to certain disease conditions such as cancer (259). Recently, Tregs were identified as responders during sepsis, with significant expansion in human and animal sepsis (230, 234). However, whether Tregs exacerbate or ameliorate pathology during sepsis is not well understood. In addition, conflicting reports about the role of Tregs in sepsis abound. While some studies have shown Tregs to be protective during sepsis (239-240), other studies have demonstrated that Tregs contribute to mortality in sepsis (232, 236-238). Additionally, studies looking at the mechanism of Treg-mediated immune response during sepsis are either few or non-existent.

In view of the above, this work was designed to delineate the immune-regulatory mechanisms involved during the pathogenesis of sepsis. In particular, the study is focused on the role of Tregs – an essential immunoregulatory cell during sepsis. Since sepsis is a condition marked by a defect in immune regulation, having a clear understanding of the role of Tregs in the overall immune response during sepsis is vital for the application of adequate therapeutic strategies and clinical management of the condition.

1.13 Global hypothesis

I hypothesize that regulatory T cells play a protective role during sepsis through prevention of exaggerated immune response.

1.14 Overarching goal

The overarching goal of this work is to elucidate the functional relevance of Tregs in the pathogenesis of sepsis and the mechanisms by which Tregs mediate their functions during sepsis.

1.14.1 Specific aims:

1) To determine the functional relevance of the increase in the number of regulatory T cells during sepsis.

2) To elucidate the mechanism by which regulatory T cells exert their protective role during sepsis.

3) To ascertain the functional relevance of Tregs during sepsis through the examination of relevant immune signaling pathways proven to be critical for Treg function, specifically – the p110δ signaling pathway.

2.0 CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Mice

Four to eight week old female C57BL/6 mice were purchased either from Charles River Laboratory, St. Constante, Quebec or from the University of Manitoba Central Animal Care Services (CACS) breeding facility. Female CD25 deficient (KO) mice (4 weeks of age), SCID, RAG1 KO and Igh KO (B cell deficient) mice all on C57BL/6 background and CD1d KO mice, SCID and TLR4 KO mice on BALB/c background and their age-matched wild-type (WT) controls were purchased from Jackson Laboratory (Bar Harbor, ME). Four to eight week old female p110δ(910A) mice on BALB/c background were also purchased from the University of Manitoba Central Animal Care Services (CACS) breeding facility. All mice were maintained in specific-pathogen free environment at the CACS, provided standard chow *ad libitum* and were used according to the guidelines stipulated by the Canadian Council for Animal Care.

2.2 Sepsis induction

Sepsis was induced by two out of the three methods listed above, namely: LPS injection and injection of live bacteria. Both LPS and bacteria were injected via the intraperitoneal route. LPS (from E. coli serotype 055:B5) was purchased from Sigma-Aldrich, Mississauga, ON, Canada. Different doses of LPS (1-30 mg/Kg) were used and are specified in each experiment varying from non-lethal to lethal as needed. In some experiments, Concanavalin A (Con A; Sigma-Aldrich, Mississauga, ON, Canada, 25 mg/Kg) was administered by intravenous injection before LPS injection. Escherichia coli (E. coli, 10^8 cfu, ATCC 25922) was used for sepsis induction. Bacteria were stored in 20% glycerol at -70 °C. For expansion, bacteria were cultured on Luria

broth (LB) agar (Invitrogen, Toronto, ON, Canada) overnight. One colony of bacteria was suspended in 3 ml of Luria broth base (Invitrogen) and was incubated in a shaking incubator (220 rpm) for 18 hrs at 37°C. The suspension was centrifuged at 6000 x *g* (5 mins, 4 °C). The pellet was washed twice with sterile PBS and then resuspended in 3 ml of sterile PBS and 10-fold dilutions were plated in LB agar. Colonies were counted after overnight incubation at 37 °C. The determined concentration of the suspension was adjusted to the desired dose using sterile PBS. Infected animals were monitored periodically for clinical signs after LPS or bacteria injection and were non-blindedly assigned arbitrary scores to indicate disease progression. Mice were monitored for movement, body condition and alertness. Disease severity was scored in a semi-quantitative fashion as follows: 0, = no abnormal clinical sign; 1, = ruffled fur but lively; 2, = ruffled fur, moving slowly, hunched, and sick; 3, = ruffled fur, squeezed eye, hardly moving, down and very sick; 4, = moribund; and 5, = dead. Clinical score 4 was used as the humane endpoint because the institutional ethical regulation does not permit score 5 in all animal experiments.

2.3 Antibodies

Rat anti-mouse CD25 (PC61) and CD4 (GK1.5) mAbs (all IgG1) were purified from hybridomas by Protein G affinity chromatography by the Manitoba Institutes of Proteomics core-facility. Mice were injected with 100 μg of antibody intraperitoneally (i.p) before LPS or bacteria challenge. Several purified and conjugated antibodies were used for this study. A summary of these, with their clones and sources are listed in Table 1

Table 1: List of antibodies

Serial No	Antibody	Clone	Company
1	Purified Rat IgG1	eBRG1	eBioscience
2	CD25 (APC, FITC)	PC61	eBioscience
3	CD4 (efluor450, APC)	GK1.5	eBioscience
4	CD3 (APC, FITC)	17A2	eBioscience
5	FOXP3 (APC)	FJK-16s	eBioscience
6	CD11b (FITC, PE)	M1/70	eBioscience
7	Gr1 (APC, PE)	RB6-8C5	eBioscience
8	TNF (PE, APC)	MP6-XT22	eBioscience
9	IFN-g (APC)	XMG1.2	eBioscience
10	CD90.2 (PE)	53-2.1	eBioscience
11	TLR4 (APC)	MTS510	eBioscience
12	CD8 (PE)	53-6.7	eBioscience
13	MCL-1	D35A5	Cell signaling

2.4 Cytokine quantification

Following sepsis induction, whole blood and peritoneal lavage fluid were collected. Blood was centrifuged and serum collected. Serum and peritoneal lavage fluids were stored at -20 °C until used for cytokine analysis. The levels of cytokines (IFN-γ, IL-1β, TNF, CCL2, IL-10 and TGF-β1, IL-6) in the serum and peritoneal lavage fluids were assayed by sandwich ELISA using paired antibodies from eBioscience (San Diego, CA) according to the manufacturer's suggested protocols. The IL-6 kit was purchased from BD Biosciences (San Jose, CA). In some cases, cytokine levels (IFN-γ, IL-1β, IL-12p70, KC, IL-10 and IL-6) in serum, peritoneal lavage fluids and lung homogenates were determined using a multiplex electrochemiluminescence detection

assay (Meso Scale Discovery Rockville, MD, USA) and read on the MSD Sector Imager 2400. The levels of cytokines (IL-6, CCL2) in supernatants from co-culture of $CD4^+$ T cells and macrophages were also determined by ELISA.

2.5 Cell purification and culture

2.5.1 Primary mouse bone marrow-derived macrophages (BMDM)

Bone marrow cells were isolated from the femur and tibia of naïve C57BL/6 mice as previously described (260). Briefly, mice were sacrificed by cervical dislocation. Femur and tibia were obtained, freed of muscle tissue and flushed with RPMI medium using 10 ml syringe and 30-gauge needle. Cells were pipetted up and down to obtain a single-cell suspension, centrifuged at 1200 rpm for 5 mins, resuspended in 10 ml RPMI medium and counted. Cells were plated at 4 x 10^6 cells/ml in 15x100 mm plates and were differentiated into macrophages using complete RPMI medium supplemented with 30% L929 cell culture supernatant. Cells were harvested on day 6 following incubation in 5% CO_2 and 37 °C.

2.5.2 Primary mouse bone marrow-derived neutrophils (BMDN)

Bone marrow cells were isolated from the femur and tibia of naïve p110δ(D910A) and WT mice as previously described (261). Briefly, mice were sacrificed by cervical dislocation. Femur and tibia were obtained, freed of muscle tissue and flushed with supplemented Hanks' balanced saline solution (HBSS; 1X HBSS, 0.5% fetal bovine serum (FBS) and 20 mM HEPES) using 10 ml syringe and 30-gauge needle. Cells were pipetted up and down to obtain a single-cell suspension, centrifuged at 1200 rpm for 5 mins and resuspended in 3 ml 45% percoll. The cells were laid over four layer percoll gradient of 81%, 62%, 55%, and 50% percoll (GE Healthcare Life Sciences, Mississauga, Ontario, Canada), respectively diluted in HBSS (100% percoll = 9

parts percoll and 1 part 10X HBSS) and centrifuged (2700 rpm, 30 mins, without braking). The neutrophils from the 81%/62% interface were collected, washed in PBS and resuspended in complete RPMI medium for use.

2.6 Co-culture experiments

2.6.1 T cell and macrophage co-culture

WT or TLR4 KO mice were sacrificed and splenocytes were obtained by a method previously described (262). T cells were isolated using a CD90.2 isolation kit (Stemcell Technologies, Vancouver, British Colombia) to a purity > 95%. The isolated T cells were incubated overnight (~ 12 hr) in complete medium (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 25 mM HEPES, and 5 x 10^{-5} M 2-ME) containing 10 μg/ml LPS. The next day, the cells were washed three times and then co-cultured with the BMDM at a ratio of 1:1 in 96-well flat-bottom plate for three days.

2.6.2 Treg and neutrophil co-culture

WT and p110δ(D910A) were sacrificed and splenocytes were obtained. Treg cells were isolated using a Treg isolation kit (Stemcell Technologies, Vancouver, British Colombia) to a purity > 95%. The purified Tregs were incubated overnight (~ 12 hr) in complete medium (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 25 mM HEPES, and 5 x 10^{-5} M 2-ME) containing 1 μg/ml LPS. The next day, BMDN were introduced at varying Treg to neutrophil ratios of 1:1, 1:2, 1:4 and 1:8 respectively and the co-culture was incubated for 12 hr in 96-well u-bottom plate.

For human Treg and neutrophil co-culture, blood was collected into heparin tubes from the peripheral vein of healthy volunteers. Neutrophils were isolated from whole blood by Dextran, Ficoll-Paque histopaque sedimentation (Amersham Pharmacia Biotech) and hypotonic lysis method as previously described (263) to a purity > 95%. Tregs were simultaneously isolated from the whole blood of volunteers using human Treg isolation kit (Stemcell Technologies). Neutrophil and Treg co-culture was done overnight in complete RPMI medium using 96-well u-bottom plate.

2.7 Western blot experiments

BMDN were isolated from WT and p110δ(D910A) as described above. The neutrophils were cultured in complete RPMI with or without LPS (1 ug/ml) for 3, 6, 12, 24 hr respectively. The cells were lysed with an NP-40 lysate buffer (Nonidet P-40, 0.1% SDS in PBS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The protein content of the lysates was estimated by Bicinchoninic acid assay (264). 10 μg of protein for each sample were resolved on polyacrylamide gels (SDS-PAGE) under denaturing conditions and were transferred onto PVDF membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5% milk in Tris-Buffered Saline and 0.1% Tween 20 and incubated with either rabbit anti-mouse Mcl-1 (Cell Signaling Technology, MA, U.S.A.), p-p38, p-Erk1/2 (R&D Systems) or mAb p-STAT3 (Santa Cruz Biotechnology), p-STAT5 (BD Bioscience) overnight at 4 °C. For loading controls, membranes were stripped for 20 mins using a glycine-based stripping buffer (2.2M Glycine, 0.5M NaCl pH4.4), blocked and subsequently incubated with polyclonal anti-Actin antibody (Sigma-Aldrich). Densitometry analysis: Band intensity was measured by the intergraded density value as determined by BIORAD Image Lab Software v4.1 on ChemiDoc MP Imaging System (BIORAD, Mississauga, Ontario, Canada).

2.8 Flow cytometry experiments

Fluorochrome-conjugated monoclonal antibodies were used in flow cytometry experiments for extracellular and intracellular staining according to the manufacturer's instructions. Briefly, cells were washed with Flow buffer (PBS containing 5% FBS and 0.01% sodium azide). The cells were incubated with Fc-block (0.25 μg/tube; eBioscience) for 10 mins on ice. Surface staining of cells was done with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CD25, CD90.2, TLR4, GR1 and CD11b obtained from eBiosciences. For intracellular staining, splenocytes were resuspended at 4 x 10^6 cells/ml in complete DMEM and plated at 1 ml per well in 24-well tissue culture plates for 48 hr. In the last 4 hr, the cultures were pulsed with PMA (50 ng/ml), ionomycin (500 ng/ml) and brefeldin A (10 μg/ml, all from Sigma-Aldrich) before surface staining. After surface staining, cells were fixed in 2% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% saponin (Sigma-Aldrich) in staining buffer and stained with specific fluorochrome-conjugated mAbs against IFN-γ, TNF or MPO (BioLegend, San Diego, CA). For MPO staining, azide free flow buffer was used. FOXP3 staining kit and fluorochrome-conjugated antibody was obtained from eBioscience. Cell viability was determined using Annexin V (eBioscience) and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Cells were acquired using BD FACS Canto II and analyzed using Flowjo software (Tree Star Inc, Ashland, OR).

2.9 Cell isolation, adoptive transfer experiments and in vivo Treg expansion

For adoptive transfer of Tregs, splenocytes were obtained from WT mice and labeled with CD4 and CD25 fluorochrome conjugated antibodies. $CD4^+CD25^+$ cells were then isolated using a BD FACS Aria III cell sorter to purity of 97%. Five million (5×10^6) Treg cells were then transferred by intravenous injection to CD25 KO mice.

For adoptive transfer of $CD4^+$ T cells, mice were sacrificed and splenocytes were obtained from WT, CD25 KO and TLR4 KO mice, respectively. $CD4^+$ T cells were selectively isolated by negative selection using Stemcell Technologies isolation kit to a purity > 95%. Five million cells were adoptively transferred into recipient SCID mice via tail vein injection. Reconstitution was assessed in peripheral blood of recipient mice by flow cytometry and was deemed to be achieved when the percentage of $CD3^+$ T cells in peripheral tail blood is similar to those of non-manipulated mice.

The expansion of Tregs in vivo was done as previously reported (265). Briefly, mice were injected with immune complex consisting of 5 μg anti-mouse IL-2 mAb and 1 μg murine IL-2 (Peprotech, NJ, U.S.A) via the intraperitoneal route everyday for 3 days. On the fourth day, mice were injected with anti-CD25 mAb (i.p) and then challenged with LPS (1 mg/Kg, i.p). Mice were monitored for clinical signs and survival following LPS injection.

2.10 Bacterial burden estimation

Bacterial clearance following depletion of Tregs was determined by estimation of bacterial burden in liver. Animals were sacrificed and liver samples collected and homogenized in 3 ml sterile PBS. The suspension was centrifuged at 6000 x *g* and the supernatant was collected. Ten

fold dilutions of the supernatant were plated in LB agar and colonies were counted after overnight incubation at 37 °C.

2.11 Proliferation assay

To measure cell proliferation, mice were sacrificed and spleen was collected. Splenocytes were processed and labeled with 5- (6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) dye (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Briefly, 4 x 10^7 cells were suspended in 5 ml of warm PBS in a 15 ml tube. An equal volume of CFSE was added and the suspension was gently rocked for 5 mins after which 5 ml of FBS was added. The suspension was centrifuged to isolate the labeled cells. The labeled cells were resuspended in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 25 mM HEPES, and 5 x 10^{-5} M 2-ME) and at 0.2 x 10^6 cells/well in 96-well flat-bottom plates and stimulated with LPS (1 μg/ml). After 72 hr, proliferation (CFSE dilution) was analyzed by flow cytometry.

2.12 Cytospin and histology experiments

Cytopreparations were permeabilized onto microscopic slides by cytospin centrifugation (ThermoShandon, Pittsburgh, PA). The slides were then stained with Hematoxylin and Eosin (H&E) solutions. Differential cell counting of stained slides was done under a Zeiss Primostar iLED microscope (Carl Zeiss, Ontario, Canada).

For histology studies, animals were sacrificed after LPS injection and animal tissue (lungs and kidney) were collected. Tissue were fixed in formalin and embedded in paraffin. 5-μm-thick tissue sections were prepared, deparaffinized in xylene, and rehydrated through graded

concentrations of alcohol to water. The sections were stained with Hematoxylin and Eosin and examined under a Zeiss Primostar iLED microscope.

2.13 Statistical analysis

All data were plotted and analyzed using GraphPad Prism software version 5.0 (La Jolla, CA). A Kaplan Meier survival curve plot was used for the survival data and the *p* values were determined using Mantel-Cox or Gehan-Breslow-Wilcoxon tests. For analyses of cytokine results, data distribution was first tested for normality using the SPSS software (Armonk NY) and a one-tailed Student's *t* or Mann Whitney tests (for normal and non-normal distributed samples, respectively) were used to compare the differences in cytokine production between different groups. Mann Whitney test was also used to compare the median range of clinical scores between different groups of mice. Differences were considered significant if $p \leq 0.05$.

2.14 Ethics statement

All animal and human studies done in this work were reviewed and approved by the University of Manitoba Human and Animal Ethics Review Boards.

3.0 CHAPTER 3

CRITICAL ROLE OF TREGS IN SURVIVAL OF SEPSIS

3.1 RATIONALE

Sepsis is a leading cause of mortality in the intensive care unit and in spite of decades in sepsis research, mortality is still unacceptably high (8). A major reason for this is that the molecular mechanisms underlying immune response during sepsis are still not well understood. Understanding of the immune regulatory pathways during sepsis will be crucial in reversing the high mortality rate associated with sepsis. The immune response during sepsis is initially tilted towards a pro-inflammatory state in response to infection (266). This normal immune response to infection becomes problematic following inability to regulate the inflammatory response and can progress to severe sepsis and septic shock. Therefore, it is pertinent to understand regulatory pathways of the immune system, which are critical for the prevention of excessive or prolonged inflammatory response.

There is unequivocal experimental evidence concerning the role of CD4+CD25+FOXP3+ T cells in the maintenance of peripheral tolerance, immune homeostasis and prevention of excessive immune response (267-268). These cells represent 5-10% of CD4+T cells in the peripheral circulation and lymphoid compartments, arise from the thymus and have an overall anti-inflammatory property (269-270). These so-called naturally occurring regulatory T cells (Tregs) have been shown to prevent autoimmunity and suppress inflammation in several animal disease models such as type 1 diabetes (271), systemic lupus erythematosus (272), inflammatory bowel disease (273), collagen-induced arthritis (274), and organ transplantation (275). Since sepsis is a disease characterized by an initial hyper-inflammatory response, it follows that Tregs, which prevent excess inflammatory response, may be important in the disease pathogenesis.

Interestingly, Tregs have been shown to increase in number in both human and clinical sepsis (230-231). However, whether Tregs contribute to immune suppression or ameliorate inflammation during sepsis is unclear with published evidence supporting both claims (236, 239). We therefore decided to investigate the role of Tregs during sepsis using a murine model. I used two murine models of sepsis, LPS injection and live bacterial infection in this study. Anti-CD25 mAb was used to deplete Tregs in mice followed by the injection of LPS or live *Escherichia coli*. I also used mice genetically lacking functional Tregs to determine the role of Tregs during sepsis. The results show that injection of LPS leads to expansion of $CD4^+CD25^+FoxP3^+$ Tregs suggesting that these cells may play an important role in immune regulation in LPS-induced acute inflammation. Indeed, genetic or immunological inhibition of Treg function using mice lacking functional Tregs (CD25 KO mice) or antibody depletion, respectively, led to acute death in an otherwise non-lethal LPS challenge. This was accompanied by exaggerated production of pro-inflammatory cytokines. Strikingly, adoptive transfer of $CD4^+CD25^+$ Tregs to CD25KO mice before LPS challenge rescues mice from death. Unlike LPS, depletion of Tregs followed by Concanavalin A (Con A) challenge does not result in mortality, suggesting that Treg depletion does not globally influence all models of acute inflammation. To further authenticate these findings, I show that depletion of Tregs leads to mortality in a non-lethal *Escherichia coli* challenge accompanied by elevated serum levels of proinflammatory cytokines. Collectively, these results indicate that in addition to regulation of LPS-induced acute inflammation, Tregs help to improve bacterial clearance and promote survival in an acute model of bacterial infection.

3.2 RESULTS

3.2.1 ***Tregs increase in mice undergoing sepsis***

I injected mice with different doses of LPS and monitored the animals for clinical signs and survival. Survival was greatly dependent on the dosage of LPS administered such that mice given 30 mg/Kg LPS showed severe clinical signs at 6 hr and recorded 100% mortality by 24 hr post LPS challenge (Fig. 3.1A). In a separate experiment, some mice were injected with 10 mg/Kg LPS and sacrificed after 6 hr and serum levels of proinflammatory cytokines were assessed by ELISA. As shown in Fig. 3.1B and consistent with previous reports (276), the serum levels of proinflammatory cytokines (IL-1β, IL-6, IL-12, CCL2 and TNF) were significantly elevated in mice given LPS compared to controls. To find out the numerical response of Tregs cells during LPS-induced acute inflammatory responses, I analyzed peripheral blood mononuclear cells and spleen cells from mice sacrificed 6 hrs after LPS (10 mg/Kg) injection and determined the percentages of Tregs ($CD4^+CD25^+Foxp3^+$) by flow cytometry. There was a significant increase in the percentage and absolute numbers of $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ T cells in the spleens of animals in response to LPS compared to control mice (Fig. 3.1C-F). As in the spleen, similar increases in the percentage and absolute numbers of Tregs were also observed in the blood of LPS-challenged animals (Fig. 3.1C, D). Thus, injection of LPS leads to acute inflammatory responses, which is accompanied by the production of elevated levels of pro-inflammatory cytokines and systemic increase in Tregs.

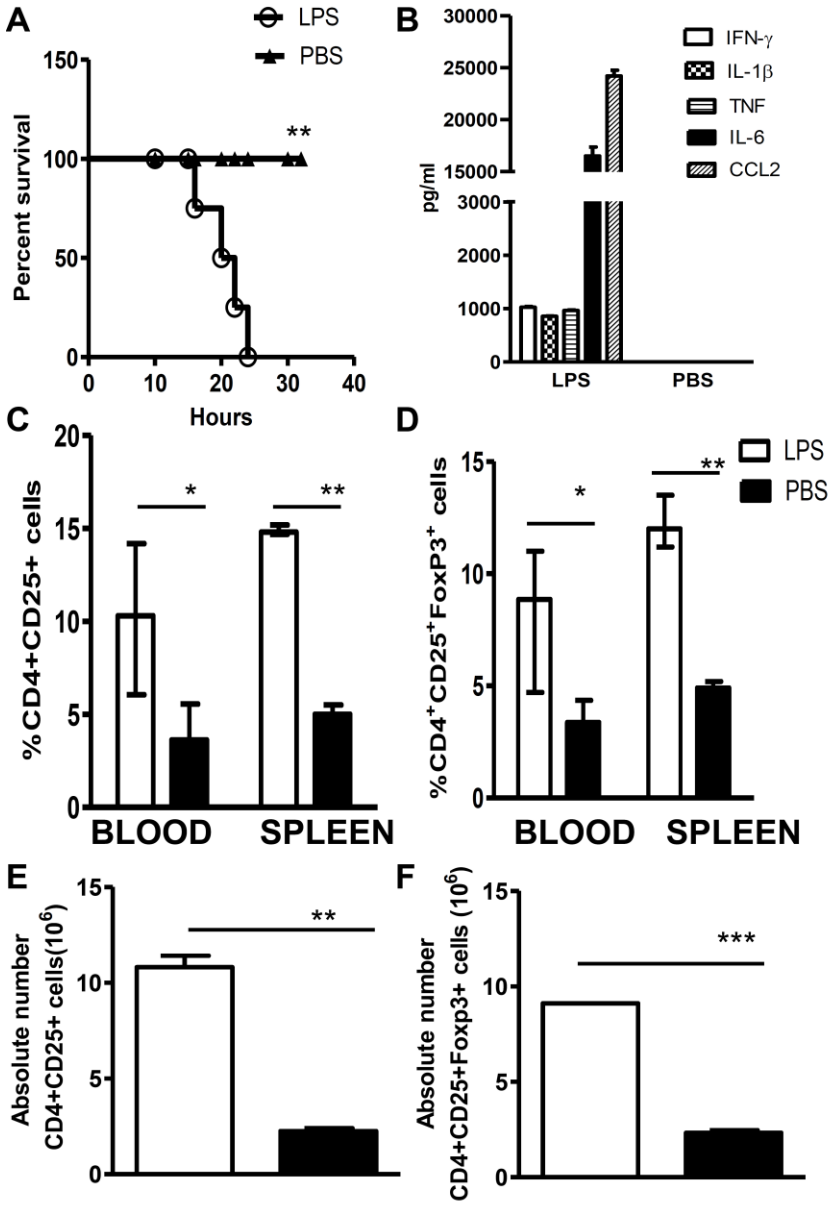


Fig 3.1 Injection of LPS leads to systemic inflammatory response and increase in regulatory T cells. Mice were injected with LPS (30 mg/Kg, i.p) or PBS and monitored for survival (A). In some experiments, mice were sacrificed after 6 hr post LPS challenge and serum levels of proinflammatory cytokines (IL-1ß, IL-6, MCP-1, TNF and IFN-γ) were determined by sandwich ELISA (B). In addition, the percentages of CD4+CD25+ (C) and CD4+CD25+FoxP3+ (D) cells in the spleen and blood were determined by flow cytometry. Also, the absolute numbers of CD4+CD25+ (E) and CD4+CD25+FoxP3+ (F) cells in the spleens were determined. The results presented are representative of 3 independent experiments (n = 4-5 mice per group) with similar results. Fig. 3.1A is a pool of 3 independent experiments (n = 12 mice). Bars show mean or median +/- standard error; **, $p < 0.01$; ***, $p < 0.001$.

3.2.2 ***Depletion of Tregs leads to acute death in an otherwise non-lethal LPS challenge.***

Previous reports have shown that the numbers of Tregs increase in both experimental models of sepsis and septic patients (230, 236). However, the exact role of these cells in the pathogenesis of sepsis and septic shock remains equivocal. To determine the role of Tregs in LPS-induced acute inflammatory responses, I injected mice with depleting anti-CD25mAb (PC61, 100 ug/mouse) to deplete Tregs, and monitored survival following non-lethal (1 mg/Kg) LPS challenge. This dose of antibody has been previously shown to cause sustained and selective depletion of natural Tregs in mice (277-280). Indeed, I found that anti-CD25 mAb treatment caused over 70-75% depletion of $CD4^{+}CD25^{+}Foxp3^{+}$ T cells (Tregs, Fig. 3.2A). Strikingly, all the mice given anti-CD25mAb before sub-lethal LPS challenge progressed rapidly to endotoxic shock as indicated by very high clinical scores (Fig. 3.2B) and attained 100% mortality within 6 hr (Fig. 3.2C). In contrast, the clinical score in mice injected with control antibody was significantly reduced and none of the mice died (Fig. 3.2B and C).

Previous reports showed that anti-CD25 mAb-mediated depletion of Tregs occurs through FcgammaRIII on macrophages (279). I therefore investigated if injection of anti-CD25 mAb resulted in the activation of macrophages which contributed to the enhanced susceptibility of the animals to LPS challenge. I cultured BMDM at minute intervals of 15, 30, 60 and 120 in the presence or absence of LPS and/or anti-CD25 mAb or isotype control and investigated the activation of the transcription factors signal transducer and activator of transcription (STAT) 1 and STAT 3. There was no difference in the expression of STAT 1 and STAT 3 by macrophages cultured in the presence of anti-CD25 mAb or isotype control (Appendix I). I therefore conclude that the enhanced mortality observed following Treg depletion and LPS challenge is not due to the enhanced activation of the macrophages by the anti-CD25 mAb.

It is worthy of note that the susceptibility to a non-lethal dose of LPS in anti-CD25 mAb-treated mice was accompanied by significantly increased serum levels of proinflammatory cytokines including IL-6 (Fig. 3.2D-F), which is a key predictor of mortality in sepsis (281). Interestingly, mice depleted of Tregs had lower levels of TGFβ1 (Fig. 3.2G), a cytokine that is associated with Tregs and plays an important role in their immunoregulatory function (194). Collectively, these observations show that depletion of Tregs enhances sensitivity and susceptibility to LPS, suggesting that Tregs play a protective role in the pathogenesis of LPS-induced acute inflammatory responses.

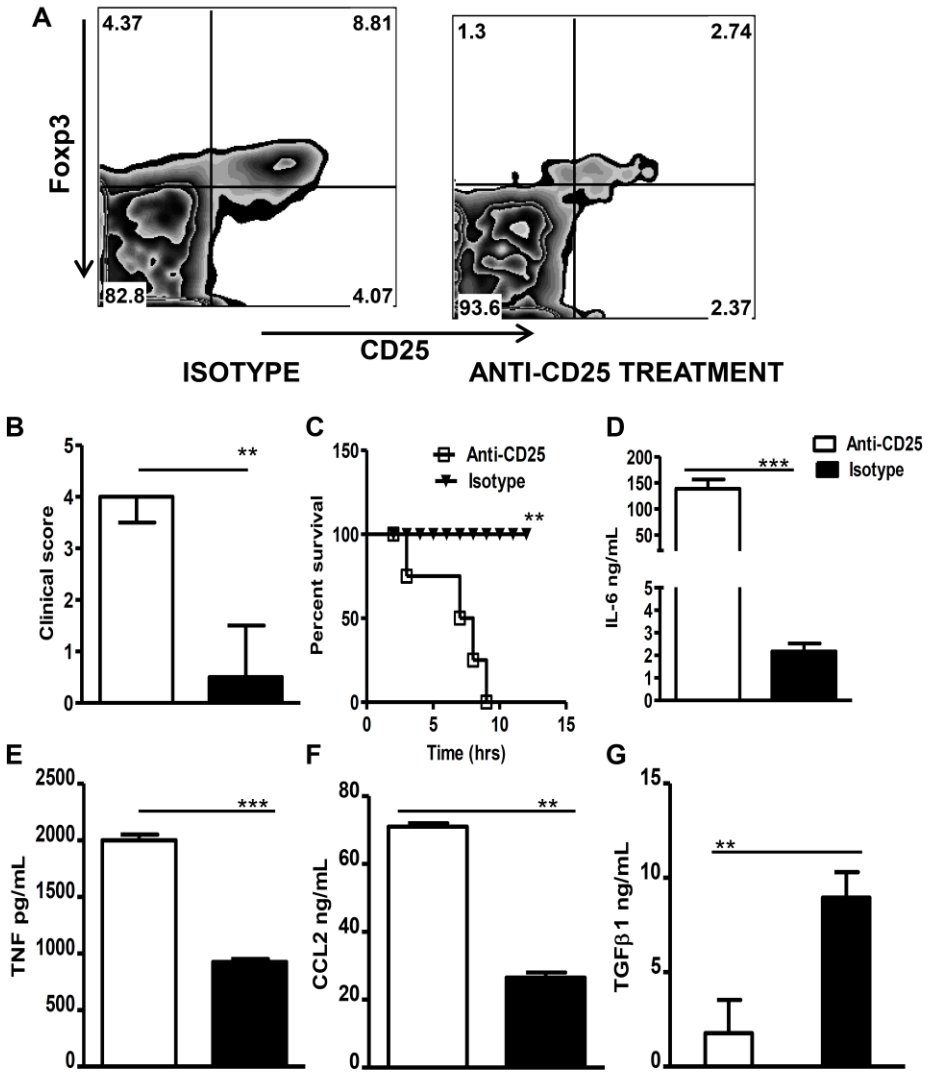


Fig. 3.2 **Depletion of Tregs leads to acute death in an otherwise non-lethal LPS challenge.** Mice were injected intraperitoneally with anti-CD25 mAb (100 μg/mouse), which causes selective and partial (~75%) depletion of Foxp3+ cells (A). Control mice received isotype-matched control mAb (100 μg/mouse). After 24 hr, mice were challenged with a sublethal dose (1 mg/Kg) of LPS and the animals were monitored for clinical signs (B) and survival (C). Some mice were sacrificed after 6 hr, and the levels of IL-6 (D), TNF (E), CCL2 (F) and TGF-β (G) in serum were determined by ELISA (D-G). The data presented are representative of 4 independent experiments (n = 4-6 mice per group; n = 12 in Fig. 2C) with similar results. Bars show mean or median +/- standard error; **, $p < 0.01$; ***, $p < 0.001$.

3.2.3 *Mice with genetically impaired Treg function also show enhanced susceptibility to LPS challenge*

Previous reports show that CD25 KO mice have impaired Treg activity due in part, to the absence of $CD4^{+}CD25^{+}Foxp3^{+}$ cells (282). Therefore, I speculated that if Tregs are protective in LPS induced acute inflammatory responses, CD25 KO mice (which lack Tregs) would be more sensitive to LPS than their WT counterpart mice. Thus, I injected CD25 KO mice with sub-lethal dose of LPS (1 mg/Kg) and compared the disease severity and survival with similarly treated WT mice. Similar to my observation with anti-CD25mAb, CD25 KO mice showed enhanced sensitivity to LPS and failed to recover from a sub-lethal dose of LPS (Fig. 3.3A, B). This enhanced sensitivity was accompanied with massive production of proinflammatory cytokines (Fig. 3.3C, D). Taken together, my data shows that genetic or immunological inhibition of Treg function is detrimental to survival in LPS induced inflammatory responses.

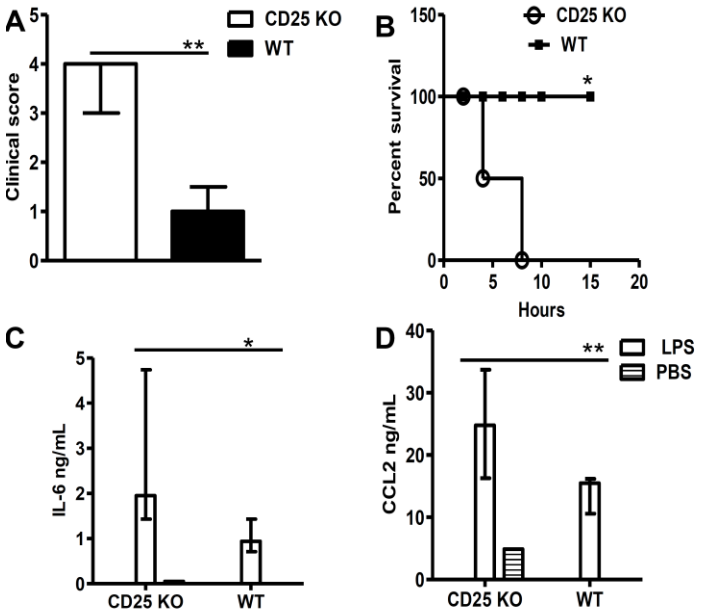


Fig. 3.3 **Mice with genetically impaired Treg function are hypersensitive to LPS challenge**. Groups of CD25 KO and WT mice were injected with sublethal dose of LPS (1 mg/Kg) and monitored for clinical signs of sepsis (A) and survival (B). In a separate experiment, some mice treated as above were sacrificed after 6 hr of LPS challenge and serum levels of proinflammatory cytokines (IL-6, C and CCL2, D) were determined by sandwich ELISA. The results presented are representative of 2 independent experiments (n = 4-5 mice per group) with similar results. Bars show mean or median +/- standard error; **, $p < 0.01$.

To further confirm the protective role of Tregs in LPS-induced acute inflammatory responses, I reconstituted CD25 KO mice with Tregs by adoptively transferring highly enriched WT $CD4^+CD25^+$ (> 94% $Foxp3^+$) Tregs (Fig. 3.4A) into CD25 KO mice 24 hr prior to the administration of sub-lethal dose of LPS. As shown in Fig. 3.4B and 3.4C, adoptive transfer of Tregs into CD25 KO mice resulted in decreased clinical signs as indicated by significantly reduced clinical scores and abrogation of LPS induced mortality (100% survival). My results suggest a potential therapeutic role for Tregs in LPS-induced acute inflammatory responses.

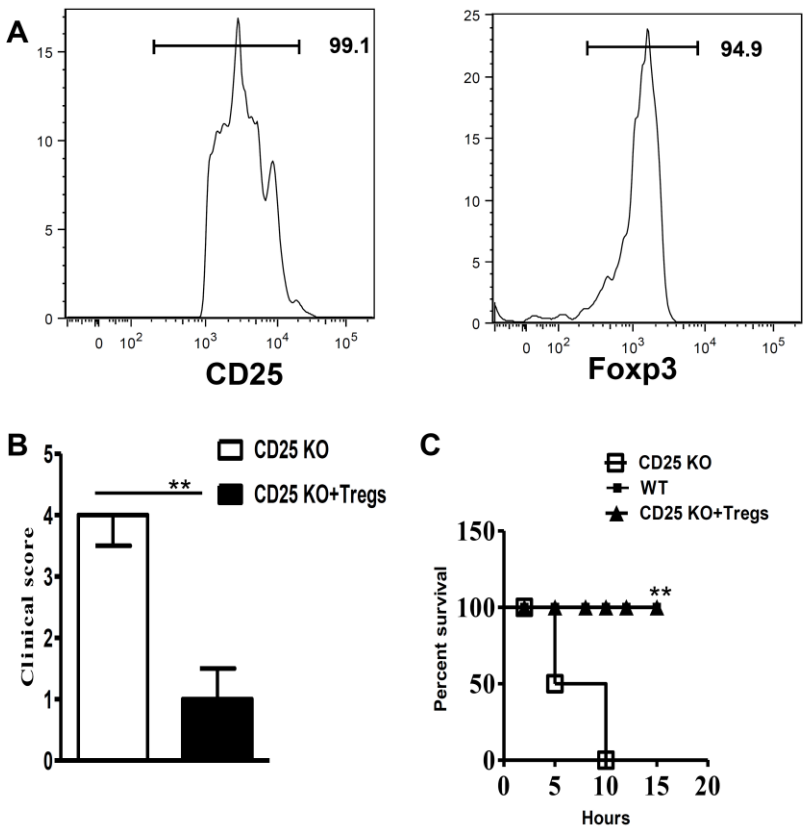


Fig. 3.4 **Adoptive transfer of WT Tregs abrogates LPS-induced sepsis mortality in CD25 KO mice**. $CD4^+CD25^+$ cells were purified from spleens of WT mice by cell sorting (> 95% purity) and injected intravenously into CD25 KO mice. (A) the purity of the sorted cells ($CD4^+CD25^+$ cells) and percent composition of $Foxp3^+$ cells ($CD4^+CD25^+Foxp3^+$ cells). Twenty-four hours after cell transfer, recipient mice where challenged with sublethal dose of LPS (1 mg/Kg) and monitored for clinical signs (B) and survival (C). The results presented are representative of 2 independent experiments (n = 4-5 mice per group) with similar results. Bars show mean or median +/- standard error; **, $p < 0.01$.

3.2.4 ***Depletion of $CD4^+CD25^-$ T cells does not lead to enhanced sensitivity to LPS***

To confirm that the enhanced susceptibility to LPS following anti-CD25 mAb treatment is strictly related to reduced number of $CD4^+CD25^+$ Tregs, I injected mice with anti-CD4 mAb (to deplete $CD4^+$ T cells) and after 24 hr challenged the mice with LPS (1 mg/Kg). Surprisingly, I found that depletion of $CD4^+$ T cells did not mirror the effect observed with depletion of Tregs, such that mice depleted of $CD4^+$ T cells neither succumbed to a non-lethal dose of LPS (Fig. 3.5A) nor showed increased serum levels of pro-inflammatory cytokines (Fig. 3.5B-D). In contrast, mice depleted of only Tregs developed severe signs of endotoxic shock, succumbed within 6 hr and contained high levels of pro-inflammatory cytokines in their serum (Fig. 3.5A-D). Taken together, these results suggest that the enhanced susceptibility of mice to LPS is specific to the depletion of Tregs.

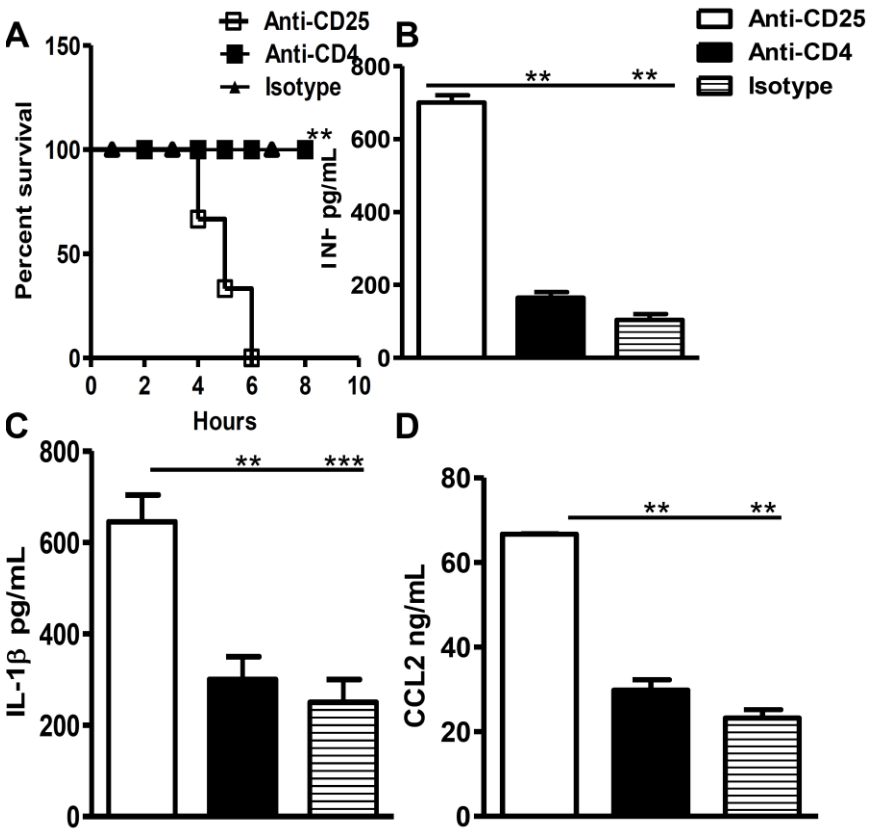


Fig. 3.5 **Depletion of CD4$^+$ T cells does not lead to enhanced susceptibility to LPS**. Mice (n = 5) were injected intraperitoneally with anti-CD25 (100 μg/mouse) or anti-CD4 (1mg/mouse) mAb, challenged with sublethal dose of LPS (1 mg/Kg) after 24 hr and survival was monitored for over 12 hr (A). In a separate experiment, some mice treated as above were sacrificed after 6 hr of LPS challenge and serum levels of TNF (B), IL-1β (C) and CCL2 (D) were determined by sandwich ELISA. The results presented are representative of 3 independent experiments (n = 4-5 mice per group) with similar results. Bars show mean +/-SEM; **, $p < 0.01$; ***, $p < 0.001$.

3.2.5 ***Depletion of Tregs does not lead to enhanced sensitivity to non-bacterial inflammation***

So far, my results have shown that depletion of Tregs leads to hypersensitivity and mortality to LPS challenge. To determine whether the enhanced sensitivity to LPS challenge is specific or a non-specific phenomenon associated with all acute inflammatory responses, I tested the expansion of Tregs and the outcome of Treg depletion in Con A-induced model of acute inflammation. I therefore injected mice anti-CD25 or control mAb and after 24hr challenged with Con A. Depletion of Tregs did not lead to hypersensitivity to Con A and resulted in 100% survival (Fig. 3.6). This was in stark contrast to the result obtained upon LPS challenge where all mice treated with anti-CD25 mAb died (Figs. 3.2C and 3.6). Collectively, these results indicate that the effects of Tregs are specific to LPS, suggesting that these cells may also play a protective role during acute bacterial infection.

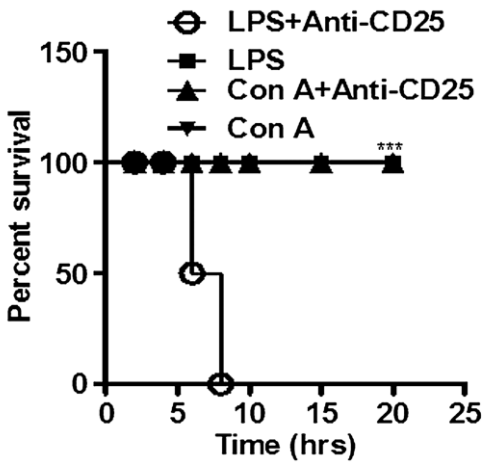


Fig. 3.6 **Depletion of Tregs does not lead to mortality in Con A-induced acute inflammation**. Groups of mice were injected with anti-CD25 or isotype-matched control mAb and after 24hr, were challenged with either LPS or Con A and the percent survival was plotted. The data is representative of 2 independent experiments (n = 4-5 mice per group) with similar results; ***, $p < 0.001$.

3.2.6 ***Depletion of Tregs leads to susceptibility to live bacterial infection***

Since depletion of Tregs leads to hypersensitivity and mortality to bacterial component LPS, it is possible that the same effect will be observed in a more physiologic model of sepsis initiated by Gram-negative bacteria infection. To investigate this, I injected mice with anti-CD25 mAb and 24 hrs later challenged them with a non-lethal dose of *E. coli* (10^8 cfu). Consistent with the findings using LPS, depletion of Tregs led to hypersensitivity to bacterial infection resulting in mortality in an otherwise non-lethal dose of *E. coli* (Fig. 3.7A). In addition, this sensitivity was accompanied by elevated levels of pro-inflammatory cytokines in the serum and peritoneal lavage fluids (Fig. 3.7B, C) and significantly higher bacterial burden in the liver (Fig. 3.7D). These results show that depletion of Tregs significantly impairs efficient bacterial clearance resulting in death to a non-lethal *E. coli* infection. They further suggest that as in LPS challenge model, Tregs are critically important for controlling bacteria-induced excessive inflammatory responses.

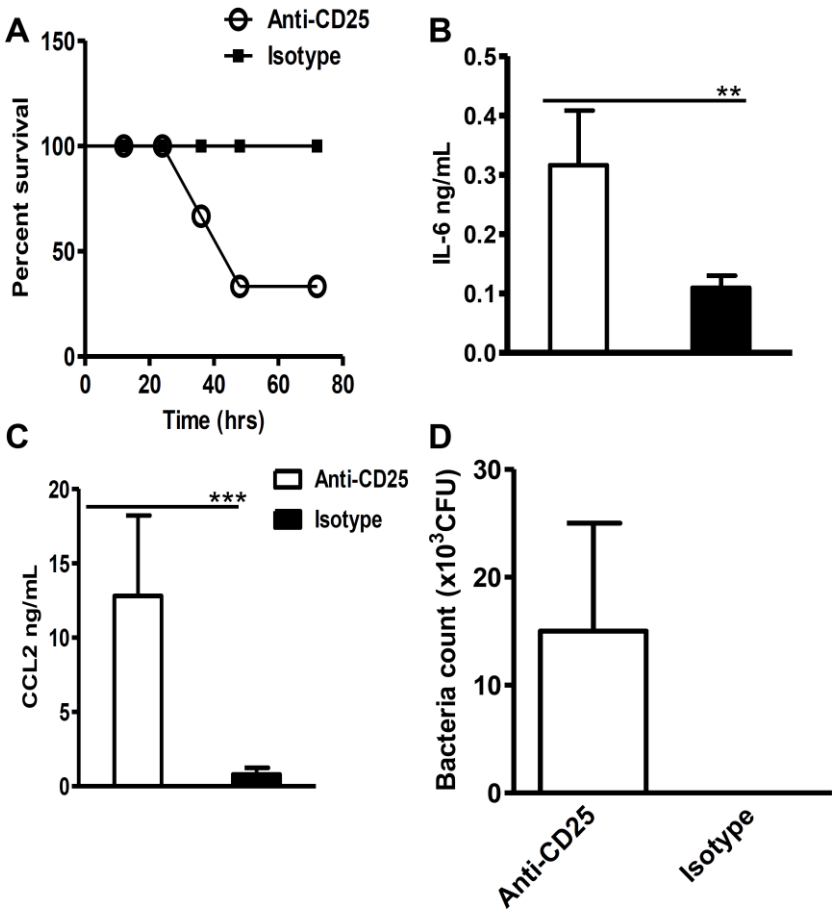


Fig. 3.7 **Depletion of Tregs leads to enhanced susceptibility to bacterial infection**. Groups of mice were injected intraperitoneally with anti-CD25 or isotype-matched control mAb. After 24hrs, mice were challenged with *E. coli* (10^8 cfu) and monitored for survival (A). In a separate experiment, mice were sacrificed at 48 hr post *E. coli* challenge and serum levels of IL-6 (B) and CCL2 (C) were determined by sandwich ELISA. At sacrifice, the liver was homogenized in PBS and bacterial burden in liver homogenate was also determined (D). The results presented are representative of 3 independent experiments (n = 4-6 mice per group) with similar results. Bars show mean +/-SEM; **, $p < 0.01$; ***, $p < 0.001$.

4.0 CHAPTER FOUR

TREGS RESTRAIN $CD4^+$ T CELLS FROM CAUSING EXAGGERATED IMMUNE ACTIVATION AND HYPERSENSITIVITY TO LPS

4.1 RATIONALE:

In the previous chapter, I investigated the role of Tregs during sepsis. The results were quite stimulating. In line with my hypothesis, I found that depletion of Tregs led to acute death to a non-lethal dose of LPS or live bacterial infection. This work is one of the first to describe the protective role of Tregs in live bacterial infection and thus provides even stronger evidence concerning the protective role of Tregs in survival of sepsis.

These findings are important and have potential for use in sepsis therapy. However, questions still abound concerning the role of Tregs during sepsis. The work from the previous chapter makes it imperative to determine the mechanism by which Treg depletion mediates sensitivity and lethality to LPS and live bacterial challenge.

In general, lymphocytes are an important component of the immune system and are generally important for the elimination of microbial pathogens and host defense. However, there are instances where immune cells have been implicated in the pathology of diseases thereby resulting in autoimmunity (283).

It is generally accepted that sepsis is due to an initial exacerbated inflammatory immune response as a result of infection (284). Also, Tregs are known to be suppressive and have been shown to prevent exaggerated effector T cell response in several disease models. For example, aberrant Treg function has been linked to pathologic effector T cell activation, which is a hallmark of several autoimmune diseases including type 1 diabetes (271), multiple sclerosis (272) and inflammatory bowel disease (273). It is possible that in the context of sepsis, Tregs

help to prevent excessive effector T cell activation. It is also conceivable that in the context of sepsis, Tregs help to prevent the excessive activation of another lymphocyte subset. Hence, it is relevant to identify which lymphocyte subset(s) Tregs help to regulate during sepsis and the particular lymphocyte subset(s) which in the absence of Tregs, contributes to sepsis mortality. This forms the basis for the work in this chapter. Here, I used different murine lymphocyte gene knock-out models to investigate the effect of Treg depletion on different lymphocyte subset(s). I found that depletion of Tregs leads to mortality to an otherwise non-lethal dose LPS challenge in wild type (WT), B cell deficient (Igh KO), NKT deficient (CD1d KO) but not in SCID or RAG1 KO mice, thereby implicating T cells as the major lymphocyte population responsible for aggravated immune response to endotoxin upon Treg depletion. In line with this observation, reconstitution of SCID mice with $CD4^+$ T cells restores enhanced LPS sensitivity and death as seen in WT mice upon Treg depletion. Additionally, depletion of Tregs leads to increased proliferation and activation of $CD4^+$T cells in response to LPS challenge. Remarkably, LPS-primed T cells induced high levels of proinflammatory cytokine production in macrophages. Furthermore, I show that a subset of $CD4^+$ T cells express toll-like receptor 4 (TLR4), the pattern recognition receptor for LPS and that signaling via TLR4 on $CD4^+$ T cells is critically important for their activation of macrophages and the subsequent enhancement of proinflammatory response following LPS challenge. Collectively, my results show that Treg depletion leads to unrestrained $CD4^+$ T cell activation leading to aggravated activation of other immune cells that contribute to host injury and poor outcome in LPS-induced acute inflammation.

4.2 RESULTS

4.2.1 *Mice lacking conventional T cells do not show enhanced sensitivity to LPS after injection of anti-CD25*

I used B cell deficient (Igh KO), NKT cell deficient (CD1d KO), SCID and RAG1 KO mice to assess the contribution of different lymphocyte subsets in enhanced sensitivity to LPS in the absence of Tregs. Interestingly, similar to WT mice, NKT and B cell deficient mice showed enhanced susceptibility to LPS upon Treg depletion and succumbed to a non-lethal dose of LPS (Fig. 4.1A and B). This was accompanied with increased levels of proinflammatory cytokines in the serum (Fig 4.1C and D). Since mice lacking B cells and NKT cells were also sensitive to LPS after Treg depletion, I concluded that B or NKT cells are not responsible for the enhanced sensitivity of WT mice to LPS after Treg depletion.

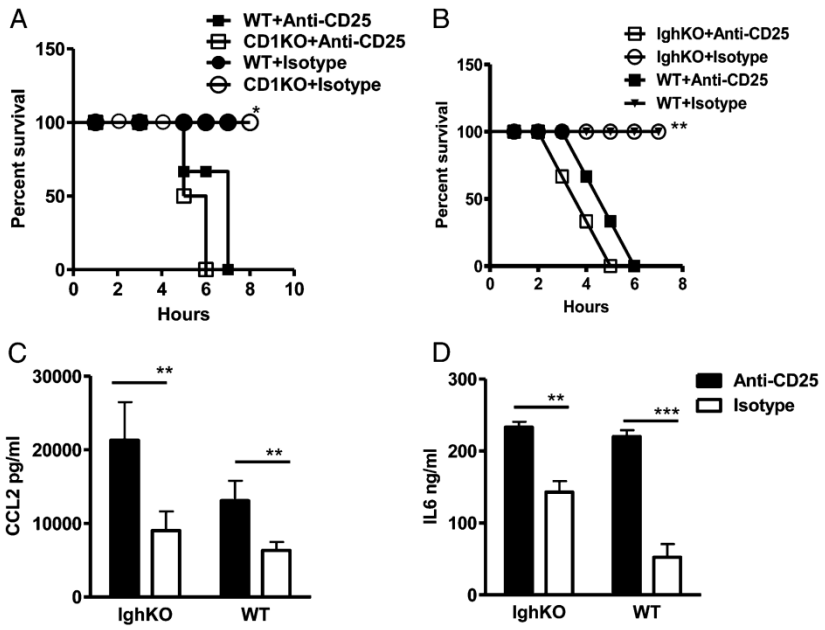


Fig. 4.1 **Mice lacking B cells or NKT cells also show enhanced susceptibility to LPS upon Treg depletion**. Wild-type (WT), CD1d KO and Igh KO mice were injected intraperitoneally with anti-CD25 mAb or isotype-matched control mAb (100 μg/mouse). After 24 hr, mice were challenged with a sublethal dose (1 mg/Kg) of LPS and the survival of CD1d KO (A) and Igh KO (B) mice was determined. Some LPS-challenged mice were sacrificed after 4 hr and the levels of CCL2 (C) and IL-6 (D) in serum were determined by ELISA. The data presented are representative of 2 independent experiments (n = 5 mice per group) with similar results. **, $p < 0.01$; ***, $p < 0.001$.

Next, I assessed the impact of injecting anti-CD25 mAb into SCID and RAG1 deficient mice that lack functional adaptive immune (B and T) cells. Surprisingly, SCID mice injected with anti-CD25 mAb and challenged with LPS did not show enhanced sensitivity as shown by decreased clinical score (Fig. 4.2A), enhanced survival (Fig 4.2B) and reduced levels of proinflammatory cytokines in peritoneal wash fluids (Fig. 4.2C and 4.2D).

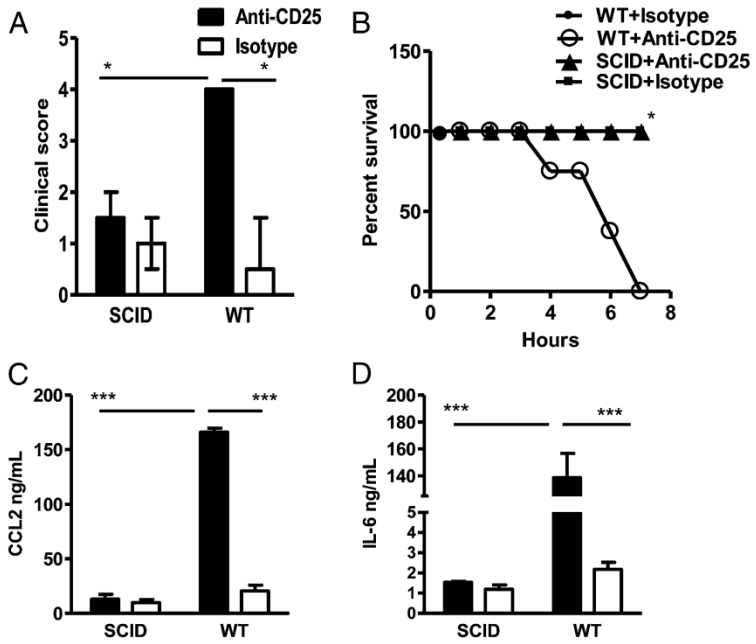


Fig. 4.2 **SCID mice do not show enhanced susceptibility to LPS upon treatment with anti-CD25 mAb**. Groups of SCID and WT mice were injected intraperitoneally with anti-CD25 mAb or isotype-matched control mAb (100 μg/mouse). After 24 hr, mice were challenged with a sublethal dose (1 mg/Kg) of LPS and monitored for clinical signs (A) and survival (B). Some mice were sacrificed after 4 hr and serum levels of CCL2 (C) and IL-6 (D) were determined by ELISA. The data presented are representative of 3 independent experiments (n = 4-6 mice per group) with similar results. *, $p < 0.05$; ***, $p < 0.001$

Because SCID mice are leaky and had been shown to possess few extraperitoneal derived T cells (285), I further verified the results using RAG1 deficient mice. Similar to SCID mice, RAG1 KO mice given anti-CD25 mAb were resistant to LPS challenge unlike anti-CD25 mAb-treated WT mice that developed severe disease and succumbed (Fig. 4.3A-D). Collectively, these results suggest that T cells are the lymphocyte population that mediates the enhanced susceptibility to LPS challenge following Treg depletion. They further show that the enhanced susceptibility to LPS following anti-CD25 mAb injection is not due to non-specific effects of the antibody on the innate immune cells.

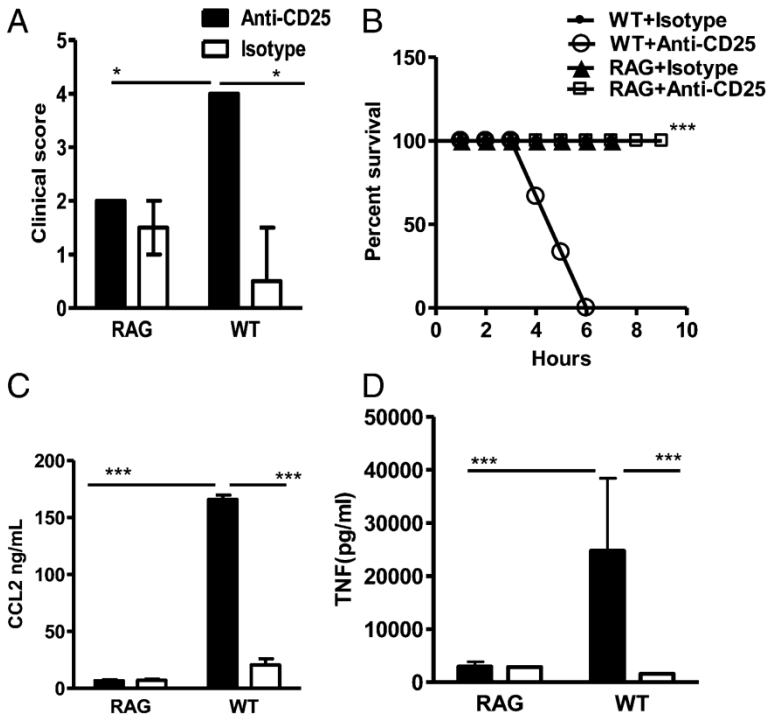


Fig. 4.3 **RAG mice do not show enhanced susceptibility to LPS upon treatment with anti-CD25 mAb**. Groups of RAG1 KO and WT mice were injected intraperitoneally with anti-CD25 mAb or isotype-matched control mAb (100 μg/mouse). After 24 hr, mice were challenged with sublethal dose (1 mg/Kg) of LPS and the animals were monitored for clinical signs (A) and survival (B). Some mice were sacrificed after 4 hr and serum levels of CCL2 (C) and IL-6 (D) were determined by ELISA. The data presented are representative of 2 independent experiments (n = 4 mice per group) with similar results. **, $p < 0.01$; ***, $p < 0.001$.

To confirm the role of T cells in the enhanced sensitivity of WT mice to LPS following depletion of Tregs, I reconstituted SCID mice with highly purified $CD4^+$ T cells from WT mice. Following repopulation of T cells after 2 weeks (Fig. 4.4A), the animals were treated with anti-CD25 mAb and challenged with LPS 24hr later. In contrast to control mice (SCID mice that did not receive T cells), those reconstituted with WT $CD4^+$ T cells showed enhanced sensitivity and succumbed to a non-lethal LPS challenge (Fig. 4.4B and C). This enhanced sensitivity to LPS was

accompanied by exaggerated levels of proinflammatory cytokines in the serum (Fig. 4.4D and E). Thus, akin to observations in WT mice, depletion of Tregs in SCID mice reconstituted with $CD4^+$ T cells led to mortality to an otherwise non-lethal LPS challenge, a phenotype that is not observed in non-reconstituted SCID mice. These results suggest that Tregs ameliorate LPS-induced acute inflammation and mortality at least in part by restraining pathogenic $CD4^+$ T cells.

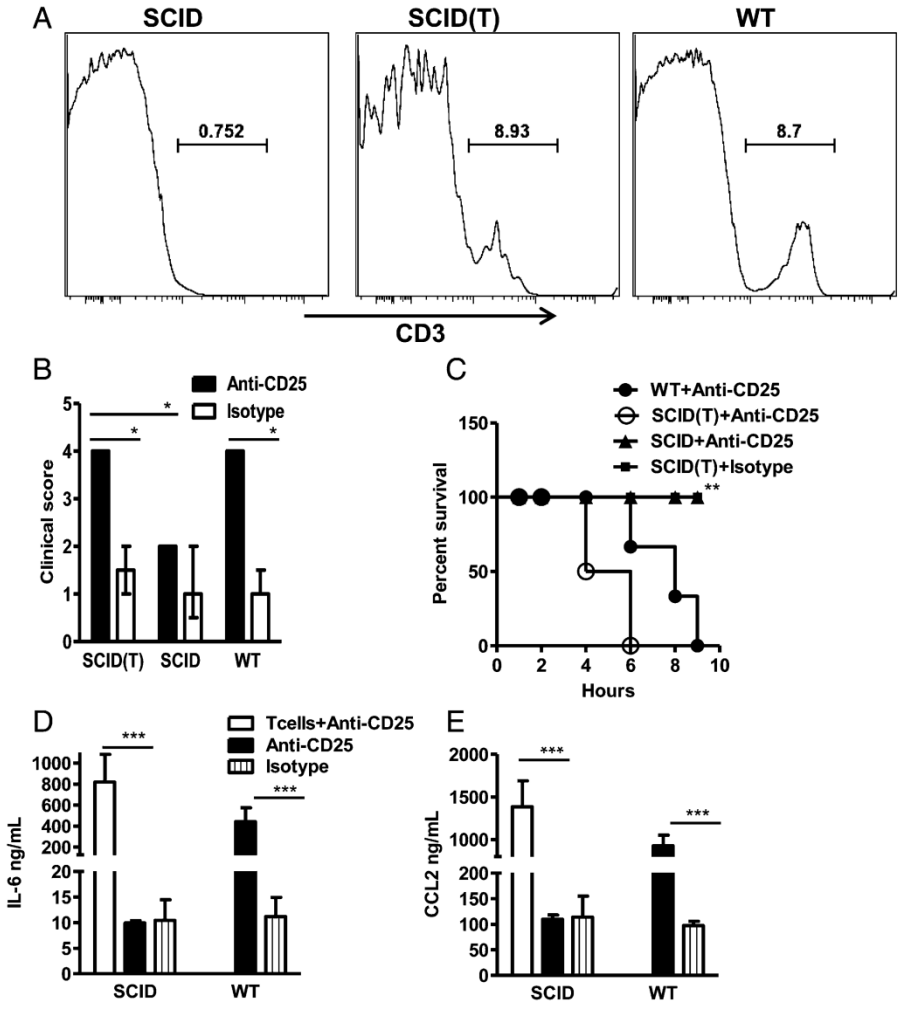


Fig. 4.4 **Reconstitution of SCID mice with CD4$^+$ T cells restores sensitivity to LPS upon anti-CD25 mAb treatment**. CD4$^+$ T cells were isolated from spleens of WT mice by negative selection using magnetic beads and injected intravenously into SCID mice. Expansion of CD4$^+$ T cells in recipient SCID mice was confirmed by flow cytometry on blood sample from tail blood (A). Recipient and control (non-reconstituted) mice were injected with anti-CD25 or isotype-matched control mAb and challenged with sublethal dose of LPS (1 mg/Kg) 24 hr later. LPS-challenged mice were monitored for clinical signs (B) and survival (C). Some animals were sacrificed after 4 hr and serum levels of IL-6 (D) and CCL2 (E) was determined by ELISA. The results presented are representative of 2 independent experiments (n = 4-6 mice per group) with similar results. *, $p < 0.05$; ***, $p < 0.001$.

To further authenticate the pathogenic effects of $CD4^+$ T cells in the absence of Tregs, I reconstituted SCID mice with $CD4^+$ T cells from WT or CD25 KO mice, which have defective Treg function (282), and challenged the animals with sub-lethal dose of LPS. SCID mice that received $CD4^+$ T cells from WT mice were normal and only exhibited sensitivity to LPS following depletion of Tregs by treatment with anti-CD25 mAb (Fig. 4.5A and B). In contrast, SCID mice reconstituted with CD25 KO $CD4^+$ T cells were highly sensitive to a non-lethal LPS challenge as shown by high clinical scores and acute death even in the absence of anti-CD25 mAb treatment (Fig. 4.5A and B). Taken together, these data show that lack of Treg function has deleterious effect on LPS challenge strictly in the presence of $CD4^+$ T cells, suggesting that Tregs may function to ameliorate LPS-induced inflammatory response and death via restraining $CD4^+$ T cell activation and/or function.

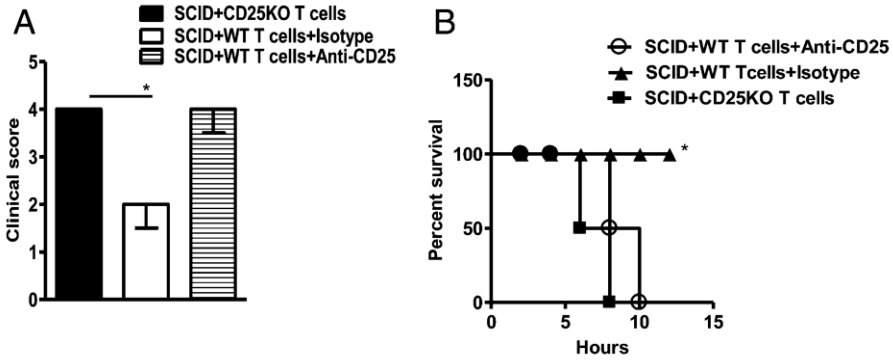


Fig. 4.5 **Reconstitution of SCID mice with $CD4^+$ T cells from CD25 KO mice also leads to sensitivity to LPS**. CD4+ T cells were isolated from spleens of CD25 KO or WT mice by negative selection using magnetic beads. Enriched cells were injected intravenously into SCID mice and allowed to expand. After full reconstitution, mice were challenged with LPS with or without prior treatment with anti-CD25 mAb and the animals were monitored for clinical signs (A) and survival (B). The results presented are representative of 2 independent experiments (n = 4-5 mice per group) with similar results. *, $p < 0.05$.

4.2.2 *Depletion of Tregs increases CD4$^+$ T cell activation and proliferation in response to LPS*

The observation that depletion of Tregs leads to CD4$^+$ T cell-mediated sensitivity to LPS suggests that CD4$^+$ T cells may be directly or indirectly activated by LPS. Therefore, I hypothesized that depletion of Tregs leads to increased activation and proliferation of CD4$^+$ T cells in the presence of LPS. To investigate this experimentally, I depleted mice of Tregs (by anti-CD25 mAb treatment), challenged them with LPS after 24 hr and sacrificed them after 4 hr to assess CD4$^+$ T cell cytokine production directly *ex vivo.* As shown in Fig. 4.6A-F, splenocytes from Treg-depleted mice (given anti-CD25 mAb) contain significantly higher percentages of TNF- and IFN-γ–producing CD4$^+$ T cells compared to isotype-matched treated controls. In addition, the mean fluorescence intensity (MFI) of these cells were significantly higher than those from isotype-matched control mAb-treated mice (Fig. 4.6C and F).

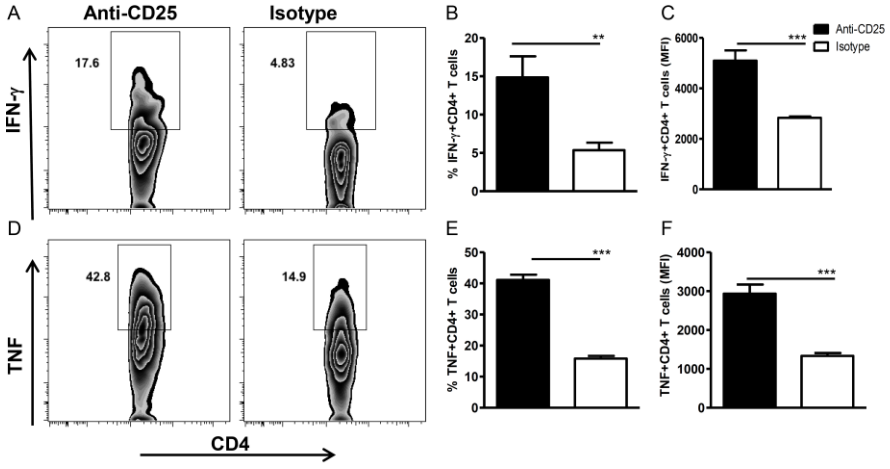


Fig.4.6 **Depletion of Tregs increases CD4$^+$ T cell cytokine production in response to LPS.** Groups of mice were treated with anti-CD25 or isotype-matched control mAb and 24 hr later challenged with LPS (1 mg/Kg). Animals were sacrificed after 4 hr and whole splenocytes were assessed for cytokine production directly *ex vivo* following 4 hr *in vitro* stimulation with PMA and ionomycin stimulation. Shown are the percentages (A, B, D and E and mean fluorescence intensities (C and F) of IFN-γ (A-C) and TNF (E-F) -producing CD4$^+$ T cells. The results presented are representative of 3 independent experiments (n = 4 mice per group) with similar results. **, $p < 0.01$; ***, $p < 0.001$

4.2.3 *$CD4^+$ T cells are directly activated by macrophages and in the absence of Tregs lead to exaggerated macrophage activation*

Following the observation that $CD4^+$ T cells from LPS-treated mice produce TNF and IFN-γ, I speculated that LPS may directly activate $CD4^+$ T cells. Therefore, I assessed $CD4^+$ T cells for their expression of TLR4, the innate pattern recognition receptor for LPS. Consistent with previous reports (286-287), I found that a sub-population of $CD4^+$ T cells expressed TLR4 and this number was upregulated following *in vitro* stimulation with LPS (Fig. 4.7A and 4.7B). Next, I assessed whether depletion of Tregs affects $CD4^+$ T cell proliferation in response to LPS following Treg depletion. Splenocytes were labeled with CFSE dye, cultured for 3 days in the presence of LPS and assessed for proliferation by flow cytometry. I found that $CD4^+$ T cells from splenocytes of mice depleted of Tregs showed greater proliferation compared to those from isotype control-treated mice (Fig. 4.7C and 4.7D). In addition and consistent with the direct *ex vivo* results, mice depleted of Tregs had greater percentage of IFN-γ-producing $CD4^+$ T cells relative to the isotype treated controls (Fig. 4.7E and F). Thus, depletion of Tregs leads to greater proliferation and cytokine production of $CD4^+$ T cells in response to LPS. Because I found that in the absence of Tregs, LPS induces $CD4^+$ T cell activation and increased production of IFN-γ, a cytokine known to potently activate macrophages (288), I hypothesized that LPS-activated $CD4^+$ T cells activate macrophages leading to proinflammatory cytokines production. I co-cultured LPS-primed or unprimed T cells with bone marrow-derived macrophages (BMDM) and after 72 hr, assessed the production of IL-6 and CCL2 by ELISA. As shown in Fig. 4.7G and H, LPS-treated T cells induced macrophages to produce increased amounts of IL-6 and CCL2. Taken together, these results suggest that the enhanced susceptibility to LPS following Treg depletion is due to hyper-activation of macrophages by overly active $CD4^+$ T cells. This is in line with

previous reports, which showed that Tregs prevent over-activation of $CD4^+$ T cells thereby preventing excessive immune activation and inflammatory responses.

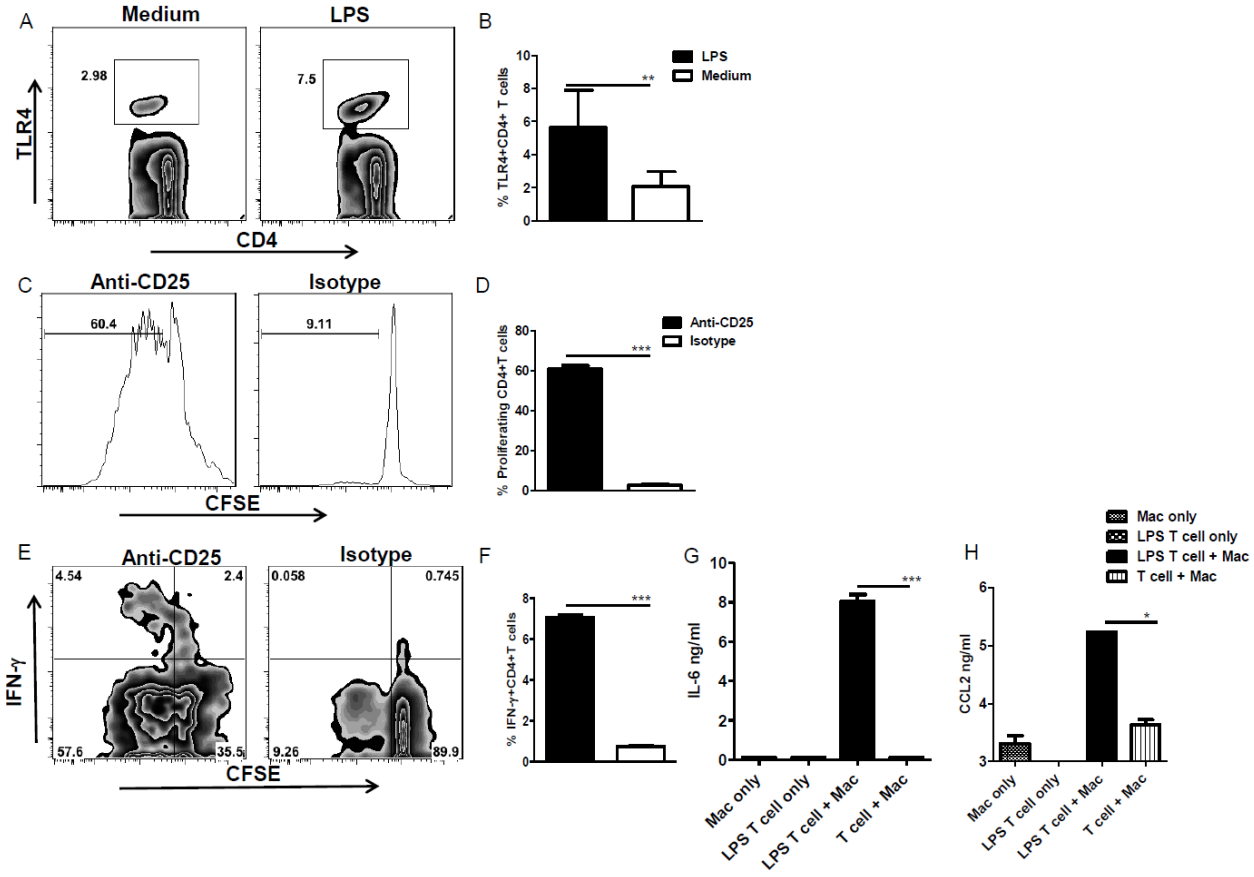


Fig. 4.7 **$CD4^+$ T cells express TLR4 and proliferate in response to LPS following Treg depletion**. Splenocytes from naïve mice were incubated for 6 hr with or without LPS analyzed for TLR4 expression (on $CD4^+$ T cells) by flow cytometry (A and B). In another experiment, groups of mice were treated with anti-CD25 or isotype-matched control mAb. After 24 hr, the mice were sacrificed and their splenocytes were labeled with CFSE and stimulated with LPS (1 μg/ml) *in vitro* for three days and $CD4^+$ T cell proliferation (C and D) and total $CD4^+IFN\text{-}\gamma^+$ cells (E and F) were analyzed by flow cytometry. In addition, some LPS-stimulated T cells were co-cultured with BMDMs for 72 hr and the culture supernatant fluids were assayed for IL-6 (G) and CCL2 (H) by ELISA. The results presented are representative of 2 independent experiments (n = 4 mice per group) with similar results. *, $p < 0.05$; ***, $p < 0.001$

4.2.4 *TLR4 signaling is important for CD4+ T cell-mediated enhancement of LPS-induced inflammatory response*

So far, my results have shown that a sub-population of $CD4^+$ T cells express TLR4 (the pattern recognition receptor for LPS) and LPS-primed $CD4^+$ T cells induced proinflammatory cytokine production by macrophages, I therefore investigated whether TLR4 signaling in $CD4^+$ T cells is necessary for the increased LPS responsiveness following Treg depletion. I co-cultured LPS-primed T cells from TLR4 KO and WT mice with BMDMs from WT mice and assessed cytokine production by ELISA. I found that LPS-primed $CD4^+$ T cells from TLR4 KO, unlike those from WT mice, were significantly impaired in their ability to induce IL-6 production by BMDMs (Fig. 4.8A). Additionally, reconstitution of SCID mice with $CD4^+$ T cells from TLR4 KO mice led to reduced clinical signs and better survival in response to LPS challenge following Treg depletion compared to reconstitution with $CD4^+$ T cells from WT mice (Fig. 4.8B, C). The improved clinical score and survival of SCID mice that received $CD4^+$ T cells from TLR4 KO mice was also accompanied by decreased levels of IL-6 in the peritoneal wash fluids (Fig. 4.8D). Collectively, these results indicate that signaling via TLR4 on $CD4^+$ T cells is important for their activation and subsequent activation of macrophages for increased proinflammatory cytokine response following LPS stimulation.

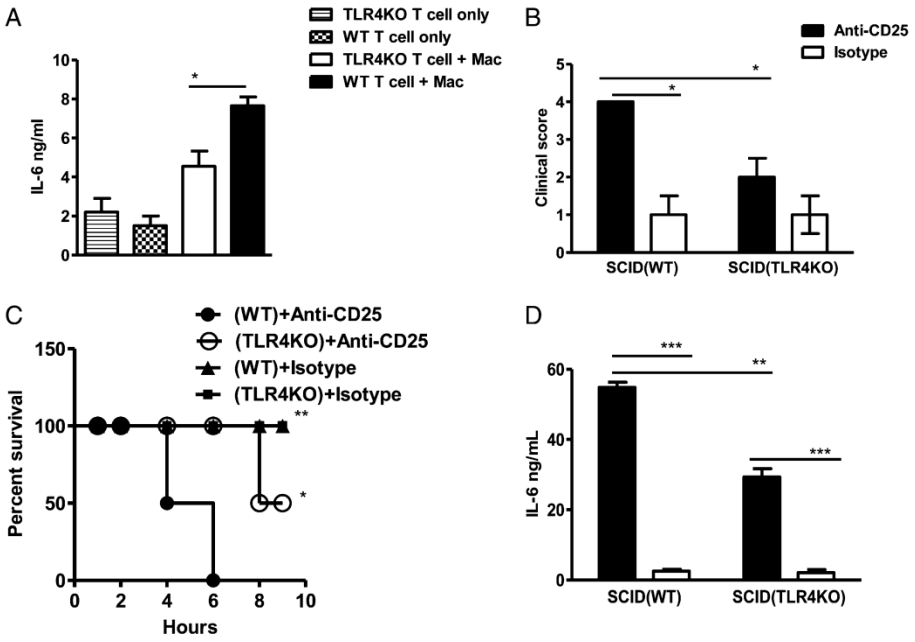


Fig. 4.8 **CD4$^+$ T cell-mediated enhancement of LPS response is TLR4 dependent.** CD4$^+$ T cells were isolated from TLR4 KO or WT mice, stimulated with LPS *in vitro* and co-cultured with WT BMDMs for 72 hr and the culture supernatant fluids were assayed for IL-6 by ELISA (A). In another experiment, highly purified CD4$^+$ T cells from WT or TLR4 KO mice were adoptively transferred to SCID mice. Following full CD4$^+$ T cell reconstitution, the recipients were treated with anti-CD25 or isotype-matched control mAb and then challenged with LPS (1 mg/kg) after 24 hr. Animals were monitored for clinical signs (B) and survival (C) and the levels of IL-6 in the peritoneal wash fluid was determined by ELISA (D). The results presented are representative of 2 independent experiments (n = 6 mice per group) with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

5.0 CHAPTER FIVE

ABSENCE OF P110 DELTA SIGNALING LEADS TO DECREASED TREG NUMBERS AND INCREASED NEUTROPHIL RECRUITMENT AND FUNCTION RESULTING IN MORTALITY TO NON-LETHAL LPS CHALLENGE

5.1 RATIONALE

The work on the previous chapters led to two important conclusions. The first is that Tregs play a protective role and promote survival in LPS-induced inflammation and live bacterial infection. The second is the unraveling of the mechanism by which Tregs confer protection during LPS-induced inflammation. A process that involves restricting aggressive immune response of $CD4^+$ T cells to LPS as described in chapter four. In this chapter, I continue to investigate the role of Tregs in sepsis using another murine model of LPS-induced inflammation.

The activity of PI3Ks leads to the activation of signaling pathways associated with numerous cell functions such as growth, differentiation, proliferation, motility, metabolism, trafficking and survival (241-243) all of which are crucial during sepsis. Hence, it is pertinent to investigate the role of PI3K signaling during sepsis. In particular, the p110δ isoform of PI3K has been shown to be relevant in immune response as it is primarily expressed on leukocytes compared to other isoforms, which are ubiquitiously expressed (289). Also, there is cross-talk between p110δ and Tregs since mice with mutation on the p110δ gene leading to non-functional p110 delta protein ($p110\delta^{D910A}$ mice; hereafter known as p110 mice) have decreased Treg numbers and function (252). Hence, the p110 mice constitute a perfect system to investigate the role of diminished Tregs numbers in sepsis. In this chapter, I show that p110 mice, which have diminished Treg numbers, are highly susceptible to LPS compared to their WT counterpart. I found that following intra-peritoneal LPS challenge, as expected, neutrophils are recruited to animal peritoneum. However, overtime, peritoneal neutrophil accumulation in p110 mice is significantly greater than

that of WT mice. I speculated that neutrophils from p110 mice survived longer than those of WT mice. Indeed, I found this to be the case. Additionally, neutrophils from p110 mice have greater activity following LPS challenge compared to WT mice. Depletion of neutrophils in p110 after but not before LPS challenge, rescued the animals from LPS mortality. Since p110 mice have characteristically diminished Treg numbers, I investigated the role of diminished Treg numbers in exaggerated activity and survival of neutrophils from p110 mice following LPS challenge. Interestingly, Tregs abrogated exaggerated neutrophil activity and increased apoptosis of neutrophils from p110 mice. Moreover, adoptive transfer of Tregs to p110 mice rescued p110 mice from LPS-induced mortality. I authenticated my findings by showing that human Tregs also regulate neutrophil function and survival. Collectively, these results indicate that Tregs regulate neutrophil activity and survival and in the absence of adequate Treg function, neutrophils exhibit exaggerated activity and increased survival in response to LPS challenge. Importantly, this work adds to the growing evidence that Tregs exert their regulatory activities on cells of the innate immune system and highlights a critical link in regulation of the immune response between innate immunity and adaptive immunity.

5.2 RESULTS

5.2.1 ***Mice lacking p110 delta signaling have decreased Treg numbers and are more susceptible to LPS***

Mice with mutation in the p110δ gene leading to non-functional p110 delta protein ($p110\delta^{D910A}$ mice; p110 mice) have been shown to have fewer Tregs compared to their WT counterpart (252). Indeed I found this to be the case (Fig. 5.1A). Absence of p110δ signaling results in almost halving of Treg numbers (Fig. 5.1A). To investigate the effect of diminished Treg numbers on the response of p110 mice to LPS, I injected p110 and WT mice with LPS and monitored the

animals for clinical signs and survival. In contrast to WT mice, p110 mice had higher clinical score (Fig. 5.1B) and were unable to recover from the LPS challenge showing 100% mortality unlike WT mice which had 75% survival (Fig. 5.1C). To verify the role of the diminished Treg numbers in the mortality of p110 mice, Tregs were isolated from WT mice and intravenously transferred into p110 mice. Next day, recipient mice were challenged with LPS and monitored for clinical signs and survival. Interestingly, adoptive transfer of WT Tregs to p110 mice resulted in lower clinical scores (Fig. 5.1D) and rescued p110 mice from LPS mortality (Fig. 5.1E). These data suggest that the susceptibility of p110 mice to LPS is associated with decreased Treg numbers and is in line with the results of the previous chapters that diminished Treg numbers leads to susceptibility to LPS-induced inflammation.

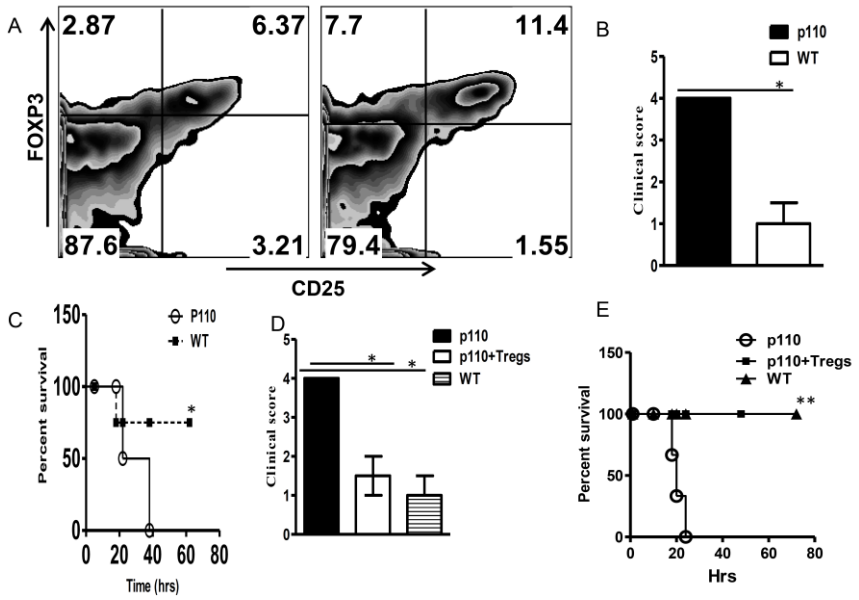


Fig. 5.1 **Mice lacking p110 delta signaling are highly sensitive to LPS challenge**. Quantification of splenic Tregs numbers in p110 (left panel) and WT(right panel) mice (A). Groups of p110 and WT mice were injected intraperitoneally with LPS (10 mg/Kg) and monitored for clinical signs (B) and survival (C). In a separate experiment, one group of p110 mice intravenously received highly purified Treg cells ($CD4^{+}CD25^{+}$) T cells from WT mice 12 hr before LPS challenge and clinical score (D) and survival (E) were also determined. The data presented are representative of at least 3 independent experiments (n = 4 mice per group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

5.2.2 Acute death of p110 mice following LPS challenge is associated with lung pathology and increased cytokine production:

A hallmark of endotoxic shock and acute inflammation is the production of proinflammatory cytokines and exaggerated production of proinflammatory cytokines is associated with poor outcome during sepsis (290). I therefore, assessed the level of proinflammatory cytokines in p110 mice. There was no difference in proinflammatory cytokine production in the serum (Fig.

5.2A-D) or peritoneal wash (Fig. 5.2E, F) of p110 and WT mice. This observation made me to do organ-specific analysis of p110 and WT mice following LPS injection .

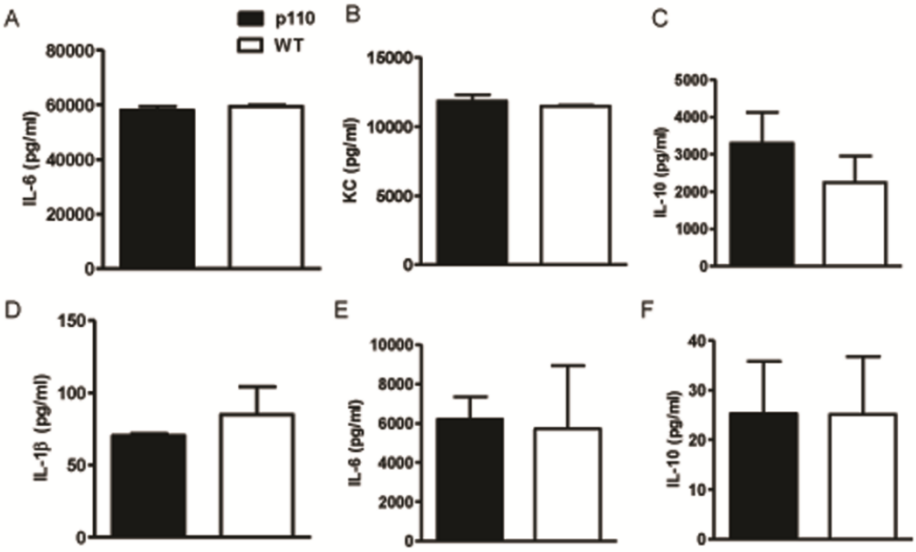


Fig. 5.2 **Comparison of proinflammatory cytokines in p110 and WT mice after LPS challenge**. Groups of p110 and WT mice were injected intraperitoneally with LPS (10 mg/Kg). After 15 hr, animals were sacrificed and the levels of proinflammatory cytokines in serum (A-D) and peritoneal wash (E, F) were determined using mesoscale discovery. The data presented are representative of at least 2 independent experiments (n = 4 mice per group) with similar results.

Given that organ dysfunction is a major problem in endotoxic shock and acute lung injury is a common occurrence in septic shock (291), I did histology on the lungs of p110 and WT mice following LPS injection. Samples from p110 mice showed greater signs of lung injury as evidenced by greater leukocyte infiltration of the airways (Fig. 5.3A). Additionally, analysis of lung pro-inflammatory cytokines revealed that p110 mice had significantly higher levels of the

cytokines IL-1β and IL-10 in the lungs (Fig. 5.3D, E). These results indicate the occurrence of acute lung injury in p110 mice.

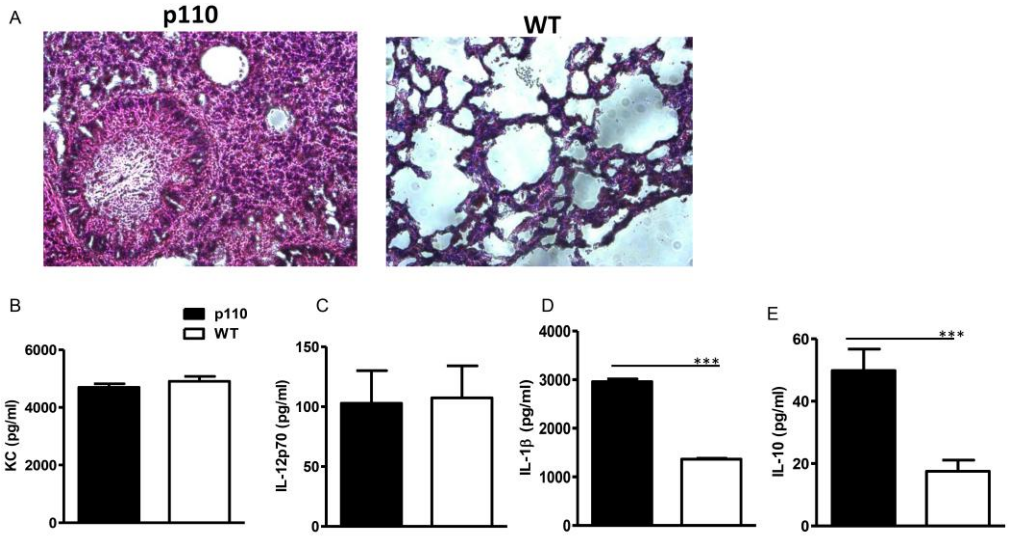


Fig. 5.3 **Evidence of acute lung injury in p110 mice after LPS challenge**. Groups of p110 and WT mice were injected intraperitoneally with LPS (10 mg/Kg). After 15 hr, lung sections were processed and stained with H&E (A). In a separate experiment, the lungs were homogenized in in 1 ml of saline and the levels of KC (B), IL-12p70 (C), IL-1b (D) and IL-10 (E) in the lung homogenate fluids were determined. The data presented are representative of a least 2 independent experiments (n = 4 mice per group) with similar results. ***, $p < 0.001$.

5.2.3 *Mice lacking p110 delta exhibit increased neutrophil influx upon LPS challenge*

Previous studies have shown that during sepsis, neutrophils are the major producers of IL-1β (292) and IL-10 (112). Based on the observations in Fig. 5.3D and 5.3E, I speculated that injection of LPS may cause increased neutrophil infiltration and activity in p110 mice. Indeed, I found this to be the case. I found greater accumulation of neutrophils in peritoneum of p110 mice compared to WT mice following LPS challenge (Fig. 5.4A, B). Since the p110 mice begin to die

at 15 hr post LPS injection, I examined neutrophil numbers at 3hr and 15 hr post LPS injection. Interestingly, there was no difference in neutrophil numbers between p110 and WT mice 3 hr after LPS injection (Fig 5.4C, D). However, at the time of death, there was significant difference in neutrophil numbers between p110 and WT mice with p110 mice having up to three times more neutrophil numbers (Fig. 5.4C, E). Hence, neutrophils accumulate in peritoneum of p110 mice following LPS challenge compared to their WT counterpart.

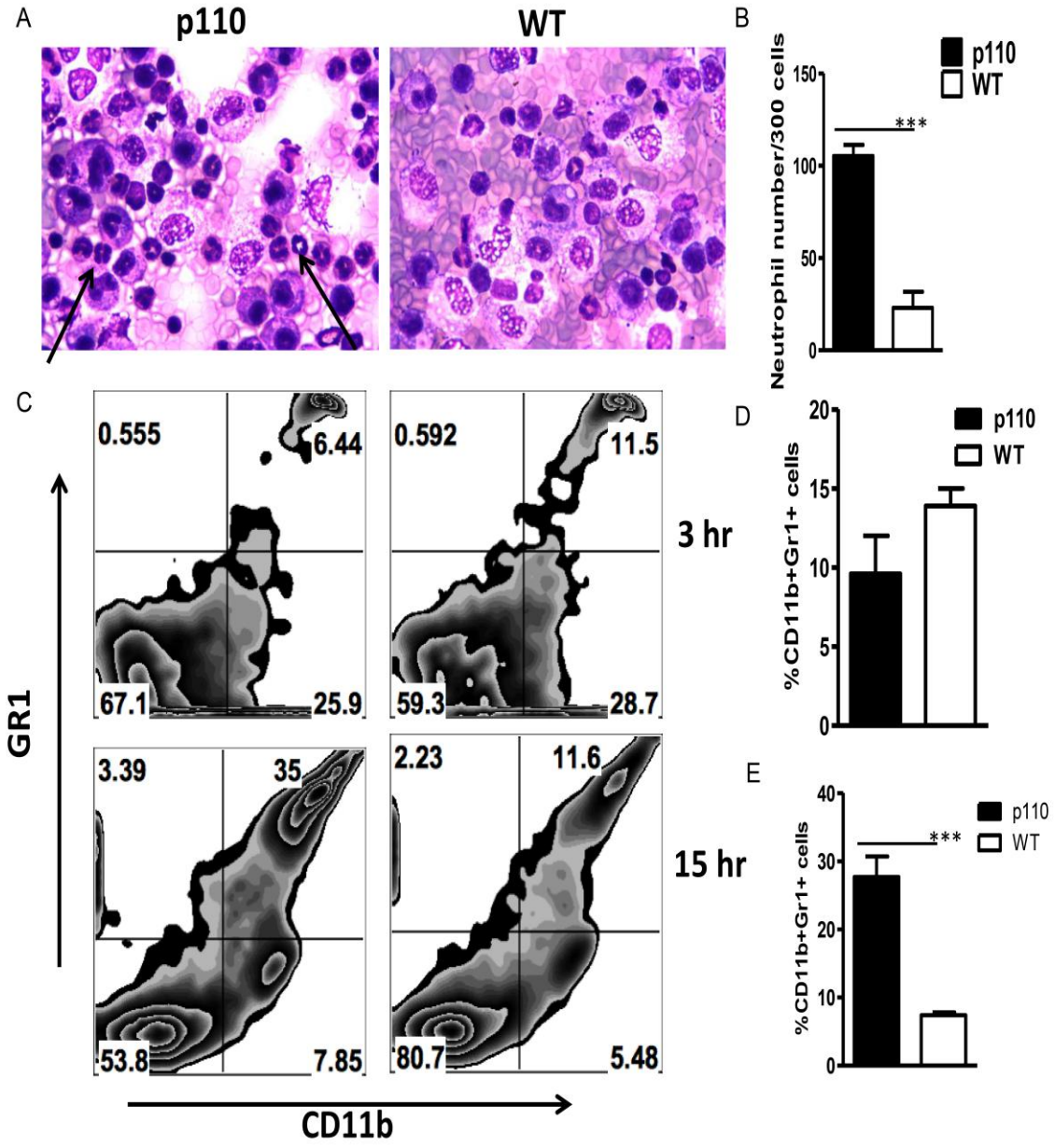


Fig. 5.4 **Increased neutrophil accumulation in peritoneum of p110 mice after LPS challenge.** Groups of p110 and WT mice were injected intraperitoneally with LPS (10 mg/Kg). After 15 hr, animals were sacrificed and cytospin preparations of peritoneal wash were stained with H&E and assessed under a light microscope (A). Bar graph represents the number of neutrophils within 300 cells (B). In addition, the numbers of neutrophils in the peritoneal wash were further analyzed by flow cytometry by assessing the percentage of $CD11b^{+}Gr^{+}$ cells at 3hr and (upper) and 15 hr (lower) panels (C-E). The data presented are representative of at least 3 independent experiments (n = 4 mice per group) with similar results. ***, $p < 0.001$.

5.2.4 *Increased neutrophil recruitment correlates with enhanced neutrophil activity and survival and contributes to sepsis mortality*

So far, I have shown that p110 mice are more susceptible to LPS challenge and that the enhanced susceptibility of p110 mice to LPS is accompanied by greater accumulation of neutrophils in their peritoneum compared to their WT counterpart. Since there was no difference in neutrophil numbers between p110 and WT mice 3 hrs after LPS challenge but there was difference at the time of death, I speculated that neutrophils from p110 mice do not undergo apoptosis as fast as neutrophils from WT mice. I therefore compared the level of apoptosis in peritoneal neutrophils from WT and p110 mice injected with LPS by flow cytometry through annexin V staining. In line with my hypothesis, neutrophils from WT mice showed greater apoptosis compared to neutrophils from p110 mice (Fig. 5.5A). Next, I wondered whether the delayed apoptosis of neutrophils correlated with increased neutrophil activity by staining for the peroxidase enzyme myeloperoxidase (MPO). I found that there was greater expression of MPO in neutrophils from p110 mice compared to neutrophils (Fig. 5.5B). These data suggest that the mortality of p110 mice may be due to enhanced neutrophil activity and survival following LPS injection.

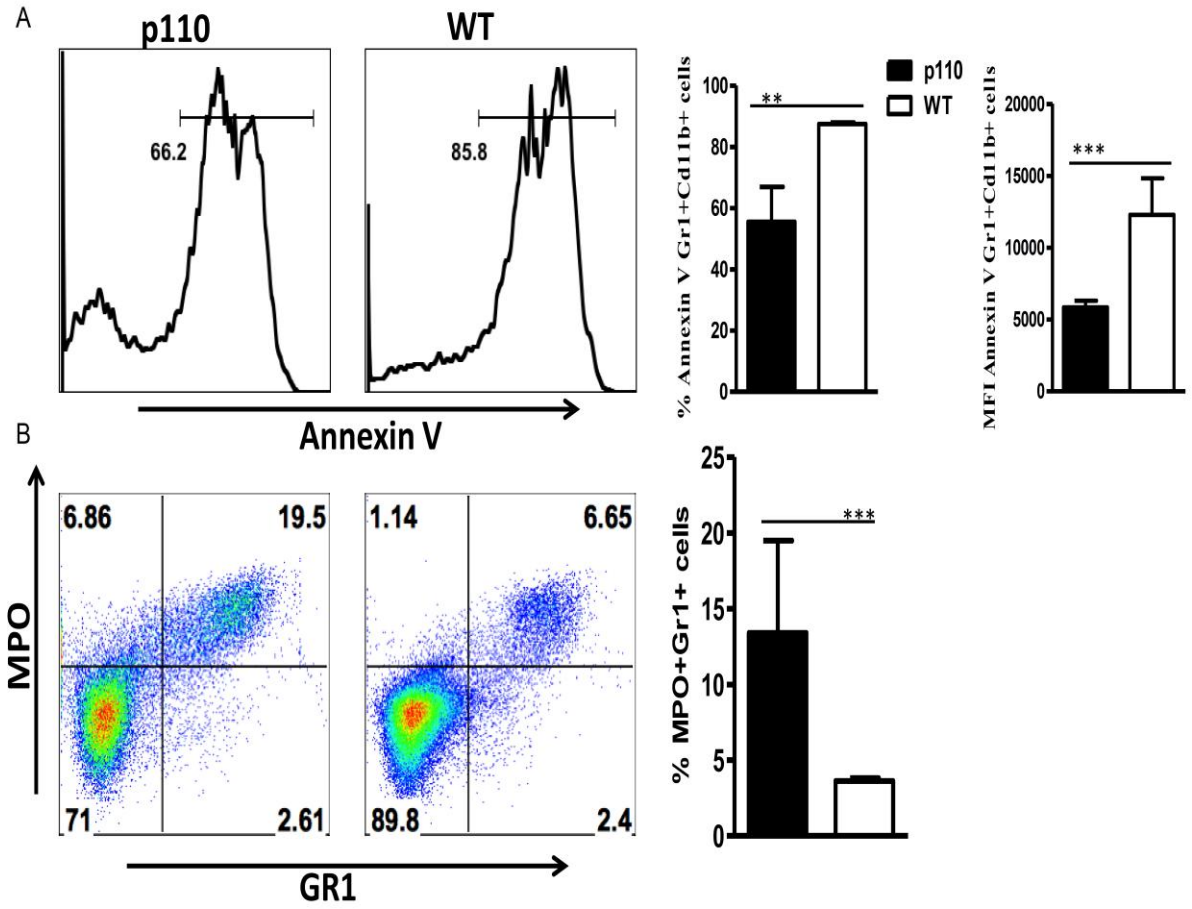


Fig. 5.5 **Impaired apoptosis and increased MPO activity in neutrophils from p110 mice following LPS challenge.** Groups of p110 and WT mice were injected intraperitoneally with LPS (10 mg/Kg). After 15 hr, the mice were sacrificed and the degree of apoptosis in peritoneal neutrophils was analyzed by flow cytometry by assessing the percentage of Annexin V positive cells (A) – gated on $CD11b^{+}Gr1^{+}$ cells. In addition, the peritoneal cells were also stained for MPO intracellularly and the percentage of $Gr1^{+}MPO^{+}$ cells (neutrophils) was analyzed by flow cytometry (B). The data presented are representative of at least 2 independent experiments (n = 4 mice per group) with similar results. **, $p < 0.01$; ***, $p < 0.001$.

5.2.5 *Neutrophils from p110 mice have prolonged survival in response to LPS challenge*

So far, my results show that neutrophils from p110 mice have delayed apoptosis and increased activity in response to LPS. Next, I used in vitro technique to compare the response of neutrophils from p110 and WT mice to LPS in a more carefully controlled setting. I enriched neutrophils from bone marrow of p110 and WT mice by a method that was previously described (261), cultured overnight them in presence of LPS and assessed apoptosis by flow cytometry. Consistent with direct ex vivo results (Fig. 5.5A), BMDN from p110 mice were less apoptotic (lower percentage of annexin V^+ cells) compared those from WT mice (Fig. 5.6A). Interestingly, this difference in cell death between BMDN from p110 and WT mice was evident only in the presence of LPS. The decreased apoptosis in p110 neutrophils correlated with increased Mcl-1 expression, an anti-apoptotic protein that belongs to the Bcl-2 family (Fig. 5.6B). Taken together, these data show that neutrophils from p110 mice have greater activity and delayed apoptosis in response to LPS and suggest that exaggerated neutrophil activity and increased survival may contribute to mortality of p110 mice following LPS challenge.

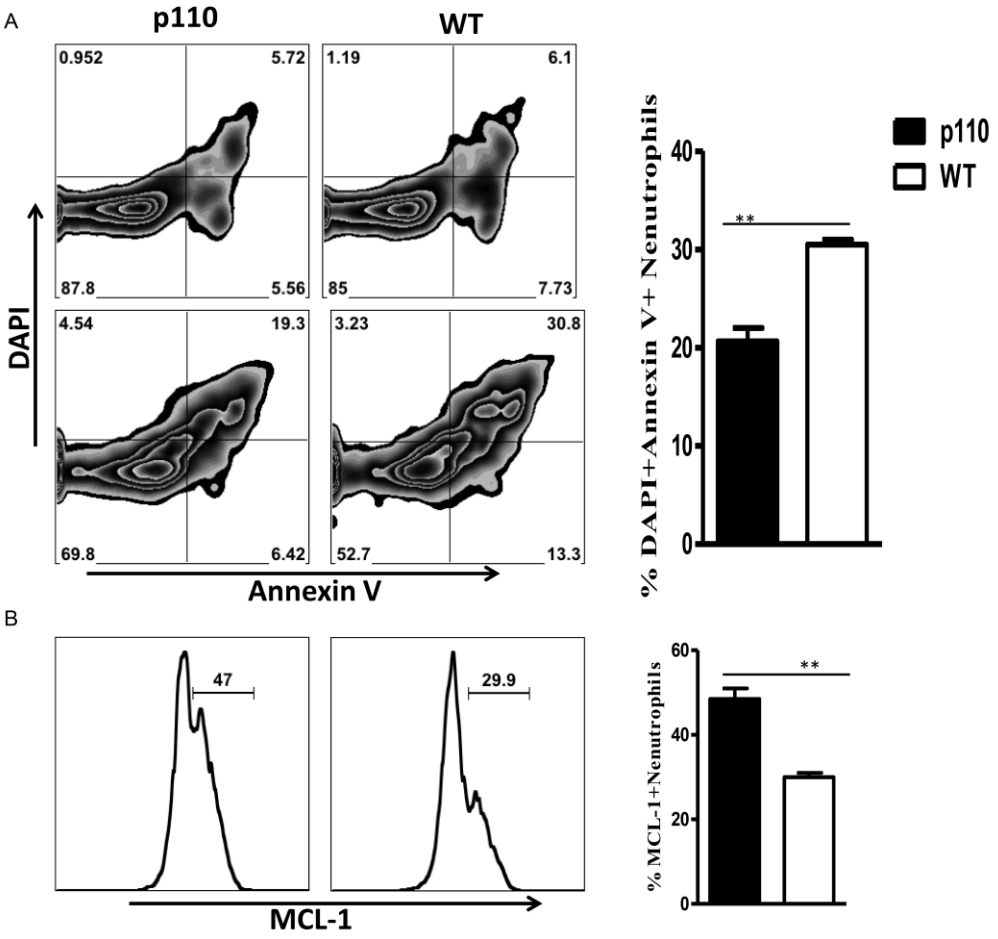


Fig. 5.6 **Reduced apoptosis in neutrophils from p110 mice following *in vitro* stimulation with LPS.** Bone marrow-derived neutrophils from p110 and WT mice were cultured in the absence (top panel) or presence (bottom panel) of LPS overnight and apoptosis (A) and survival (B) was analyzed by flow cytometry. The data presented are representative of at least 2 independent experiments with similar results. **, $p < 0.01$.

5.2.6 ***Depletion of neutrophils rescues p110 mice from LPS mortality***

Neutrophils are one of the first responder cells to bacterial infection and are necessary for the maintenance of anti-bacterial immunity (99). However, exaggerated neutrophil activity is detrimental and has been implicated in the pathology of several diseases (103). To investigate the role of exaggerated neutrophil activity and survival in the mortality of p110 mice, I injected groups of p110 mice with anti-GR1 depleting antibody to deplete neutrophils, before and six hours after LPS challenge. Interestingly, depletion of neutrophils before LPS challenge resulted in mortality of p110 mice following LPS challenge (Fig. 5.7). However, depletion of neutrophils six hours after LPS challenge rescued p110 mice from LPS mortality (Fig. 5.7). These results indicate that the mortality of p110 mice following LPS challenge is due in part to exaggerated neutrophil activity. They further support my hypothesis that neutrophils are important in sepsis but exaggerated neutrophil activity is detrimental to survival from sepsis.

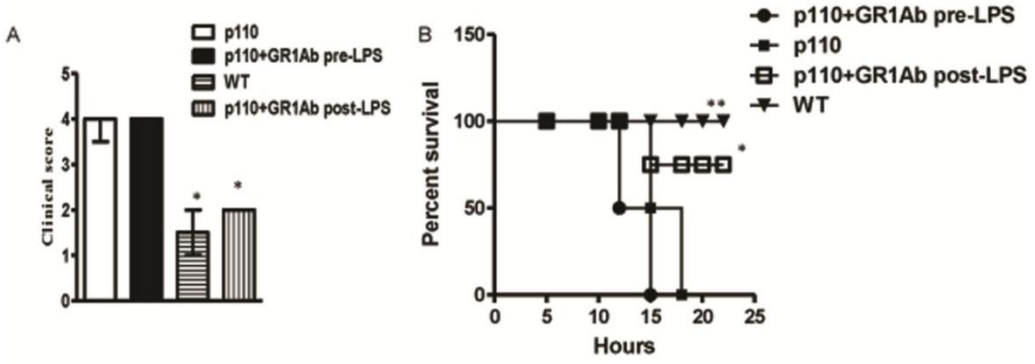


Fig. 5.7 **Depletion of neutrophils after LPS challenge rescues p110 mice from LPS mortality.** Groups of p110 mice were injected with anti-GR1 antibody either overnight before or 6 hr after LPS challenge and monitored for clinical signs (A) and survival (B). The data presented are representative of at least 2 independent experiments (n = 4 mice per group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

5.2.7 *Tregs regulate neutrophil function and reverse LPS-induced survival of neutrophils from p110 mice*

So far, I have shown that p110 mice have diminished Treg numbers and that decreased Treg numbers contribute to the mortality of p110 mice as adoptive transfer of Tregs rescues p110 mice from LPS mortality. In a similar vein, p110 mice have delayed neutrophil apoptosis and enhanced neutrophil activity following LPS challenge and these contribute to mortality as depletion of neutrophils rescues p110 mice from LPS mortality. It remains to be determined whether there is any link between reduced Treg numbers and enhanced neutrophil activity in p110 mice following LPS challenge. In other words, does Tregs regulate neutrophil activity and survival?

To investigate this, I isolated Tregs ($CD4^+CD25^+$) or effector T cells ($CD4^+CD25^-$) from the spleens of p110 and WT mice, co-cultured them with BMDN from p110 mice in the presence or absence of LPS and examined apoptosis by annexin V staining. Strikingly, both p110 and WT

Tregs were able to reverse LPS-induced delayed apoptosis of neutrophils from p110 mice as shown by increased annexin V expression and decreased Mcl-1 expression (Fig. 5.8). This reversal of delayed apoptosis of p110 neutrophils was dose dependent (Fig. 5.8B) and specific to Tregs as effector T cells were not able to produce similar effect (Fig. 5.8A). Additionally, Tregs dramatically reduced proinflammatory cytokine production by neutrophils (Fig. 5.9A-D). Taken together, these data suggest that the diminished Treg numbers in p110 mice may in part contribute to the exaggerated neutrophil activity and survival in response to LPS. These data also suggest that Tregs regulate neutrophil activity and survival and highlights a possible mechanism of regulation of innate immunity by adaptive immunity.

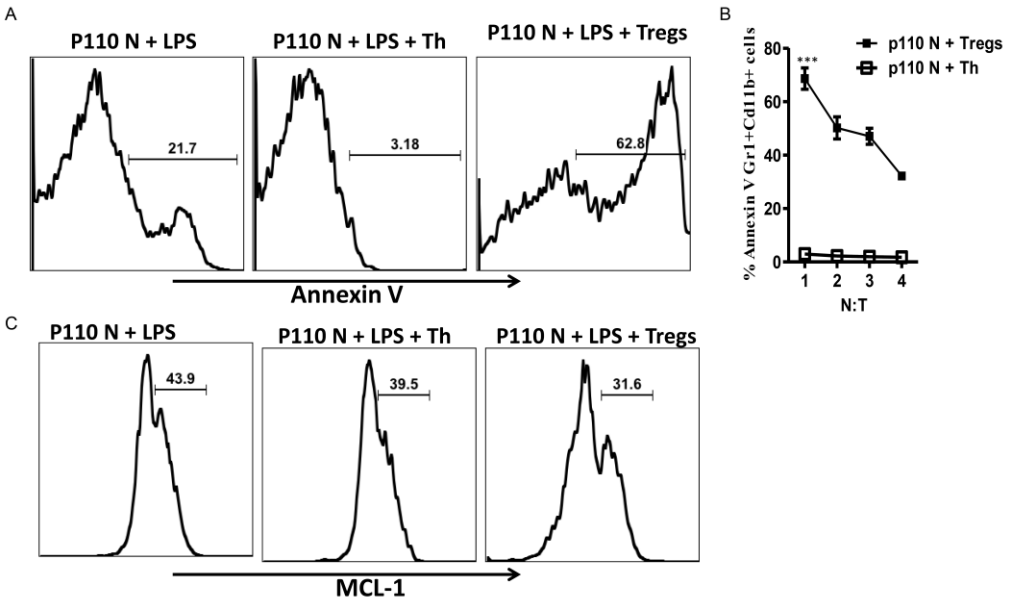


Fig. 5.8 **Tregs reverse LPS-induced survival of neutrophils from p110 mice.** BMDN from p110 mice were co-cultured with varying ratios of either Tregs or T helper cells (Th) of 1:1, 1:2, 1:4 and 1:8 overnight. The next day, neutrophil apoptosis (A, B) and survival (C) were analyzed by flow cytometry. The data presented are representative of at least 3 independent experiments with similar results. ***, $p < 0.001$.

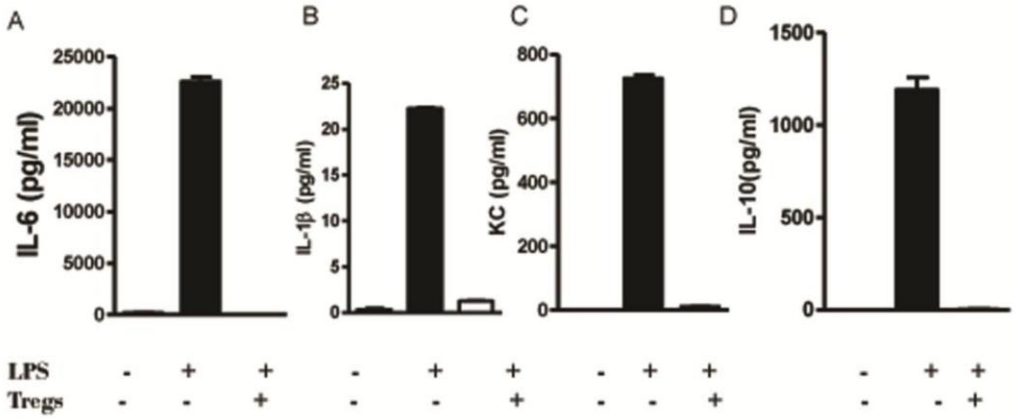


Fig. 5.9 **Tregs reverse LPS-induced exaggerated activity of neutrophils from p110 mice.** BMDN from p110 mice were co-cultured with Tregs overnight. The next day, the levels of proinflammatory cytokines (A-D) were analyzed by mesoscale. The data presented are representative of at least 2 independent experiments with similar results.

The above results are compelling. Following these observations, I wondered the clinical implication of the regulation of neutrophil activity by Tregs. I isolated neutrophils and Tregs from healthy volunteers and co-cultured neutrophils and autologous Tregs in the presence or absence of LPS overnight. Similar to the results obtained in mice, human Tregs were able to increase neutrophil apoptosis and decrease neutrophil activity in response to LPS (Fig. 10A, B). Therefore I conclude that Tregs regulate neutrophil activity and survival and that the regulation of neutrophil activity by Tregs could play an important role in the resolution of bacterial infection.

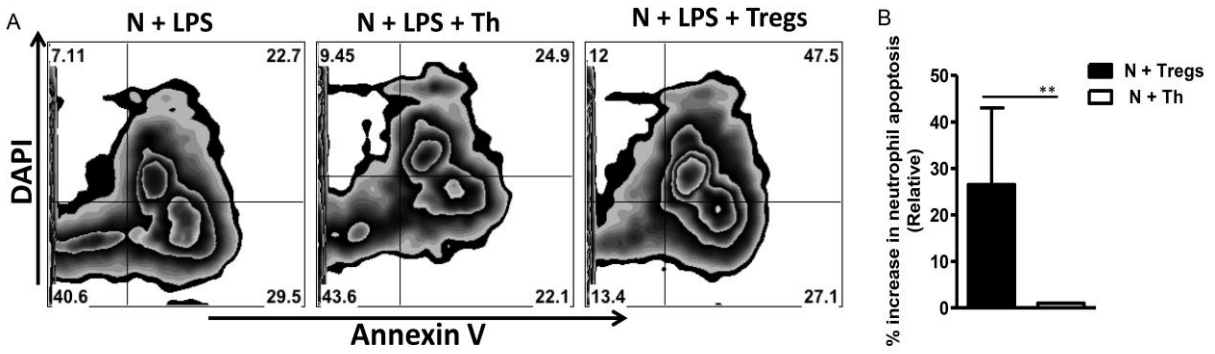


Fig. 5.10 **Human Tregs also reverse LPS-induced survival of neutrophils from p110 mice.** Neutrophils were isolated from the blood of healthy volunteers and co-cultured with either autologous Tregs or helper T cells in LPS overnight. Neutrophil apoptosis was then analyzed by flow cytometry (A, B). The data presented are representative of 3 independent experiments with similar results. *** , $p < 0.001$.

6.0 CHAPTER SIX

GENERAL DISCUSSION

6.1 CRITICAL ROLE OF TREGS IN SURVIVAL OF SEPSIS

Regulatory T cells (Tregs) play a crucial role in maintenance of peripheral tolerance and prevention of aggressive immune response and their potent suppressive function are being investigated for in the treatment of several autoimmune and inflammatory diseases (293). However, their suppressive function can also be detrimental and lead to inability of the host to clear infection (294). Increased numbers of $CD4^+CD25^+$ Tregs has been reported in septic shock patients and these cells have been proposed to contribute to the so called 'immunoparalysis' in individuals with septic shock conditions (230). In line with this proposal, Hiraki et al reported that a decrease in the numbers of $CD4^+CD25^+$ Tregs led to improved survival of septic mice (237). However, this study did not directly assess the impact of $CD4^+CD25^+$ Tregs as mice were treated with anti-IL-10 and/or anti-TGF-β, and this was shown to indirectly reduce the numbers of Tregs as well as their function. Conversely, Heuer et al reported that in a murine model of septic shock induced by cecal ligation and perforation (CLP), adoptive transfer of *ex vivo* stimulated $CD4^+CD25^+$ Tregs improved survival (239). This was the first study demonstrating a beneficial role for $CD4^+CD25^+$ Tregs in a clinically relevant model of sepsis.

Herein, I report an increase in $CD4^+CD25^+$ Tregs following LPS-induced acute inflammatory responses and that depletion of these cells leads to enhanced sensitivity and mortality to LPS challenge. I showed that this effect was specific to LPS as it was not reproduced in another model of acute inflammation following Con A administration. Consistent with the protective role of Tregs, I showed that CD25 KO mice, which do not have functional Tregs, are hypersensitive to LPS challenge. Adoptive transfer of Tregs was able to rescue CD25 KO mice from LPS-

induced mortality. More importantly, I showed that Tregs play a protective role during Gram-negative bacteria (*E. coli*) infection; depletion of Tregs resulted in acute death from an otherwise sub-lethal dose of LPS or *E. coli.* Collectively, my data show that Tregs dampen LPS-induced acute inflammatory response and protect from an acute experimental model of bacterial infection.

Why does LPS injection lead to increase in Treg numbers?

My findings show increase in Treg numbers in spleen and blood of mice injected with LPS (Fig. 3.1). It is worthy of note that increase in Treg numbers have been reported in patients with sepsis (230). It is conceivable that injection of LPS leads to the induction of Tregs from naïve $CD4^+CD25^-$ T cells in the periphery. It is also possible that injection of LPS leads to the migration of Tregs to lymphoid organs. Alternatively, the activation of the inflammation cascade may be compensated for by an increase in Treg numbers in the periphery. In any case, the overwhelming evidence indicates increase in Treg numbers following LPS challenge and during sepsis.

How do Tregs mediate their protective effect following acute bacteria and/or LPS challenge? Previous reports show that $CD4^+$ T cells (particularly Tregs) express TLR4 and respond to LPS stimulation (287, 295). The binding of LPS to TLR4 on innate immune cells triggers a cascade of intracellular signaling events leading to the release of cytokine and non-cytokine inflammatory mediators including TNF, IL-1, IL-6, eicosanoids, and platelet activation factor (58, 296). Collectively, these events lead to mobilization of more inflammatory cells, particularly neutrophils and macrophages to the site of the infection. Dysfunction in regulation of these factors leads to diffuse cellular activation, excessive systemic inflammatory response and death.

Since $CD4^+CD25^+Foxp3^+$ Tregs play a critical role in maintaining immune homeostasis by preventing excessive activation of $CD4^+$ T cells (297), it is conceivable that their depletion following LPS challenge leads to hyper-activation of T cells, leading to overall excessive immune activation and enhanced susceptibility. Consistent with this, I found that depletion of $CD4^+CD25^-$ T cells does not lead to susceptibility to LPS (Fig. 3.5 A-D). In addition, I have found that depletion of Tregs leads to increased proliferation and cytokine production by $CD4^+$ T cells following LPS challenge (Figs. 4.5, 4.6), suggesting that Tregs may help to prevent over-activation of $CD4^+$ T cells. More studies are needed to fully determine the mechanism through which Tregs regulate over-activation of the immune system following LPS challenge.

The immunoregulatory properties of Tregs have been shown to occur via contact dependent and independent mechanisms. The contact dependent mechanism is mediated by direct cell-to-cell contact through cytotoxic T-lymphocyte antigen 4 (CTLA4) expression (173). In contrast, contact independent mechanism is mediated by the production of immunoregulatory cytokines IL-10 and TGFβ (221). Interestingly, I found that the acute death and exaggerated serum levels of proinflammatory cytokines in anti-CD25 mAb-treated mice challenged with sublethal dose of LPS was associated with significantly lower serum levels of TGFβ1 (Fig. 3.2F) while IL-10 levels were unaffected. It is conceivable that the diminished levels of TGFβ1 may be due to depletion of Tregs leading to detrimental outcome in LPS-induced acute inflammatory responses. This is not surprising since TGFβ1, (a potent immunoregulatory cytokine), has been shown to prevent aggressive T cell responses (298) and its deficiency leads to autoimmune lymphoproliferative disease as seen in TGFβ1 null mice (194). More work is needed to properly decipher the role of Treg derived TGFβ1 in immune regulation upon LPS challenge.

In contrast to my findings, Scumpia et al reported that inhibition of $CD4^+CD25^+$ Tregs activity either by antibody-mediated depletion or genetic deletion does not affect survival outcome in a CLP model of sepsis (234). LPS infusion or live bacterial injection are both more acute animal models of sepsis compared to CLP (299). This may explain the difference between my findings and those of Scumpia et al but also strongly agrees with my proposal that $CD4^+CD25^+$ Tregs are initially protective in sepsis but this beneficial activity may be lost over time. However, Heuer et al (239) showed that adoptive transfer of *ex vivo* stimulated $CD4^+CD25^+$ Tregs improved survival in CLP model of sepsis an observation that is in agreement with my findings. Thus, although more work is needed to delineate the role of $CD4^+CD25^+$ Tregs in sepsis, the overwhelming evidence suggests a potential beneficial role for these cells in several experimental models of sepsis. In conclusion, my results show that depletion of Tregs resulted in high mortality in a non-lethal LPS challenge and E *coli* infection. Collectively, my results suggest that Tregs may help to suppress inflammation, improve bacterial clearance and promote survival in an acute model of LPS challenge or bacterial infection.

6.2 TREGS RESTRAIN $CD4^+$ T CELLS FROM CAUSING EXAGGERATED IMMUNE ACTIVATION AND HYPERSENSITIVITY TO LPS

Previously, I showed that depletion of Tregs leads to enhanced sensitivity to LPS that is characterized by acute death to an otherwise non-lethal dose of LPS challenge or E. coli infection and production of high levels of proinflammatory cytokines. Following that observation, I investigated the mechanism by which Treg depletion results in enhanced sensitivity to LPS. Using different murine lymphocyte gene knock-out models, I showed that in the absence of T cells, the enhanced sensitivity to LPS is abolished thereby implicating T cells as the drivers of unrestrained host immune response to LPS following Treg depletion. Critically, I showed that reconstitution of SCID mice with $CD4^+$ T cells restores enhanced sensitivity to LPS following

Treg depletion. Furthermore, reconstitution of SCID mice with $CD4^+$ T cells from CD25 KO mice (which lack functional Tregs) results in acute death to a non-lethal LPS challenge. Collectively, my results implicate $CD4^+$ T cells as the lymphocyte subset that drives aggressive immune response to LPS in the absence of Tregs. This novel protective role of Tregs in LPS-induced acute inflammatory response is consistent with the numerous reports showing that Tregs prevent over-activation of $CD4^+$ T cells and that aberrant Treg function leads to T cell-mediated autoimmune diseases in both human and animal studies (300-302).

Previous reports have shown a significant increase in the percentage (and absolute numbers) of Tregs in both human and animal models of sepsis (232, 236). However, the role of Tregs in sepsis remains controversial with some reports suggesting they may contribute to mortality by mediating immunosuppression (232). On the contrary, my data indicates that during bacterial infection and in LPS model of acute inflammation, Tregs are important for survival and that diminished Treg numbers leads to excessive $CD4^+$ T cell activation and acute death to an otherwise non-lethal dose challenge. In agreement with my findings and using a more relevant clinical model of sepsis, Heuer et al showed that Tregs improve survival in a murine model of polymicrobial sepsis (239).

Studies have shown that depletion of Tregs can efficiently reduce chronic retroviral loads and that therapy targeting Tregs may be a promising approach for the treatment of chronic infectious diseases (303). In addition, it has been suggested that since Tregs contribute to tumor-mediated immunosuppression in cancer patients (304), therapies targeting Treg depletion may be beneficial in managing the condition (305). My findings highlight Tregs as essential regulators of the inflammatory milieu in response to LPS challenge or bacterial infection. Additionally, my

data shows that the use of such Treg-targeted therapies may lead to an unwanted susceptibility to bacterial infection.

How do $CD4^+$ T cells contribute to susceptibility to LPS challenge? I speculated that following their interaction with LPS, a subset of TLR-4-expressing $CD4^+$ T cells become activated and produce IFN-γ and TNF (Fig. 4.6A and D), cytokines that are potent activators of macrophages (288). These cytokines activate macrophages, which in turn contribute to the inflammation milieu by producing inflammatory mediators such as the proinflammatory cytokines TNF, IL-6 and CCL2 (306). As has been previously shown in several models, Tregs are critically important in keeping $CD4^+$ T cells under tight check and regulation to avoid their excessive activation. Thus, it follows that the hyper-activation of $CD4^+$ T cells as a result of Treg depletion, will indirectly lead to hyper-activation of macrophages. The end-result of such an aggressive immune response is host injury and mortality to an otherwise non-lethal challenge with LPS or with live bacteria. Hence, Treg depletion is detrimental to survival in a murine model of acute inflammation and a sepsis model of live *E. coli* infection.

Pattern recognition receptors (PRRs), such as TLR4, have long been identified as essential receptors of the innate immune system. However, accumulating evidence suggest that these receptors are not exclusive to innate cells but are also present on cells of the adaptive immune system. Indeed, it was recently reported that human T cells express TLR4 (287, 307). Similarly, it has been shown that a subset of mouse T cells, including Tregs, express TLR4 (286). In line with these observations, I found that $CD4^+$ T cells express TLR4 and are activated by LPS *in vivo* and *in vitro* resulting in proliferation and enhanced IFN-γ and TNF production (Fig. 5.7A-F). Additionally, I show that LPS-stimulated T cells (but not unstimulated cells) activate bone marrow-derived macrophages to produce inflammatory cytokines (Fig. 5.7G and H).

Importantly, I demonstrate that absence of TLR4 expression on $CD4^+$ T cells leads to significantly diminished response to LPS in both *in vitro* and *in vivo* studies (Fig. 5.8A-D). Thus, my findings corroborate the results of other studies that show that T cells possess germline encoded pattern recognition receptors such as TLR4 and are able to respond to pathogen associated molecular patterns such as LPS. More work is needed to delineate the signaling pathways involved in $CD4^+$ T cell activation by LPS.

How do Tregs restrain $CD4^+$ T cells from becoming overly activated and thereby prevent sensitivity to LPS? Although not directly assessed in this study, certain observations provide possible clues to this question. Previous reports have shown that some subsets of Tregs express TLR4 and respond to LPS (286). It is conceivable that as for $CD4^+$ T cells, TLR4-expressing Tregs may become activated to produce inhibitory and/or regulatory cytokines, including IL-10 and TGF-β, upon recognizing LPS. As has been shown in other studies (221), these immunoregulatory cytokines may directly or indirectly regulate $CD4^+$ T cell activation and/or inhibit proinflammatory cytokine production by T cells and macrophages. Indeed, it was shown that LPS-activated Tregs were able to prevent wasting disease caused by naïve $CD4^+$ T cells in alymphoid recipients (286). In line with this, I found that depletion of Tregs was associated with dramatic reduction in TGF-β production following LPS or E. coli challenge (Fig. 3.2).

In conclusion, my data shows that in the absence of Tregs, $CD4^+$ T cells drive systemic inflammatory response following LPS challenge that is detrimental to survival and injurious to the host. Collectively, these data are consistent with previous reports that demonstrate a beneficial role of Tregs in several experimental models of sepsis (239-240). They further suggest that these cells may help suppress inflammatory responses and promote survival in an acute model of LPS challenge.

6.3 ABSENCE OF P110 DELTA SIGNALING LEADS TO DECREASED TREG NUMBERS AND INCREASED NEUTROPHIL RECRUITMENT AND FUNCTION RESULTING IN MORTALITY TO NON-LETHAL LPS CHALLENGE

The results obtained in the previous chapters showed that Tregs are able to respond to LPS and help to prevent excessive activation of effector T cells in response to LPS. Hence, depletion of Tregs leads to enhanced susceptibility and mortality to non-lethal LPS challenge or bacterial infection. Mice lacking p110δ signaling have characteristically diminished Treg numbers and therefore constitute a suitable model for the investigation of the role of diminished Treg numbers in LPS challenge (252). I therefore used these animals to investigate the effect of diminished Treg numbers in LPS challenge. Consistent with the results in previous chapters, I found that p110 mice are susceptible to LPS challenge and succumb to a non-lethal dose of LPS (Fig. 5.1A-C). This susceptibility is directly related to decreased Treg numbers as adoptive transfer of WT Tregs rescues p110 mice from LPS-induced mortality (Fig. 5.1D, E). Thus, the p110 mice model confirms my previous findings (using anti-CD25 mAb) that diminished Treg numbers is associated with susceptibility to LPS challenge. This also further provides evidence of the protective role of Tregs in bacterial infection.

The upregulation of neutrophil survival in the presence of LPS has been well described as a necessary mechanism for the elimination of infection by neutrophils (308). While there is no difference in survival of neutrophils from p110 and WT mice in the absence of LPS, neutrophils from p110 mice show delayed apoptosis and increased survival in response to LPS. Hence, it appears that the absence of p110δ signaling leads to delayed apoptosis of neutrophils in response to LPS. Since mice lacking p110δ signaling also have diminished Treg numbers (252), it is conceivable that the absence of p110δ signaling and diminished Treg numbers constitute a dual hit that leads to the susceptibility of p110 mice to LPS challenge.

Majority of the regulatory function of Tregs has been ascribed to their ability to regulate $CD4^+$ T cells (207). Recently, there is emerging evidence of the suppressive function of Tregs on $CD8^+$ T cells (205), B cells (208), monocytes (210), mast cells (212) and neutrophils (213). In this study, I found that p110 mice, which have diminished Treg numbers and are more susceptible to LPS challenge, have exaggerated neutrophil activity and increased neutrophil survival. This enhanced neutrophil activity contributes to mortality of p110 mice as depletion of neutrophils abrogates this mortality (Fig. 5.7). Strikingly, co-culture of Tregs and neutrophils resulted in reduced neutrophil activity and increased neutrophil apoptosis. These results demonstrate that Tregs regulate neutrophil function and survival and illustrates a mechanism for the regulation of innate immunity by adaptive immunity.

It is well known that neutrophils are important for the elimination of bacterial infection. For example a mechanism for antibacterial activity by neutrophils is the process of respiratory burst, which involves the production of superoxide anion from molecular oxygen to yield microbicides (115). Hence, patients with chronic granulomatous disease – a genetic disease that affects the respiratory bursts of phagocytes are susceptible to bacterial infections (309). Paradoxically, neutrophil activity has been implicated in the pathogenesis of several diseases. For example, Bullous pemphigoid (BP) is a skin disease caused by autoimmunity to the epidermal protein BP 180. Studies in animal models revealed that depletion of neutrophils resulted in resistance to BP (310). Interestingly, BP is triggered by the injection of anti-BP 180 antibodies in WT mice but not in mice deficient in the neutrophilic granular protein – Gelatinase B, although there was normal recruitment of neutrophils and pathogenic deposition of IgG (311). These experiments demonstrate that neutrophil activity is a major contributor to the pathogenesis of BP. In the same vein, neutrophils have been implicated in the pathogenesis of vasculitis (312), rheumatoid

arthritis (313) and systemic lupus erythematosus (314). Interestingly, all these diseases have been associated with defective Treg function (315). These observations are intriguing and show that in many cases, defective Treg function is usually associated with exacerbated neutrophil activity. This further lends credence to this work that diminished Treg activity in p110 mice is accompanied by exaggerated neutrophil activity.

A major mechanism of maintenance of immune homeostasis and function involves the programmed elimination of immune cells through apoptosis. Dysfunction in the regulation of apoptosis constitutes a major problem for the immune system and can lead to immune suppression (in the case where there is too much apoptosis) or cancer (in the case where apoptosis is too low). Hence, there are immunoregulatory mechanisms that are in place to ensure the maintenance of optimum apoptosis and immune balance. In this work, I identify Tregs as a major regulatory cell responsible for the regulation of neutrophil apoptosis particularly in response to bacteria associated inflammatory response. Since increased neutrophil survival is a major consequence of neutrophil activation by LPS (308), it is conceivable that Tregs ensure programmed cell death of neutrophils following activation by LPS. In fact, my results suggest this to be the case. This work is also in agreement with the findings of Lewkowicz et al, which showed that Tregs inhibit neutrophil function and promote their apoptosis (213). So far, these are the only two publications indicating Treg/neutrophil interactions.

Tregs play a major role in the maintenance of peripheral tolerance and immune homeostasis. Indeed, several theories have been put forward to explain Treg-mediated immune suppression and most of them point to the fact that the mechanisms of Treg-mediated immune suppression are not well understood. Tregs have been shown to suppress the function of other immune cells via direct cell-to-cell contact through cytotoxic T-lymphocyte antigen 4 (CTLA4) expression

(173) or by the production of immunoregulatory cytokines IL-10 and TGFβ (221). Another important mechanism by which Tregs mediate their suppressive function is by promoting apoptosis of the target cell. Indeed, Tregs have been shown to mediate apoptosis of T cells (202), B cells (203), monocytes (202) and neutrophils (316). Hence, apoptosis of immune cells is a well-established process of maintaining immune homeostasis by Tregs.

How does Tregs mediate apoptosis of target cells? Studies in animal and human models show that Tregs mediate apoptosis through the Fas/FasL interaction. Tregs have also been shown to mediate apoptosis by the direct release of perforin and granzyme (202-203). This work did not explore the mechanism by which Tregs mediate neutrophil apoptosis. However, Lewkowicz et al demonstrated that Treg suppression of neutrophil function is partially dependent on the cytokines, IL-10 and TGF-β (316).

The distinction between the innate and adaptive immune systems is a central dogma in immunology (317). The inter-dependency of both arms of the immune system is well established. For example, antigen presentation and the requirement for co-stimulation provide evidence of the dependence of the adaptive immune system on innate immune response. In a similar vein, cytokines produced by the adaptive immune system are known to affect the cells of the innate immune system. This work portrays the interaction of Tregs (cells of the adaptive immune system) with neutrophils (cells of the innate immune system) as an essential mechanism of immune regulation during infection. Hence, the results present a new paradigm in the regulation of the innate immune system by the adaptive immune system.

The results presented here have implications in the clinic for sepsis treatment. Neutrophil activity is known to be important in sepsis and has been proposed as a reliable biomarker for sepsis

(318). The results of this work indicate that neutrophil activity is a potential clinical predictor of outcome during sepsis. Furthermore, it is conceivable that inhibition of neutrophil activity can also be a veritable approach in sepsis treatment as demonstrated here. Indeed, Hasan et al have already shown that targeting neutrophil CD44 prevented lung damage in abdominal sepsis (319). Hence, there is an opportunity here for clinical translation of this work.

Overall, this work illustrates the significance of Tregs in regulation of bacterial infection and highlights the cross-talk between innate and adaptive immunity.

6.4 OVERALL DISCUSSION

6.4.1 Tregs and regulation of sepsis

There is unequivocal experimental evidence in human and animal models regarding the paramount role of Tregs in maintenance of immune homeostasis (219). Hence, this work adds to the numerous evidences illustrating the role of Tregs in immune regulation. Tregs were discovered as immune cells that were essential for tolerance to self-antigens and prevention of autoimmunity (160). However, since their discovery, there has been an explosion in the role of Tregs in immune function from fetal-maternal tolerance to asthma (219). Since Tregs are suppressor cells, it is paradoxical that they will be important in pathogen-induced immune response. However, this function of Tregs has been demonstrated in several studies. For example, depletion of Tregs resulted in increased susceptibility to herpes simplex virus and lymphocytic choriomeningitis virus indicating a protective role for Tregs in virus-induced immunopathology (218). Also, Cambos et al showed that Tregs help to control excessive inflammation in lethal *plasmodium chabaudi adami* infection in which mortality is associated with systemic inflammatory response (240). In line with the above studies, this work shows that Tregs are important in the regulation of bacteria associated inflammatory responses. Specifically, diminished Treg numbers leads to mortality to a non-lethal dose of LPS and bacteria challenge (Fig. 3.2, 3.7) In view of these observations, in the case of pathogen-induced immune response, it is pertinent to regard Tregs as regulatory cells and not suppressor cells. This is because although Tregs help to maintain immune homeostasis by ‘suppressing’ the immune response, their ‘suppressor’ function is indeed a regulatory mechanism that is indispensable in the immune response to pathogen invasion. Removal of this regulatory mechanism as has been demonstrated in this work can be catastrophic to the host analogous to a moving automobile without brakes.

However, in light of the well known suppressive function of Tregs, there is a school of thought that is associated the increased Treg numbers found during sepsis with immune suppression that is associated with increased mortality. Monneret et al associated increased Treg numbers in septic shock patients with sepsis-induced immunosuppression (230). They showed that Tregs actually induce apoptosis in monocytes and conclude that this is a mechanism by which Tregs contribute to immune suppression during sepsis (210). For one thing, their study agrees with mine in the ability of Tregs to regulate the function of innate immune cells, in their own case monocytes. In contrast, I hold that Tregs are protective during sepsis as the data in this work has demonstrated. I propose that the role of Tregs in sepsis can be rightly described in two words: dosage and timing. The increase in Tregs observed in patients with sepsis and in experimental sepsis is protective during the initial stages of the disease. It is also possible that these Tregs contribute to immune suppression in the later stages of sepsis. However, patients with sepsis are already in a state of immune dysfunction and the suppressed immune state cannot be due to the presence of Tregs alone.

It is generally accepted that sepsis results from excessive and dysregulated immune response to infection which can culminate in tissue damage and organ dysfunction (284). However, studies show that the hyper inflammatory response is often accompanied by an opposite and/or equivalent anti-inflammatory response, a phenomenon that can be likened to Newton's law of momentum in physics (64). In addition, sepsis is a complex, heterogeneous and dynamic syndrome consisting of three stages: sepsis, severe sepsis and septic shock. This complexity makes results from clinical and experimental research involving sepsis usually difficult to interpret (320).

The results presented here show a beneficial role of Tregs in sepsis. In light of the increase in knowledge of the immune response during sepsis, there is a gradual shift in paradigm on the major consequence of a suppressed immune state in sepsis compared to the inflammatory response. While the beneficial role of Tregs has been reported in several immune pathologies like type 1 diabetes (271), systemic lupus erythematosus (272) and inflammatory bowel disease (273), the immune suppressive role of Tregs is detrimental in certain cases like cancer (321). For example, adoptive transfer of Tregs leads to reduced anti-tumor immune response in mice (322). In their recent review, Hotchkiss and Moldawer drew an analogy between cancer and infectious disease (323). They argue that the immune response in cancer and chronic infectious disease are similar, and marked by persistent inflammation and immune activation which can ultimately lead to 'immune exhaustion'. They suggest that therapies aimed at boosting the immune system are ideal for sepsis treatment (323). Also, as mentioned earlier, Monneret et al associated increased Treg numbers in septic shock patients with sepsis-induced immunosuppression (230). These findings argue against the potential use of Treg therapy for sepsis patients. I agree that there is a concern of Treg-mediated immune suppression in sepsis which is in contrast with the major findings of this work. However, there is no doubt that inflammation contributes to sepsis mortality (324). Hence, Treg therapy may be beneficial for a select group of patients and should be investigated further.

As shown in this work (Fig. 4.7A and B) and in other studies (287, 307), pattern recognition receptors such as TLR4, which have long been identified as essential receptors of the innate immune system, are also present on cells of the adaptive immune system. Accordingly, Caramalho et al showed more than a decade ago that Tregs possess TLR4 and are able to respond to LPS (286). The fact that Tregs respond to LPS strongly suggests that they are

activated during bacterial infection. Since Tregs suppress inflammation, depletion of Tregs or diminished Treg function will contribute to excessive inflammation in response to bacterial infection as shown in this work.

The inflammatory response has long been implicated in the pathogenesis of sepsis. Indeed sepsis is defined as systemic inflammatory response to infection. As already mentioned earlier, the changes which the immune system undergoes during the inflammatory response – the release of proinflammatory cytokines, aggregation of immune cells, activation of immune cells, changes in endothelial permeability and vasodilation, can be detrimental to the host (324).

Several studies have implicated the inflammatory response in the pathogenesis of several diseases, notably, allergy and cancer (325-326). This work adds to the growing body of evidence of the paradoxical role of the immune system in disease pathogenesis. Hence, the data shows that in the absence of adequate Treg function, two immune cells – $CD4^+$ T cells (Fig. 4.6, 4.7) and neutrophils (Fig. 5.4) become the culprit in LPS-induced mortality. In general $CD4^+$ T cells have been shown to contribute to the pathogenesis of other diseases (327). On the same note, neutrophils have also been implicated in disease pathology in many studies (328). In view of the above evidences and as shown in this work, the immune response – our arsenal for fighting infections can evidently be a major contributor to mortality.

The quality of the immune response is the ultimate decider of susceptibility and resistance of the host following pathogen invasion. Hence, following bacterial infection, an optimum immune response is needed for pathogen elimination. An immune response below the optimum will lead to pathogen colonization of the host and susceptibility to infection. In contrast, an immune response above the optimum will lead to organ damage and mortality. The problem of

elucidating factors that determine an optimum immune response is a basic problem in immunology and beyond the scope of this work. This work however, highlights the fine line between susceptibility and survival and implicates immune cells as contributors to pathology during sepsis.

6.4.2 Cross-talk between innate and adaptive immunity

The term immunity, which is the ability of a living organism to resist microbial colonization, has many different attributes. Classically, the immune system is broadly divided into two: innate and adaptive immunity. Cells of the innate immune system are characterized by a rapid response to microbial products, and having a finite repertoire of germline-encoded receptors. On the other hand, cells of the adaptive immune system have a time lag to respond following infection and are able to expand in a clonal manner producing a large repertoire of antigen-specific receptors by somatic recombination. The cells of the adaptive immune system, T and B cells, encounter antigen in lymphoid organs and then undergo clonal expansion differentiating into effector cells. These two arms of the immune system are notably different in three areas: speed of response, specificity of receptors and memory. Memory is a distinct property of the adaptive immune system arising from the fact that antigen-specific T and B cells can persist-long term and can clonally expand rapidly producing stronger effector functions upon secondary challenge. While there are valid reasons for this distinction between innate and adaptive immunity, emerging evidence suggests that the separation between innate and adaptive immunity is indeed blurred.

This work further highlights the blurry line dividing innate and adaptive immunity. For one thing, my results show that a subpopulation of $CD4^+$ T cells possesses TLR4 (Fig. 4.7A, B). This in itself is not surprising since Tregs have been shown to possess TLR4 more than a decade ago

(286). However, this finding of the germline-encoded PRRs on T cells as reported in this work and in other studies (287, 307) brings to light many unanswered questions. If T cells respond to LPS through TLR4, does that imply rapid immune response and elimination of the lag phase by adaptive immune cells in response to primary infection? Does the T cell response to LPS through TLR4 eliminate the requirement for co-stimulation and antigen presentation? What are the genetic markers and distinct phenotype of the subset of T cells that respond to LPS? What are the implications of the presence of PRRs on cells of the adaptive immune system? These questions remain to be answered and are the subject of different immunological research going on all over the world. The final question was answered in part through this study. I showed that T cells are able to respond directly to LPS (Fig. 4.6, 4.7) and that the response of Tregs to LPS is a critical regulatory mechanism for the control of responses of other immune cells, particularly T cells and neutrophils.

Critics of the separation of both arms of the immune system point to numerous studies that further show a significant overlap in their functions. For example, germline encoded receptors of the innate immune system although limited in diversity show 'specificity' in their ability to detect specific microbial products. Hence TLR4 will detect LPS (82) but not double-stranded RNA, which is recognized by TLR3 (329). Also, the notion of the lack of memory in innate immune cells has been challenged by recent studies. There is evidence of immune memory in primitive organisms that do not have adaptive immunity (330). Natural killer (NK) cells are cells of the innate immune system that have been shown to have memory-like attributes. NK cells were previously shown to have a half-life of about 2 weeks (331) but recent evidence has challenged that assertion. For example, NK cells have been shown to have memory-like attributes in hapten-induced contact hypersensitivity (CHS) in mice. CHS was previously

thought to be mediated by $CD4^+$ T cells (332). However, (Rag-2)–deficient mice, which lack T and B cells but possess NK cells were shown to exhibit NK cell–mediated CHS response (333). The 'memory-like' NK cells showed hapten-specific responses and were detected 4 weeks after hapten priming. Interestingly, adoptive transfer of hapten-primed NK cells could induce CHS in recipient mice (333-334). Other studies have shown evidence of immune memory using NK cells (335-337).

The interdependency of the innate and adaptive immune system is well established. The requirement for co-stimulation to mount an effective immune response by T and B cells emphasizes this. Hence cells of the innate immune system have long been known to influence adaptive immunity. Studies have shown that activated neutrophils release several chemokines like CCL20, CXCL10, CXCL9 and CCL2 that can attract T cells to sites of inflammation (338). Likewise, activated T cells can release CXCL8, which is a neutrophil chemoattractant (338). Also, activated T cells produce cytokines like IFN-γ which modulates neutrophil function and survival (339). In particular, Tregs produce CXCL8, which is a strong neutrophil chemoattractant (340). Recent findings suggest that neutrophils shape adaptive immunity significantly more than previously thought (341). Neutrophils have been shown to migrate to lymph nodes following antigen encounter suggesting the possibility of antigen presentation to T and B cells (342). In vitro, murine neutrophils have been induced by T cells to express MHC class II molecules (343). Also, injection of neutrophils pulsed with antigen into mice resulted in the differentiation of naïve $CD8^+$ T cells into effector cytotoxic cells (344). These studies highlight the expanding role of neutrophils in shaping adaptive immunity and vice versa. Therefore, it is not surprising that Tregs regulate neutrophil function as found in this study since

the regulatory activity of Tregs can lead to the production of cytokines like IL-10 and TGF-β which affects neutrophil function (345-346).

As mentioned earlier, this work highlights the interdependency of the innate and adaptive immune system. Sepsis is well known to be a consequence of innate immune cells to infection. However, it is possible for cells of the adaptive immune system to contribute to the pathology of sepsis. The importance of T cells in sepsis is emphasized in this work. In particular, the regulatory role of Tregs is highlighted. This work shows that in the absence of Tregs, there is enhanced susceptibility and increased mortality in response to infection (Fig. 3.2, 3.7). This enhanced susceptibility and increased mortality in response to infection is caused by exaggerated T cell responses in the absence of Tregs (Fig. 4.6, 4.7). Exaggerated T cell response, marked by enhanced production of cytokines like TNF and IFN-γ (Fig. 4.6) will in no doubt affect innate immune cells like neutrophils and macrophages (Fig. 4.7G, H) leading to organ damage and LPS mortality. Therefore, it is possible for the excessive inflammatory response of innate immune cells to bacterial infection to originate from the signals they receive from cells of the adaptive immune system as shown in this work.

CHAPTER SEVEN

SIGNIFICANCE AND IMPLICATIONS

7.1 SIGNIFICANCE

The work in this thesis constitutes a significant contribution to immunology and infection. Firstly, it expounds the role of Tregs in LPS and E. coli-induced acute inflammation. Since their discovery, Tregs have proven to be paramount for normal immune function and maintenance of homeostasis. Interestingly, Tregs were originally identified as immune cells necessary for the maintenance of peripheral tolerance (160). This inherently meant that Tregs have the function of suppressing immune response. Indeed, it is paradoxical that this subtype of suppressor cells is important for host immunity to infection. Nevertheless, recent evidence has shown that Tregs are essential for host immunity to infections (218, 240). As shown in this work, depletion of Tregs leads to susceptibility to *E. coli* infection. Heuer et al previously showed that in a murine model of septic shock induced by CLP, adoptive transfer of *ex vivo* stimulated $CD4^+CD25^+$ Tregs improved survival (239). However, the work reported here is the first demonstration of the importance of Tregs in live bacterial infection. My results affirm that depletion of Tregs leads to susceptibility to live bacterial infection (Fig. 3.2B, C) and thus has contributed to our knowledge of the role of Tregs in anti-bacterial immunity.

Another important contribution of this work is its elucidation of the role of Tregs in the regulation of neutrophil function and survival. Neutrophil activity is critical in several immune functions and has been implicated in several immunopathologies. Interestingly, most diseases associated with neutrophil activity have been shown to involve defective Treg function. For example, neutrophils are known to contribute to the progression of rheumatoid arthritis (RA)

(347), at the same time diminished Treg function is associated with the progression of RA (274). In spite of these evidences suggesting a link between exaggerated neutrophil activity and defective Treg function, only one study had previously looked at the ability of Tregs to regulate neutrophil function and survival (213). This work therefore highlights the important cross-talk between neutrophils and Tregs and the catastrophic implication of diminished Treg function on neutrophil activity and survival. It holds implications not only for sepsis but several immunopathologies in which inflammation is driven by exaggerated neutrophil function.

7.2 IMPLICATIONS

Previous reports show that Tregs increase in patients with sepsis (231) but whether Tregs contribute to sepsis pathology through immunosuppression or helps to prevent excessive inflammation during sepsis is dogged with controversy. I report here that Tregs are protective during sepsis and that depletion of Tregs leads to susceptibility and mortality to non-lethal LPS and bacterial challenge (Fig. 3.2 B, C; Fig. 3.7). Importantly, I show that Treg therapy is able to rescue mice from LPS-induced inflammation (Fig. 3.4 B, C). This finding is significant and has implications for sepsis treatment in the clinic. Hence from my findings, it is conceivable that Treg therapy may be a veritable approach in sepsis treatment. Treg therapy has also been established in animal models of several immune-mediated inflammatory diseases like type 1 diabetes (348) and graft-versus-host-disease (GVHD) (349). Recently, Treg therapy has found its application in the clinic. Di Ianni and co-workers performed the first human Treg infusion therapy for the prevention of GVHD in patients with HLA unmatched donors in bone marrow transplantation (350). Similar work has also been reported by Brunstein and co-workers (351). The use of Treg therapy for clinical sepsis is foreseeable in the future especially in the early stages of sepsis when excessive inflammation is a major concern. Hence, following the diagnosis

of severe sepsis, a patient can be infused with autologous Tregs or with Tregs from HLA-matched donors.

A major finding in this work is the role of immune cells in immunopathology during LPS-driven inflammation. In particular, this work shows that exaggerated neutrophil activity is associated with poor outcome from LPS challenge. The results presented here have implications in the clinic for sepsis treatment. Neutrophil activity is known to be important in sepsis and has been proposed as a reliable biomarker for sepsis (318). The results of this work indicate that neutrophil activity is a potential clinical predictor of outcome during sepsis. Furthermore, it is conceivable that inhibition of neutrophil activity can also be a veritable approach in sepsis treatment as demonstrated here. Indeed, Hasan et al have already shown that targeting neutrophil CD44 prevented lung damage in abdominal sepsis (319). Hence, this work implicates neutrophils as a major contributor to sepsis in the case of diminished Treg numbers. This implies that neutrophil activity can be used as a marker of disease severity in sepsis and indeed in certain inflammatory disorders. In this regard, a clinical study investigating neutrophil function in sepsis survivors vs non-survivors will be essential. Also, it will be important to determine the relationship between the numbers of Tregs and neutrophils in sepsis survivors vs non-survivors.

The data presented here highlights that the suppressive nature of Tregs is beneficial during infection. In contrast, the suppressive function of Tregs in certain clinical conditions like cancer is known to be detrimental (305). In the case where Treg suppressive function is not desired, Treg depletion is advocated. The results of this work imply that Treg depletion in patients may result to susceptibility to infections. Overall, the results presented here are of major clinical significance and may have implications for the clinical treatment of sepsis.

7.3 FUTURE DIRECTIONS

As stated earlier, the work presented here is a significant contribution on the role of Tregs in infection and immunity. However, there are certain questions that remain to be answered in this project which were not addressed due to time constraints. For example, this work did not address the mechanism by which Tregs mediate its protective activity during sepsis. In particular, I report here that Tregs regulate neutrophil function and survival (Figs. 5.8, 5.9, 5.10). It remains to be determined if Tregs regulate neutrophil function and survival in a contact dependent or contact independent manner. Trans-well experiments will be necessary in future to answer this question. If the regulation of neutrophil activity by Tregs is contact dependent, it will be imperative to determine the receptors by which Tregs mediate this function. If it is contact independent, what is the secreted protein or cytokine by which Tregs regulate neutrophil activity and survival? These questions will be answered in future.

The work presented here shows that Tregs are protective in LPS driven inflammation and live *E.coli* infection. Hence this work highlights the importance of Tregs in Gram-negative infections. Although Gram-negative infections are the major cause of sepsis in the clinic (13), Gram-positive infections still pose a significant problem. Therefore it is imperative that future work will investigate whether depletion of Tregs will result in susceptibility to Gram-positive bacterial infection. It will also be important to validate the findings in this work with CLP which is a more clinically relevant model of sepsis.

The results of this work also highlight some clinical studies that will be pursued in future. As I mentioned earlier, a clinical study investigating neutrophil function in survivors of sepsis vs non-survivors will be essential. Also, it will be important to determine the relationship between

the numbers of Tregs and neutrophils in survivors of sepsis vs non-survivors. Finally, it will be important to conduct a clinical study investigating Treg therapy in patients with sepsis. The results of this work strongly suggest a potential benefit of Treg therapy to patients with sepsis. However, the potential of Treg-induced immune suppression cannot be ruled out. Hence, any clinical study investigating the role of Tregs in sepsis should first exclude patients that exhibit signs of immune suppression such as diminished lymphocyte response to antigenic stimulation. This will make it possible to identify a select group of patients that can benefit from Treg therapy in sepsis. It is hoped that my findings in conjunction with future research efforts will lead to better outcome for patients with sepsis.

CHAPTER EIGHT

References

1. Geroulanos S, Douka ET. Historical perspective of the word "sepsis". Intensive Care Med. 2006 Dec;32(12):2077.
2. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101(6):1644-55.
3. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Critical care medicine. 2003;31(4):1250-6.
4. Adhikari NK, Fowler RA, Bhagwanjee S, Rubenfeld GD. Critical care and the global burden of critical illness in adults. Lancet. 2010 Oct 16;376(9749):1339-46.
5. Husak L, Marcuzzi A, Herring J, Wen E, Yin L, Capan DD, et al. National analysis of sepsis hospitalizations and factors contributing to sepsis in-hospital mortality in Canada. Healthc Q. 2010;13 Spec No:35-41.
6. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003 Apr 17;348(16):1546-54.
7. Gaieski DF, Edwards JM, Kallan MJ, Carr BG. Benchmarking the incidence and mortality of severe sepsis in the United States. Crit Care Med. 2013 May;41(5):1167-74.
8. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Critical care medicine. 2001;29(7):1303-10.
9. Finfer S, Bellomo R, Lipman J, French C, Dobb G, Myburgh J. Adult-population incidence of severe sepsis in Australian and New Zealand intensive care units. Intensive Care Med. 2004 Apr;30(4):589-96.
10. Kaukonen KM, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. JAMA. 2014 Apr 2;311(13):1308-16.
11. Heyland DK, Hopman W, Coo H, Tranmer J, McColl MA. Long-term health-related quality of life in survivors of sepsis. Short Form 36: a valid and reliable measure of health-related quality of life. Critical care medicine. 2000;28(11):3599-605.
12. Mayr FB, Yende S, Linde-Zwirble WT, Peck-Palmer OM, Barnato AE, Weissfeld LA, et al. Infection rate and acute organ dysfunction risk as explanations for racial differences in severe sepsis. JAMA. 2010 Jun 23;303(24):2495-503.
13. Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, et al. International study of the prevalence and outcomes of infection in intensive care units. JAMA. 2009 Dec 2;302(21):2323-9.
14. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013 Nov 21;369(21):2063.
15. Chapman SJ, Hill AV. Human genetic susceptibility to infectious disease. Nat Rev Genet. 2012 Mar;13(3):175-88.
16. Garred P, Madsen HO. Genetic Susceptibility to Sepsis: A Possible Role for Mannose-binding Lectin. Curr Infect Dis Rep. 2004 Oct;6(5):367-73.

17. Wang H, Wei Y, Zeng Y, Qin Y, Xiong B, Qin G, et al. The association of polymorphisms of TLR4 and CD14 genes with susceptibility to sepsis in a Chinese population. BMC Med Genet. 2014;15:123.
18. Poeze M, Ramsay G, Gerlach H, Rubulotta F, Levy M. An international sepsis survey: a study of doctors' knowledge and perception about sepsis. Critical care (London, England). 2004;8(6):R409-13.
19. Gauer RL. Early recognition and management of sepsis in adults: the first six hours. Am Fam Physician. 2013 Jul 1;88(1):44-53.
20. Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. High serum procalcitonin concentrations in patients with sepsis and infection. Lancet. 1993 Feb 27;341(8844):515-8.
21. Chiesa C, Pacifico L, Rossi N, Panero A, Matrunola M, Mancuso G. Procalcitonin as a marker of nosocomial infections in the neonatal intensive care unit. Intensive Care Med. 2000 Mar;26 Suppl 2:S175-7.
22. Tang BM, Eslick GD, Craig JC, McLean AS. Accuracy of procalcitonin for sepsis diagnosis in critically ill patients: systematic review and meta-analysis. Lancet Infect Dis. 2007 Mar;7(3):210-7.
23. Gibot S, Bene MC, Noel R, Massin F, Guy J, Cravoisy A, et al. Combination biomarkers to diagnose sepsis in the critically ill patient. Am J Respir Crit Care Med. 2012 Jul 1;186(1):65-71.
24. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. Crit Care Med. 2013 Feb;41(2):580-637.
25. Levy MM, Dellinger RP, Townsend SR, Linde-Zwirble WT, Marshall JC, Bion J, et al. The Surviving Sepsis Campaign: results of an international guideline-based performance improvement program targeting severe sepsis. Crit Care Med. 2010 Feb;38(2):367-74.
26. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med. 2006 Jun;34(6):1589-96.
27. Paule W, Conte NF. Staphylococcal septicemia; report of a case with hyponatremia and eventual recovery through use of multiple antibiotics and adrenal steroids. AMA Arch Intern Med. 1959 May;103(5):796-8.
28. Heller AR, Heller SC, Borkenstein A, Stehr SN, Koch T. Modulation of host defense by hydrocortisone in stress doses during endotoxemia. Intensive Care Med. 2003 Sep;29(9):1456-63.
29. Tsao CM, Ho ST, Chen A, Wang JJ, Li CY, Tsai SK, et al. Low-dose dexamethasone ameliorates circulatory failure and renal dysfunction in conscious rats with endotoxemia. Shock. 2004 May;21(5):484-91.
30. Annane D, Sebille V, Charpentier C, Bollaert PE, Francois B, Korach JM, et al. Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. JAMA : the journal of the American Medical Association. 2002;288(7):862-71.
31. Keh D, Boehnke T, Weber-Cartens S, Schulz C, Ahlers O, Bercker S, et al. Immunologic and hemodynamic effects of "low-dose" hydrocortisone in septic shock: a double-blind, randomized, placebo-controlled, crossover study. Am J Respir Crit Care Med. 2003 Feb 15;167(4):512-20.
32. Annane D, Bellissant E, Bollaert PE, Briegel J, Confalonieri M, De Gaudio R, et al. Corticosteroids in the treatment of severe sepsis and septic shock in adults: a systematic review. JAMA. 2009 Jun 10;301(22):2362-75.
33. Jackson CJ, Xue M. Activated protein C--an anticoagulant that does more than stop clots. The international journal of biochemistry & cell biology. 2008;40(12):2692-7.

34. Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. The Journal of biological chemistry. 2001;276(14):11199-203.
35. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. The New England journal of medicine. 2001;344(10):699-709.
36. Abraham E, Laterre PF, Garg R, Levy H, Talwar D, Trzaskoma BL, et al. Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. The New England journal of medicine. 2005;353(13):1332-41.
37. Marti-Carvajal AJ, Sola I, Lathyris D, Cardona AF. Human recombinant activated protein C for severe sepsis. Cochrane database of systematic reviews (Online). 2011;(4)(4):CD004388.
38. Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem. 1977;46:897-930.
39. Endo A. The discovery and development of HMG-CoA reductase inhibitors. J Lipid Res. 1992 Nov;33(11):1569-82.
40. Janda S, Young A, Fitzgerald JM, Etminan M, Swiston J. The effect of statins on mortality from severe infections and sepsis: a systematic review and meta-analysis. J Crit Care. 2010 Dec;25(4):656 e7-22.
41. Freeman DJ, Norrie J, Sattar N, Neely RD, Cobbe SM, Ford I, et al. Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. Circulation. 2001 Jan 23;103(3):357-62.
42. Poynter JN, Gruber SB, Higgins PD, Almog R, Bonner JD, Rennert HS, et al. Statins and the risk of colorectal cancer. N Engl J Med. 2005 May 26;352(21):2184-92.
43. Merx MW, Liehn EA, Graf J, van de Sandt A, Schaltenbrand M, Schrader J, et al. Statin treatment after onset of sepsis in a murine model improves survival. Circulation. 2005 Jul 5;112(1):117-24.
44. De Loecker I, Preiser JC. Statins in the critically ill. Ann Intensive Care. 2012;2(1):19.
45. Dobesh PP, Klepser DG, McGuire TR, Morgan CW, Olsen KM. Reduction in mortality associated with statin therapy in patients with severe sepsis. Pharmacotherapy. 2009 Jun;29(6):621-30.
46. Almog Y, Novack V, Eisinger M, Porath A, Novack L, Gilutz H. The effect of statin therapy on infection-related mortality in patients with atherosclerotic diseases. Crit Care Med. 2007 Feb;35(2):372-8.
47. Al Harbi SA, Tamim HM, Arabi YM. Association between statin therapy and outcomes in critically ill patients: a nested cohort study. BMC clinical pharmacology. 2011;11(Journal Article):12.
48. Kruger P, Bailey M, Bellomo R, Cooper DJ, Harward M, Higgins A, et al. A multicenter randomized trial of atorvastatin therapy in intensive care patients with severe sepsis. Am J Respir Crit Care Med. 2013 Apr 1;187(7):743-50.
49. Kruger PS, Harward ML, Jones MA, Joyce CJ, Kostner KM, Roberts MS, et al. Continuation of statin therapy in patients with presumed infection: a randomized controlled trial. Am J Respir Crit Care Med. 2011 Mar 15;183(6):774-81.
50. Fernandez R, De Pedro VJ, Artigas A. Statin therapy prior to ICU admission: protection against infection or a severity marker? Intensive Care Med. 2006 Jan;32(1):160-4.
51. Sommers MS. The cellular basis of septic shock. Critical care nursing clinics of North America. 2003;15(1):13-25.
52. Brodsky IE, Medzhitov R. Targeting of immune signalling networks by bacterial pathogens. Nature cell biology. 2009;11(5):521-6.
53. van der Poll T, Opal SM. Host-pathogen interactions in sepsis. Lancet Infect Dis. 2008 Jan;8(1):32-43.

54. Bone RC. Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS). Annals of Internal Medicine. 1996;125(8):680-7.
55. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunology today. 1992;13(1):11-6.
56. Clarke TB, Weiser JN. Intracellular sensors of extracellular bacteria. Immunological reviews. 2011;243(1):9-25.
57. Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends in immunology. 2006;27(8):352-7.
58. van Deventer SJ, Buller HR, ten Cate JW, Aarden LA, Hack CE, Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood. 1990;76(12):2520-6.
59. Miyoshi T, Yamashita K, Arai T, Yamamoto K, Mizugishi K, Uchiyama T. The role of endothelial interleukin-8/NADPH oxidase 1 axis in sepsis. Immunology. 2010 Nov;131(3):331-9.
60. Heller A, Koch T, Schmeck J, van Ackern K. Lipid mediators in inflammatory disorders. Drugs. 1998;55(4):487-96.
61. Duma D, Fernandes D, Bonini MG, Stadler K, Mason RP, Assreuy J. NOS-1-derived NO is an essential triggering signal for the development of systemic inflammatory responses. European journal of pharmacology. 2011;668(1-2):285-92.
62. van der Poll T. Tissue factor as an initiator of coagulation and inflammation in the lung. Critical care (London, England). 2008;12 Suppl 6(Journal Article):S3.
63. Gando S, Sawamura A, Hayakawa M. Trauma, shock, and disseminated intravascular coagulation: lessons from the classical literature. Annals of Surgery. 2011;254(1):10-9.
64. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Critical care medicine. 1996;24(7):1125-8.
65. Hotchkiss RS, Coopersmith CM, McDunn JE, Ferguson TA. The sepsis seesaw: tilting toward immunosuppression. Nature medicine. 2009;15(5):496-7.
66. Vannier E, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(9):4076-80.
67. Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest. 2000;117(4):1162-72.
68. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. Journal of immunology (Baltimore, Md: 1950). 1991;146(10):3444-51.
69. Ninomiya C, Spiegelberg HL. IL-4 and transforming growth factor-beta suppress human immunoglobulin secretion in vitro by surface IgD- B cells. Clinical and experimental immunology. 1992;89(2):261-8.
70. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. The Journal of experimental medicine. 1986;163(5):1037-50.
71. Finder J, Stark WW, Jr., Nakayama DK, Geller D, Wasserloos K, Pitt BR, et al. TGF-beta regulates production of NO in pulmonary artery smooth muscle cells by inhibiting expression of NOS. Am J Physiol. 1995 May;268(5 Pt 1):L862-7.
72. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359(6397):693-9.
73. Thomas L. Germs. N Engl J Med. 1972 Sep 14;287(11):553-5.

74. Strieter RM, Lynch JP, 3rd, Basha MA, Standiford TJ, Kasahara K, Kunkel SL. Host responses in mediating sepsis and adult respiratory distress syndrome. Semin Respir Infect. 1990 Sep;5(3):233-47.
75. Cohen J. The immunopathogenesis of sepsis. Nature. 2002 Dec 19-26;420(6917):885-91.
76. Imler JL, Hoffmann JA. Signaling mechanisms in the antimicrobial host defense of Drosophila. Curr Opin Microbiol. 2000 Feb;3(1):16-22.
77. Stein D, Roth S, Vogelsang E, Nusslein-Volhard C. The polarity of the dorsoventral axis in the Drosophila embryo is defined by an extracellular signal. Cell. 1991 May 31;65(5):725-35.
78. Pandey S, Kawai T, Akira S. Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. Cold Spring Harb Perspect Biol. 2015 Jan;7(1):a016246.
79. O'Neill LA, Dinarello CA. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol Today. 2000 May;21(5):206-9.
80. Ulevitch RJ, Tobias PS. Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr Opin Immunol. 1999 Feb;11(1):19-22.
81. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol. 2005 Jan;3(1):36-46.
82. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998 Dec 11;282(5396):2085-8.
83. Gioannini TL, Weiss JP. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. Immunol Res. 2007;39(1-3):249-60.
84. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008 May;42(2):145-51.
85. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med. 1999 Jun 7;189(11):1777-82.
86. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007 May;7(5):353-64.
87. Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997 Nov 28;278(5343):1612-5.
88. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity. 1999 Jul;11(1):115-22.
89. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol. 2001 Nov 15;167(10):5887-94.
90. Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature. 2002 Apr 18;416(6882):750-6.
91. Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. J Immunol. 2004 Sep 1;173(5):2913-7.
92. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001 Mar 1;410(6824):37-40.
93. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol. 2004 May;5(5):503-7.

94. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature. 2006 Jan 12;439(7073):208-11.
95. Guo B, Cheng G. Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. J Biol Chem. 2007 Apr 20;282(16):11817-26.
96. Perry AK, Chen G, Zheng D, Tang H, Cheng G. The host type I interferon response to viral and bacterial infections. Cell Res. 2005 Jun;15(6):407-22.
97. Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006 Mar;6(3):173-82.
98. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest. 2000 May;80(5):617-53.
99. Deng Q, Harvie EA, Huttenlocher A. Distinct signalling mechanisms mediate neutrophil attraction to bacterial infection and tissue injury. Cell Microbiol. 2012 Apr;14(4):517-28.
100. Tavares-Murta BM, Zaparoli M, Ferreira RB, Silva-Vergara ML, Oliveira CH, Murta EF, et al. Failure of neutrophil chemotactic function in septic patients. Crit Care Med. 2002 May;30(5):1056-61.
101. Alves-Filho JC, Spiller F, Cunha FQ. Neutrophil paralysis in sepsis. Shock. 2010 Sep;34 Suppl 1:15-21.
102. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, et al. Shock and tissue injury induced by recombinant human cachectin. Science. 1986 Oct 24;234(4775):470-4.
103. Wang L, Taneja R, Razavi HM, Law C, Gillis C, Mehta S. Specific role of neutrophil inducible nitric oxide synthase in murine sepsis-induced lung injury in vivo. Shock. 2012 May;37(5):539-47.
104. Delano MJ, Kelly-Scumpia KM, Thayer TC, Winfield RD, Scumpia PO, Cuenca AG, et al. Neutrophil mobilization from the bone marrow during polymicrobial sepsis is dependent on CXCL12 signaling. J Immunol. 2011 Jul 15;187(2):911-8.
105. Kovach MA, Standiford TJ. The function of neutrophils in sepsis. Curr Opin Infect Dis. 2012 Jun;25(3):321-7.
106. Bazzoni F, Cassatella MA, Laudanna C, Rossi F. Phagocytosis of opsonized yeast induces tumor necrosis factor-alpha mRNA accumulation and protein release by human polymorphonuclear leukocytes. J Leukoc Biol. 1991 Sep;50(3):223-8.
107. Malyak M, Smith MF, Jr., Abel AA, Arend WP. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1 beta. J Clin Immunol. 1994 Jan;14(1):20-30.
108. Takahashi GW, Andrews DF, 3rd, Lilly MB, Singer JW, Alderson MR. Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. Blood. 1993 Jan 15;81(2):357-64.
109. Kasama T, Strieter RM, Standiford TJ, Burdick MD, Kunkel SL. Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha. J Exp Med. 1993 Jul 1;178(1):63-72.
110. Kita H, Ohnishi T, Okubo Y, Weiler D, Abrams JS, Gleich GJ. Granulocyte/macrophage colony-stimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. J Exp Med. 1991 Sep 1;174(3):745-8.
111. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today. 1995 Jan;16(1):21-6.
112. Kasten KR, Muenzer JT, Caldwell CC. Neutrophils are significant producers of IL-10 during sepsis. Biochem Biophys Res Commun. 2010 Feb 26;393(1):28-31.
113. Fava RA, Olsen NJ, Postlethwaite AE, Broadley KN, Davidson JM, Nanney LB, et al. Transforming growth factor beta 1 (TGF-beta 1) induced neutrophil recruitment to synovial tissues:

implications for TGF-beta-driven synovial inflammation and hyperplasia. J Exp Med. 1991 May 1;173(5):1121-32.
114. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annu Rev Immunol. 2002;20:825-52.
115. Babior BM. The respiratory burst of phagocytes. J Clin Invest. 1984 Mar;73(3):599-601.
116. Malech HL, Gallin JI. Current concepts: immunology. Neutrophils in human diseases. N Engl J Med. 1987 Sep 10;317(11):687-94.
117. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532-5.
118. Meng W, Paunel-Gorgulu A, Flohe S, Hoffmann A, Witte I, MacKenzie C, et al. Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice. Crit Care. 2012;16(4):R137.
119. Gao X, Hao S, Yan H, Ding W, Li K, Li J. Neutrophil extracellular traps contribute to the intestine damage in endotoxemic rats. J Surg Res. 2014 Dec 19.
120. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol. 2002 Jul;283(1):R7-28.
121. Classen A, Lloberas J, Celada A. Macrophage activation: classical versus alternative. Methods Mol Biol. 2009;531:29-43.
122. Suzuki T, Hashimoto S, Toyoda N, Nagai S, Yamazaki N, Dong HY, et al. Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. Blood. 2000 Oct 1;96(7):2584-91.
123. Unanue ER. Antigen-presenting function of the macrophage. Annu Rev Immunol. 1984;2:395-428.
124. Bjorkbacka H, Fitzgerald KA, Huet F, Li X, Gregory JA, Lee MA, et al. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. Physiol Genomics. 2004 Nov 17;19(3):319-30.
125. Hashimoto S, Morohoshi K, Suzuki T, Matsushima K. Lipopolysaccharide-inducible gene expression profile in human monocytes. Scand J Infect Dis. 2003;35(9):619-27.
126. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 2009 Oct;30(10):475-87.
127. Manjuck J, Saha DC, Astiz M, Eales LJ, Rackow EC. Decreased response to recall antigens is associated with depressed costimulatory receptor expression in septic critically ill patients. J Lab Clin Med. 2000 Feb;135(2):153-60.
128. Monneret G, Finck ME, Venet F, Debard AL, Bohe J, Bienvenu J, et al. The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration. Immunol Lett. 2004 Sep;95(2):193-8.
129. Boisrame-Helms J, Kremer H, Schini-Kerth V, Meziani F. Endothelial dysfunction in sepsis. Curr Vasc Pharmacol. 2013 Mar 1;11(2):150-60.
130. Faure E, Thomas L, Xu H, Medvedev A, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. J Immunol. 2001 Feb 1;166(3):2018-24.
131. Jirik FR, Podor TJ, Hirano T, Kishimoto T, Loskutoff DJ, Carson DA, et al. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. J Immunol. 1989 Jan 1;142(1):144-7.
132. Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol. 1986 Aug 15;137(4):1270-4.

133. Leeuwenberg JF, Smeets EF, Neefjes JJ, Shaffer MA, Cinek T, Jeunhomme TM, et al. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. Immunology. 1992 Dec;77(4):543-9.
134. Somers WS, Tang J, Shaw GD, Camphausen RT. Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. Cell. 2000 Oct 27;103(3):467-79.
135. Stephens KE, Ishizaka A, Larrick JW, Raffin TA. Tumor necrosis factor causes increased pulmonary permeability and edema. Comparison to septic acute lung injury. Am Rev Respir Dis. 1988 Jun;137(6):1364-70.
136. Boughton-Smith NK, Evans SM, Laszlo F, Whittle BJ, Moncada S. The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. Br J Pharmacol. 1993 Nov;110(3):1189-95.
137. Yanada M, Kojima T, Ishiguro K, Nakayama Y, Yamamoto K, Matsushita T, et al. Impact of antithrombin deficiency in thrombogenesis: lipopolysaccharide and stress-induced thrombus formation in heterozygous antithrombin-deficient mice. Blood. 2002 Apr 1;99(7):2455-8.
138. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science. 1993 Apr 23;260(5107):547-9.
139. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986 Apr 1;136(7):2348-57.
140. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73.
141. Hoag KA, Lipscomb MF, Izzo AA, Street NE. IL-12 and IFN-gamma are required for initiating the protective Th1 response to pulmonary cryptococcosis in resistant C.B-17 mice. Am J Respir Cell Mol Biol. 1997 Dec;17(6):733-9.
142. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. J Immunol. 1990 Dec 1;145(11):3796-806.
143. Guillen C, McInnes IB, Vaughan DM, Kommajosyula S, Van Berkel PH, Leung BP, et al. Enhanced Th1 response to Staphylococcus aureus infection in human lactoferrin-transgenic mice. J Immunol. 2002 Apr 15;168(8):3950-7.
144. Geyer MB, Radhakrishnan K, Van de Ven C, Suri MS, Ayello J, Cairo MS. Enhanced survival during experimental Listeria monocytogenes sepsis in neonatal mice prophylactically treated with Th1 and macrophage immunoregulatory cytokines and mediators. Pediatr Infect Dis J. 2014 Dec;33(12):e330-7.
145. Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM, et al. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. J Exp Med. 2007 Jun 11;204(6):1463-74.
146. Hotchkiss RS, Swanson PE, Knudson CM, Chang KC, Cobb JP, Osborne DF, et al. Overexpression of Bcl-2 in transgenic mice decreases apoptosis and improves survival in sepsis. J Immunol. 1999 Apr 1;162(7):4148-56.
147. Shelley O, Murphy T, Paterson H, Mannick JA, Lederer JA. Interaction between the innate and adaptive immune systems is required to survive sepsis and control inflammation after injury. Shock. 2003 Aug;20(2):123-9.
148. Martignoni A, Tschop J, Goetzman HS, Choi LG, Reid MD, Johannigman JA, et al. CD4-expressing cells are early mediators of the innate immune system during sepsis. Shock. 2008 May;29(5):591-7.

149. Kasten KR, Tschop J, Goetzman HS, England LG, Dattilo JR, Cave CM, et al. T-cell activation differentially mediates the host response to sepsis. Shock. 2010 Oct;34(4):377-83.
150. Llewelyn M, Cohen J. Superantigens: microbial agents that corrupt immunity. Lancet Infect Dis. 2002 Mar;2(3):156-62.
151. Spaulding AR, Salgado-Pabon W, Kohler PL, Horswill AR, Leung DY, Schlievert PM. Staphylococcal and streptococcal superantigen exotoxins. Clin Microbiol Rev. 2013 Jul;26(3):422-47.
152. Sherwood ER, Lin CY, Tao W, Hartmann CA, Dujon JE, French AJ, et al. Beta 2 microglobulin knockout mice are resistant to lethal intraabdominal sepsis. Am J Respir Crit Care Med. 2003 Jun 15;167(12):1641-9.
153. Wesche-Soldato DE, Chung CS, Gregory SH, Salazar-Mather TP, Ayala CA, Ayala A. CD8+ T cells promote inflammation and apoptosis in the liver after sepsis: role of Fas-FasL. Am J Pathol. 2007 Jul;171(1):87-96.
154. von Knethen A, Sha LK, Knape T, Kuchler L, Giegerich AK, Schulz M, et al. Activation of the peroxisome proliferator-activated receptor gamma counteracts sepsis-induced T cell cytotoxicity toward alloantigenic target cells. J Mol Med (Berl). 2015 Jan 6.
155. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology. 1970 May;18(5):723-37.
156. Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T cells. J Immunol. 1972 Mar;108(3):586-90.
157. Baker PJ, Barth RF, Stashak PW, Amsbaugh DF. Enhancement of the antibody response to type 3 pneumococcal polysaccharide in mice treated with antilymphocyte serum. J Immunol. 1970 May;104(5):1313-5.
158. Green DR, Flood PM, Gershon RK. Immunoregulatory T-cell pathways. Annu Rev Immunol. 1983;1:439-63.
159. Kronenberg M, Steinmetz M, Kobori J, Kraig E, Kapp JA, Pierce CW, et al. RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. Proc Natl Acad Sci U S A. 1983 Sep;80(18):5704-8.
160. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995 Aug 1;155(3):1151-64.
161. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell. 2000 May 26;101(5):455-8.
162. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol. 1999 May 1;162(9):5317-26.
163. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol. 2002 Jun;2(6):389-400.
164. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med. 1996 Jul 1;184(1):19-29.
165. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 2003 Dec 15;198(12):1875-86.
166. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med. 2000 Nov 6;192(9):1213-22.
167. Apostolou I, von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. J Exp Med. 2004 May 17;199(10):1401-8.

168. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity. 2003 May;18(5):605-17.
169. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003 Apr;4(4):330-6.
170. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003 Feb 14;299(5609):1057-61.
171. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001 Jan;27(1):68-73.
172. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001 Jan;27(1):20-1.
173. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med. 2000 Jul 17;192(2):303-10.
174. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med. 2000 Jul 17;192(2):295-302.
175. McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity. 2002 Feb;16(2):311-23.
176. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. Immunity. 2004 Oct;21(4):503-13.
177. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006 Jul 10;203(7):1693-700.
178. Robb RJ, Munck A, Smith KA. T cell growth factor receptors. Quantitation, specificity, and biological relevance. J Exp Med. 1981 Nov 1;154(5):1455-74.
179. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1994 Aug;1(5):405-13.
180. Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, et al. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. Proc Natl Acad Sci U S A. 1997 Jun 10;94(12):6216-21.
181. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med. 1990 May 1;171(5):1393-405.
182. Aerts NE, Dombrecht EJ, Ebo DG, Bridts CH, Stevens WJ, De Clerck LS. Activated T cells complicate the identification of regulatory T cells in rheumatoid arthritis. Cell Immunol. 2008 Feb;251(2):109-15.
183. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol. 2000 Jan 1;164(1):183-90.
184. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol. 2007 Dec;8(12):1353-62.
185. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. J Immunol. 2006 Oct 1;177(7):4376-83.

186. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. Trends Mol Med. 2007 Mar;13(3):108-16.
187. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol. 2006 Jun 1;176(11):6752-61.
188. Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, et al. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J Immunol. 2004 Jan 15;172(2):834-42.
189. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med. 2005 Oct 17;202(8):1075-85.
190. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H, et al. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J Exp Med. 2002 Jul 15;196(2):237-46.
191. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. Blood. 2009 May 21;113(21):5125-33.
192. Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J Exp Med. 2008 Sep 1;205(9):1975-81.
193. Huber S, Schramm C, Lehr HA, Mann A, Schmitt S, Becker C, et al. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. J Immunol. 2004 Dec 1;173(11):6526-31.
194. Bommireddy R, Babcock GF, Singh RR, Doetschman T. TGFbeta1 deficiency does not affect the generation and maintenance of CD4+CD25+FOXP3+ putative Treg cells, but causes their numerical inadequacy and loss of regulatory function. Clinical immunology (Orlando, Fla). 2008;127(2):206-13.
195. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature. 1997 Oct 16;389(6652):737-42.
196. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol. 2005 Sep 1;175(5):3025-32.
197. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, et al. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol. 2006 Nov 1;177(9):5852-60.
198. Kryczek I, Wei S, Zou L, Zhu G, Mottram P, Xu H, et al. Cutting edge: induction of B7-H4 on APCs through IL-10: novel suppressive mode for regulatory T cells. J Immunol. 2006 Jul 1;177(1):40-4.
199. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol. 2001 Mar 1;166(5):3008-18.
200. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol. 2002 Feb 1;168(3):1080-6.
201. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood. 2004 Nov 1;104(9):2840-8.

202. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. 2004 Oct;21(4):589-601.
203. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood. 2006 May 15;107(10):3925-32.
204. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol. 2005 Feb 15;174(4):1783-6.
205. Strauss L, Bergmann C, Whiteside TL. Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis. J Immunol. 2009 Feb 1;182(3):1469-80.
206. Weber SE, Harbertson J, Godebu E, Mros GA, Padrick RC, Carson BD, et al. Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. J Immunol. 2006 Apr 15;176(8):4730-9.
207. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol. 2008 Mar;9(3):239-44.
208. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. J Immunol. 2005 Oct 1;175(7):4180-3.
209. Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H. Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. Cancer Res. 2003 Aug 1;63(15):4516-20.
210. Venet F, Pachot A, Debard AL, Bohe J, Bienvenu J, Lepape A, et al. Human CD4+CD25+ regulatory T lymphocytes inhibit lipopolysaccharide-induced monocyte survival through a Fas/Fas ligand-dependent mechanism. J Immunol. 2006 Nov 1;177(9):6540-7.
211. Hubert P, Jacobs N, Caberg JH, Boniver J, Delvenne P. The cross-talk between dendritic and regulatory T cells: good or evil? J Leukoc Biol. 2007 Oct;82(4):781-94.
212. Lu LF, Lind EF, Gondek DC, Bennett KA, Gleeson MW, Pino-Lagos K, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature. 2006 Aug 31;442(7106):997-1002.
213. Lewkowicz N, Klink M, Mycko MP, Lewkowicz P. Neutrophil--CD4+CD25+ T regulatory cell interactions: a possible new mechanism of infectious tolerance. Immunobiology. 2013 Apr;218(4):455-64.
214. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat Med. 2002 Sep;8(9):1024-32.
215. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science. 1994 Aug 26;265(5176):1237-40.
216. Zhang X, Izikson L, Liu L, Weiner HL. Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. J Immunol. 2001 Oct 15;167(8):4245-53.
217. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol. 2004 Mar;5(3):266-71.
218. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. Science. 2008 May 30;320(5880):1220-4.
219. Corthay A. How do regulatory T cells work? Scand J Immunol. 2009 Oct;70(4):326-36.
220. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol. 2005 Dec;6(12):1219-27.
221. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunological reviews. 2001;182(Journal Article):68-79.

222. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunological reviews. 2001;182(Journal Article):207-14.
223. Lee YH, Ishida Y, Rifa'i M, Shi Z, Isobe K, Suzuki H. Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. J Immunol. 2008 Jan 15;180(2):825-32.
224. Endharti AT, Okuno Y, Shi Z, Misawa N, Toyokuni S, Ito M, et al. CD8+CD122+ regulatory T cells (Tregs) and CD4+ Tregs cooperatively prevent and cure CD4+ cell-induced colitis. J Immunol. 2011 Jan 1;186(1):41-52.
225. Rifa'i M, Shi Z, Zhang SY, Lee YH, Shiku H, Isobe K, et al. CD8+CD122+ regulatory T cells recognize activated T cells via conventional MHC class I-alphabetaTCR interaction and become IL-10-producing active regulatory cells. Int Immunol. 2008 Jul;20(7):937-47.
226. Chen W, Ford MS, Young KJ, Cybulsky MI, Zhang L. Role of double-negative regulatory T cells in long-term cardiac xenograft survival. J Immunol. 2003 Feb 15;170(4):1846-53.
227. Ford MS, Young KJ, Zhang Z, Ohashi PS, Zhang L. The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. J Exp Med. 2002 Jul 15;196(2):261-7.
228. Priatel JJ, Utting O, Teh HS. TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. J Immunol. 2001 Dec 1;167(11):6188-94.
229. Johansson M, Lycke N. A unique population of extrathymically derived alpha beta TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. J Immunol. 2003 Feb 15;170(4):1659-66.
230. Monneret G, Debard AL, Venet F, Bohe J, Hequet O, Bienvenu J, et al. Marked elevation of human circulating CD4+CD25+ regulatory T cells in sepsis-induced immunoparalysis. Crit Care Med. 2003 Jul;31(7):2068-71.
231. Venet F, Pachot A, Debard AL, Bohe J, Bienvenu J, Lepape A, et al. Increased percentage of CD4+CD25+ regulatory T cells during septic shock is due to the decrease of CD4+CD25- lymphocytes. Crit Care Med. 2004 Nov;32(11):2329-31.
232. Wisnoski N, Chung CS, Chen Y, Huang X, Ayala A. The contribution of CD4+ CD25+ T-regulatory-cells to immune suppression in sepsis. Shock. 2007 Mar;27(3):251-7.
233. Hein F, Massin F, Cravoisy-Popovic A, Barraud D, Levy B, Bollaert PE, et al. The relationship between CD4+CD25+CD127- regulatory T cells and inflammatory response and outcome during shock states. Critical care (London, England). 2010;14(1):R19.
234. Scumpia PO, Delano MJ, Kelly KM, O'Malley KA, Efron PA, McAuliffe PF, et al. Increased natural CD4+CD25+ regulatory T cells and their suppressor activity do not contribute to mortality in murine polymicrobial sepsis. Journal of immunology (Baltimore, Md: 1950). 2006;177(11):7943-9.
235. Carrigan SO, Yang YJ, Issekutz T, Forward N, Hoskin D, Johnston B, et al. Depletion of natural CD4+CD25+ T regulatory cells with anti-CD25 antibody does not change the course of Pseudomonas aeruginosa-induced acute lung infection in mice. Immunobiology. 2009;214(3):211-22.
236. Venet F, Chung CS, Kherouf H, Geeraert A, Malcus C, Poitevin F, et al. Increased circulating regulatory T cells (CD4(+)CD25 (+)CD127 (-)) contribute to lymphocyte anergy in septic shock patients. Intensive care medicine. 2009;35(4):678-86.
237. Hiraki S, Ono S, Tsujimoto H, Kinoshita M, Takahata R, Miyazaki H, et al. Neutralization of interleukin-10 or transforming growth factor-beta decreases the percentages of CD4(+)CD25(+)Foxp3(+) regulatory T cells in septic mice, thereby leading to an improved survival. Surgery. 2011(Journal Article).

238. Ai YH, Yao YM, Dai XG. Effect of apoptosis of CD4+ CD25+ regulatory T cells on proliferation as well as secretion of effector T cells and interventional activity of Xuebijing injection in septic rats. Zhonghua wai ke za zhi [Chinese journal of surgery]. 2009;47(1):58-61.
239. Heuer JG, Zhang T, Zhao J, Ding C, Cramer M, Justen KL, et al. Adoptive transfer of in vitro-stimulated CD4+CD25+ regulatory T cells increases bacterial clearance and improves survival in polymicrobial sepsis. Journal of immunology (Baltimore, Md: 1950). 2005;174(11):7141-6.
240. Cambos M, Belanger B, Jacques A, Roulet A, Scorza T. Natural regulatory (CD4+CD25+FOXP+) T cells control the production of pro-inflammatory cytokines during Plasmodium chabaudi adami infection and do not contribute to immune evasion. International journal for parasitology. 2008;38(2):229-38.
241. Cantley LC. The phosphoinositide 3-kinase pathway. Science (New York, NY). 2002;296(5573):1655-7.
242. Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. The Journal of biological chemistry. 1999;274(13):8347-50.
243. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nature reviewsGenetics. 2006;7(8):606-19.
244. Whitman M, Downes CP, Keeler M, Keller T, Cantley L. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature. 1988;332(6165):644-6.
245. Wrann CD, Tabriz NA, Barkhausen T, Klos A, van Griensven M, Pape HC, et al. The phosphatidylinositol 3-kinase signaling pathway exerts protective effects during sepsis by controlling C5a-mediated activation of innate immune functions. Journal of immunology (Baltimore, Md: 1950). 2007;178(9):5940-8.
246. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. The Journal of biological chemistry. 2002;277(35):32124-32.
247. Schabbauer G, Tencati M, Pedersen B, Pawlinski R, Mackman N. PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24(10):1963-9.
248. Zhang WJ, Wei H, Hagen T, Frei B. Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(10):4077-82.
249. Ong E, Gao XP, Predescu D, Broman M, Malik AB. Role of phosphatidylinositol 3-kinase-gamma in mediating lung neutrophil sequestration and vascular injury induced by E. coli sepsis. American journal of physiologyLung cellular and molecular physiology. 2005;289(6):L1094-103.
250. Martin EL, Souza DG, Fagundes CT, Amaral FA, Assenzio B, Puntorieri V, et al. Phosphoinositide-3 kinase gamma activity contributes to sepsis and organ damage by altering neutrophil recruitment. American journal of respiratory and critical care medicine. 2010;182(6):762-73.
251. Aksoy E, Taboubi S, Torres D, Delbauve S, Hachani A, Whitehead MA, et al. The p110delta isoform of the kinase PI(3)K controls the subcellular compartmentalization of TLR4 signaling and protects from endotoxic shock. Nat Immunol. 2012 Nov;13(11):1045-54.
252. Patton DT, Garden OA, Pearce WP, Clough LE, Monk CR, Leung E, et al. Cutting edge: the phosphoinositide 3-kinase p110 delta is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. Journal of immunology (Baltimore, Md: 1950). 2006;177(10):6598-602.
253. Doi K, Leelahavanichkul A, Yuen PS, Star RA. Animal models of sepsis and sepsis-induced kidney injury. The Journal of clinical investigation. 2009;119(10):2868-78.
254. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nature protocols. 2009;4(1):31-6.

255. Iskander KN, Craciun FL, Stepien DM, Duffy ER, Kim J, Moitra R, et al. Cecal ligation and puncture-induced murine sepsis does not cause lung injury. Crit Care Med. 2013 Jan;41(1):159-70.
256. Starr ME, Steele AM, Saito M, Hacker BJ, Evers BM, Saito H. A new cecal slurry preparation protocol with improved long-term reproducibility for animal models of sepsis. PLoS One. 2014;9(12):e115705.
257. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A. 2013 Feb 26;110(9):3507-12.
258. Leelahavanichkul A, Huang Y, Hu X, Zhou H, Tsuji T, Chen R, et al. Chronic kidney disease worsens sepsis and sepsis-induced acute kidney injury by releasing High Mobility Group Box Protein-1. Kidney Int. 2011 Dec;80(11):1198-211.
259. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. Nature. 2011 Jul 14;475(7355):226-30.
260. Weischenfeldt J, Porse B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc. 2008;2008:pdb prot5080.
261. Boxio R, Bossenmeyer-Pourie C, Steinckwich N, Dournon C, Nusse O. Mouse bone marrow contains large numbers of functionally competent neutrophils. J Leukoc Biol. 2004 Apr;75(4):604-11.
262. Kruisbeek AM. Isolation of mouse mononuclear cells. Curr Protoc Immunol. 2001 May;Chapter 3:Unit 3 1.
263. Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. FASEB J. 2001 Apr;15(6):940-9.
264. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985 Oct;150(1):76-85.
265. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. The Journal of experimental medicine. 2009;206(4):751-60.
266. Lewis DH, Chan DL, Pinheiro D, Armitage-Chan E, Garden OA. The immunopathology of sepsis: pathogen recognition, systemic inflammation, the compensatory anti-inflammatory response, and regulatory T cells. J Vet Intern Med. 2012 May-Jun;26(3):457-82.
267. Attridge K, Walker LS. Homeostasis and function of regulatory T cells (Tregs) in vivo: lessons from TCR-transgenic Tregs. Immunol Rev. 2014 May;259(1):23-39.
268. Shen S, Ding Y, Tadokoro CE, Olivares-Villagomez D, Camps-Ramirez M, Curotto de Lafaille MA, et al. Control of homeostatic proliferation by regulatory T cells. J Clin Invest. 2005 Dec;115(12):3517-26.
269. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med. 2005 Mar 7;201(5):723-35.
270. Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. J Clin Invest. 2004 Nov;114(9):1209-17.
271. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med. 2004 Jun 7;199(11):1455-65.

272. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol. 2006 Aug 1;177(3):1451-9.
273. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol. 2003 Apr 15;170(8):3939-43.
274. Morgan ME, Sutmuller RP, Witteveen HJ, van Duivenvoorde LM, Zanelli E, Melief CJ, et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum. 2003 May;48(5):1452-60.
275. Zheng SG, Meng L, Wang JH, Watanabe M, Barr ML, Cramer DV, et al. Transfer of regulatory T cells generated ex vivo modifies graft rejection through induction of tolerogenic CD4+CD25+ cells in the recipient. Int Immunol. 2006 Feb;18(2):279-89.
276. Hadjiminas DJ, McMasters KM, Peyton JC, Cheadle WG. Tissue tumor necrosis factor mRNA expression following cecal ligation and puncture or intraperitoneal injection of endotoxin. The Journal of surgical research. 1994;56(6):549-55.
277. Stephens LA, Anderton SM. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells". Journal of immunology (Baltimore, Md: 1950). 2006;177(4):2036; author reply 7-8.
278. Zelenay S, Demengeot J. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells". Journal of immunology (Baltimore, Md: 1950). 2006;177(4):2036-7; author reply 7-8.
279. Setiady YY, Coccia JA, Park PU. In vivo depletion of CD4+FOXP3+ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcgammaRIII+ phagocytes. European journal of immunology. 2010;40(3):780-6.
280. Okwor I, Muleme H, Jia P, Uzonna JE. Altered proinflammatory cytokine production and enhanced resistance to Trypanosoma congolense infection in lymphotoxin beta-deficient mice. The Journal of infectious diseases. 2009;200(3):361-9.
281. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. Shock. 2002 Jun;17(6):463-7.
282. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med. 2002 Sep 16;196(6):851-7.
283. Theofilopoulos AN. The basis of autoimmunity: Part I. Mechanisms of aberrant self-recognition. Immunol Today. 1995 Feb;16(2):90-8.
284. Remick DG. Pathophysiology of sepsis. Am J Pathol. 2007 May;170(5):1435-44.
285. Bosma GC, Fried M, Custer RP, Carroll A, Gibson DM, Bosma MJ. Evidence of functional lymphocytes in some (leaky) scid mice. J Exp Med. 1988 Mar 1;167(3):1016-33.
286. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med. 2003 Feb 17;197(4):403-11.
287. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. Journal of immunology (Baltimore, Md: 1950). 2002;168(9):4531-7.
288. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med. 1983 Sep 1;158(3):670-89.
289. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science. 2002 Aug 9;297(5583):1031-4.

290. Cavaillon JM, Adib-Conquy M, Fitting C, Adrie C, Payen D. Cytokine cascade in sepsis. Scand J Infect Dis. 2003;35(9):535-44.
291. Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, et al. Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A2. Nat Immunol. 2000 Jul;1(1):42-6.
292. Cho JS, Guo Y, Ramos RI, Hebroni F, Plaisier SB, Xuan C, et al. Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against Staphylococcus aureus in mice. PLoS Pathog. 2012;8(11):e1003047.
293. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nature reviewsImmunology. 2007;7(8):585-98.
294. Mendez S, Reckling SK, Piccirillo CA, Sacks D, Belkaid Y. Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. The Journal of experimental medicine. 2004;200(2):201-10.
295. Kabelitz D. Expression and function of Toll-like receptors in T lymphocytes. Current opinion in immunology. 2007;19(1):39-45.
296. Lefer AM. Significance of lipid mediators in shock states. Circulatory shock. 1989;27(1):3-12.
297. Tran DQ. In vitro suppression assay for functional assessment of human regulatory T cells. Methods Mol Biol. 2013;979:199-212.
298. Zhang N, Bevan MJ. TGF-beta signaling to T cells inhibits autoimmunity during lymphopenia-driven proliferation. Nat Immunol. 2012 Jul;13(7):667-73.
299. Remick DG, Ward PA. Evaluation of endotoxin models for the study of sepsis. Shock. 2005 Dec;24 Suppl 1:7-11.
300. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med. 2004 Aug 2;200(3):277-85.
301. Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, Perroni L, et al. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J Clin Invest. 2006 Jun;116(6):1713-22.
302. Long SA, Buckner JH. CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game. J Immunol. 2011 Sep 1;187(5):2061-6.
303. Dietze KK, Zelinskyy G, Liu J, Kretzmer F, Schimmer S, Dittmer U. Combining regulatory T cell depletion and inhibitory receptor blockade improves reactivation of exhausted virus-specific CD8+ T cells and efficiently reduces chronic retroviral loads. PLoS Pathog. 2013;9(12):e1003798.
304. Grauer OM, Nierkens S, Bennink E, Toonen LW, Boon L, Wesseling P, et al. CD4+FoxP3+ regulatory T cells gradually accumulate in gliomas during tumor growth and efficiently suppress antiglioma immune responses in vivo. Int J Cancer. 2007 Jul 1;121(1):95-105.
305. Curiel TJ. Tregs and rethinking cancer immunotherapy. J Clin Invest. 2007 May;117(5):1167-74.
306. Fujiwara N, Kobayashi K. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy. 2005 Jun;4(3):281-6.
307. Caron G, Duluc D, Fremaux I, Jeannin P, David C, Gascan H, et al. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. J Immunol. 2005 Aug 1;175(3):1551-7.
308. Dick EP, Prince LR, Prestwich EC, Renshaw SA, Whyte MK, Sabroe I. Pathways regulating lipopolysaccharide-induced neutrophil survival revealed by lentiviral transduction of primary human neutrophils. Immunology. 2009 Jun;127(2):249-55.
309. Gallin JI, Buescher ES, Seligmann BE, Nath J, Gaither T, Katz P. NIH conference. Recent advances in chronic granulomatous disease. Ann Intern Med. 1983 Nov;99(5):657-74.
310. Liu Z, Giudice GJ, Zhou X, Swartz SJ, Troy JL, Fairley JA, et al. A major role for neutrophils in experimental bullous pemphigoid. J Clin Invest. 1997 Sep 1;100(5):1256-63.

311. Liu Z, Shipley JM, Vu TH, Zhou X, Diaz LA, Werb Z, et al. Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid. J Exp Med. 1998 Aug 3;188(3):475-82.
312. Brooks CJ, King WJ, Radford DJ, Adu D, McGrath M, Savage CO. IL-1 beta production by human polymorphonuclear leucocytes stimulated by anti-neutrophil cytoplasmic autoantibodies: relevance to systemic vasculitis. Clin Exp Immunol. 1996 Nov;106(2):273-9.
313. Edwards SW, Hallett MB. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. Immunol Today. 1997 Jul;18(7):320-4.
314. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011 Mar 9;3(73):73ra19.
315. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev. 2008 Jun;223:371-90.
316. Lewkowicz P, Lewkowicz N, Sasiak A, Tchorzewski H. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. J Immunol. 2006 Nov 15;177(10):7155-63.
317. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. Nat Immunol. 2004 Oct;5(10):971-4.
318. Elawady S, Botros SK, Sorour AE, Ghany EA, Elbatran G, Ali R. Neutrophil CD64 as a diagnostic marker of sepsis in neonates. J Investig Med. 2014 Mar;62(3):644-9.
319. Hasan Z, Palani K, Rahman M, Thorlacius H. Targeting CD44 expressed on neutrophils inhibits lung damage in abdominal sepsis. Shock. 2011 Jun;35(6):567-72.
320. Natanson C, Esposito CJ, Banks SM. The sirens' songs of confirmatory sepsis trials: selection bias and sampling error. Critical care medicine. 1998;26(12):1927-31.
321. Oleinika K, Nibbs RJ, Graham GJ, Fraser AR. Suppression, subversion and escape: the role of regulatory T cells in cancer progression. Clin Exp Immunol. 2013 Jan;171(1):36-45.
322. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol. 2005 Mar 1;174(5):2591-601.
323. Hotchkiss RS, Moldawer LL. Parallels between cancer and infectious disease. N Engl J Med. 2014 Jul 24;371(4):380-3.
324. Bosmann M, Ward PA. The inflammatory response in sepsis. Trends Immunol. 2013 Mar;34(3):129-36.
325. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008 Jul 24;454(7203):445-54.
326. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Mol Cancer Res. 2006 Apr;4(4):221-33.
327. Wildner G, Kaufmann U. What causes relapses of autoimmune diseases? The etiological role of autoreactive T cells. Autoimmun Rev. 2013 May 16.
328. Nemeth T, Mocsai A. The role of neutrophils in autoimmune diseases. Immunol Lett. 2012 Mar 30;143(1):9-19.
329. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001 Oct 18;413(6857):732-8.
330. Kurtz J. Specific memory within innate immune systems. Trends Immunol. 2005 Apr;26(4):186-92.
331. Jamieson AM, Isnard P, Dorfman JR, Coles MC, Raulet DH. Turnover and proliferation of NK cells in steady state and lymphopenic conditions. J Immunol. 2004 Jan 15;172(2):864-70.

332. Marchal G, Seman M, Milon G, Truffa-Bachi P, Zilberfarb V. Local adoptive transfer of skin delayed-type hypersensitivity initiated by a single T lymphocyte. J Immunol. 1982 Sep;129(3):954-8.
333. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol. 2006 May;7(5):507-16.
334. Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol. 2010 Dec;11(12):1127-35.
335. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. Nat Immunol. 2001 Oct;2(10):951-6.
336. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. Nature. 2009 Jan 29;457(7229):557-61.
337. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. Proc Natl Acad Sci U S A. 2009 Feb 10;106(6):1915-9.
338. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood. 2010 Jan 14;115(2):335-43.
339. Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. J Leukoc Biol. 2010 Dec;88(6):1163-70.
340. Himmel ME, Crome SQ, Ivison S, Piccirillo C, Steiner TS, Levings MK. Human CD4+ FOXP3+ regulatory T cells produce CXCL8 and recruit neutrophils. Eur J Immunol. 2011 Feb;41(2):306-12.
341. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol. 2011 Aug;11(8):519-31.
342. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, et al. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. Blood. 2005 Sep 1;106(5):1843-50.
343. Abi Abdallah DS, Egan CE, Butcher BA, Denkers EY. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. Int Immunol. 2011 May;23(5):317-26.
344. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guermonprez P, et al. Neutrophils efficiently cross-prime naive T cells in vivo. Blood. 2007 Oct 15;110(8):2965-73.
345. Reibman J, Meixler S, Lee TC, Gold LI, Cronstein BN, Haines KA, et al. Transforming growth factor beta 1, a potent chemoattractant for human neutrophils, bypasses classic signal-transduction pathways. Proc Natl Acad Sci U S A. 1991 Aug 1;88(15):6805-9.
346. Sun L, Guo RF, Newstead MW, Standiford TJ, Macariola DR, Shanley TP. Effect of IL-10 on neutrophil recruitment and survival after Pseudomonas aeruginosa challenge. Am J Respir Cell Mol Biol. 2009 Jul;41(1):76-84.
347. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. Nat Rev Rheumatol. 2014 Oct;10(10):593-601.
348. Xiao F, Ma L, Zhao M, Huang G, Mirenda V, Dorling A, et al. Ex vivo expanded human regulatory T cells delay islet allograft rejection via inhibiting islet-derived monocyte chemoattractant protein-1 production in CD34+ stem cells-reconstituted NOD-scid IL2rgammanull mice. PLoS One. 2014;9(3):e90387.
349. Nguyen VH, Shashidhar S, Chang DS, Ho L, Kambham N, Bachmann M, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. Blood. 2008 Jan 15;111(2):945-53.
350. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011 Apr 7;117(14):3921-8.

351. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood. 2011 Jan 20;117(3):1061-70.

List of publications

1. **E.B Okeke,** and J. E Uzonna. In search of a cure for sepsis: taming the monster in critical care medicine. *J. Innate Immun.* **2016** DOI: 10.1159/000442469.

2. **E.B Okeke,** I Okwor and J. E Uzonna. Regulatory T cells restrain CD4+ T cells from causing unregulated immune activation and hypersensitivity to lipopolysaccharide challenge. *J. Immunol.* **2014** *193(2)* 655-62. PMID 24943218

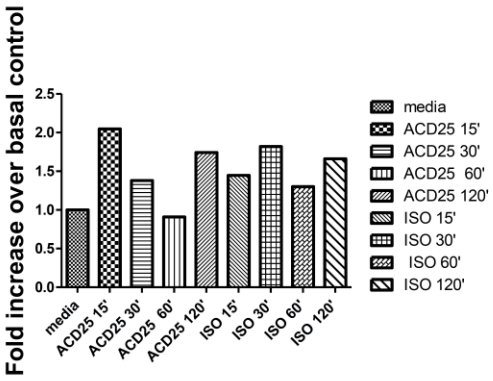
3. **E.B Okeke,** I Okwor, Z Mou, P Jia and J. E Uzonna. CD4+CD25+ regulatory T cells attenuate lipopolysaccharide-induced systemic inflammatory responses and promotes survival in murine *Escherichia coli* infection. *Shock* **2013** *40(1)* 65-73. PMID 23635849

4. S. Kuriakose, H. Muleme, C. Onyilagha, **E. Okeke** and J. Uzonna. Diminazene aceturate (Berenil) modulates LPS induced pro-inflammatory cytokine production by inhibiting phosphorylation of MAPKs and STAT proteins. *Innate Immun*. **2013**. PMID 24179040

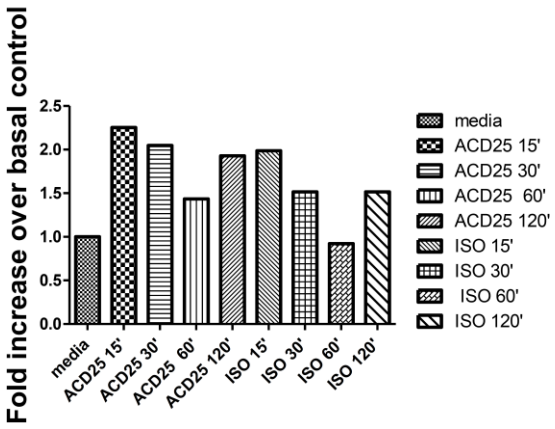
5. **E.B Okeke** and D.V Soldatov. Coordination and Inclusion Compounds Formed by Addition of Quinoline (Q) or Isoquinoline (Iq) to a Metal(II) Dibenzoylmethanate (Co, Ni, Zn, Cd): Composition, Structure and Thermal Dissociation Properties. *J. Therm. Anal. Cal.* **2010** *100* 801-810.

Appendix I

A



B



There is no difference in upregulation of STAT signaling in BMDM treated with anti-CD25 monoclonal antibody (anti-CD25 mAb) or isotype control antibody. BMDM were cultured in the presence of LPS 1 ug/ml and/or anti-CD25 mAb or isotype control antibody for 15, 30, 60 and 120 mins respectively. Cells were lysed following which STAT 1 (A) and STAT 3 (B) protein expression was examined by western blot. Plots show relative protein expression with respect to basal loading control.