UNDERSTANDING ADAPTIVE IMMUNE SYSTEM DYSFUNCTION IN MURINE SEPSIS

By

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By

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Sepsis is the result of an overwhelming infection that causes a dramatic systemic inflammatory response. Sepsis induces profound dysfunction in both the innate and adaptive immune systems. We studied how sepsis affects the adaptive immune system. Specifically, we studied the roles and function of different adaptive immune system cells (dendritic cells, $CD4^+$ helper T cells, and $CD4^+CD25^+$ regulatory T cells) in sepsis. We examined whether these cells contribute to mortality and adaptive immune dysfunction in the murine cecal ligation and puncture (CLP) model of sepsis. Using transgenic mice that can be depleted of dendritic cells (DCs), as well as a plasmacytoid DC-depleting antibody, we found that conventional dendritic cells, but not plasmacytoid DCs, are absolutely required for survival in sepsis. Using a CD25-depleting antibody as well as CD25 knockout mice, we observed that loss of regulatory T cells does not affect outcome to sepsis. Interestingly, we found that depletion of the total $CD4^+$ population cell population also does not affect sepsis mortality. However, we observed considerable antigen non-specific and antigen specific $CD4^+$ effector T cell dysfunction, and hypothesized that this dysfunction precludes their contribution to survival from sepsis. We treated mice with an agonistic antibody against glucocorticoid induced tumor necrosis factor receptor (GITR) that stimulates $CD4^+$ T cell activation just prior to sepsis, and we found that

anti-GITR treatment improves T cell function and leads to an improvement in outcome from sepsis. Importantly, depletion of $CD4^+$ T cells abrogates the protective effect of anti-GITR treatment, indicating that $CD4^+$ T cells are required for the anti-GITR induced improvement in survival. These findings illustrate the importance of maintaining adaptive immune system function in sepsis, and may represent a novel therapeutic strategy to improve outcome in sepsis.

CHAPTER 1
INTRODUCTION

This introduction discusses the incidence, individual and social impact, and characteristics of sepsis. In addition, this introduction will present background on the role of the adaptive immune system in infection, in general, as well as discuss the importance of the decreased ability of the adaptive immune system to function in patients or animals that are suffering from sepsis. Then, this introduction will provide a scientific rationale for studying cellular contributions to immune dysfunction, particularly those involving dendritic cells, $CD4^+$ helper T cells, regulatory T cells (Treg), and B cells in the potential pathogenesis of the disease.

Sepsis

Traditionally, sepsis has been defined as a clinical syndrome when a severe microbial infection is accompanied by a systemic inflammatory response characterized by fever/hypothermia, leukocytosis/leukopenia, elevated cardiac output, and reduced systemic vascular resistance (1). The infection can originate anywhere, and sepsis may be accompanied by a positive blood culture, although the use of antibiotics may limit the appearance of the causative agent in the blood. Although the clinical appearance of systemic inflammatory response syndrome (SIRS) can occur in the absence of infection (such is the case in severe hemorrhage, pancreatitis, organ ischemia or reperfusion injury, and thermal injury), for the purposes of this study, the definition of sepsis is the association of an infection with SIRS.

Epidemiology

In the United States, sepsis remains the leading cause of death in the non-cardiac intensive care unit affecting over 750,000 patients in the United States and causing approximately 210,000 deaths annually (2). The incidence of sepsis has increased from 82.7 cases per 100,000 population in 1979 to 240.4 cases per 100,000 population in 2001. Significant advancements in

our understanding of sepsis pathophysiology, supportive care, and immune dysfunction have occurred during this time period (2). Unfortunately, mortality from severe sepsis has only declined modestly, and with the increasing incidence of sepsis caused by the aging of the population and patients on immunosuppressive therapies, the resultant total mortality has increased over 3-fold during the aforementioned study period, and is expected to increase even more in the future (2).

Treatment Options

Although our understanding of sepsis pathophysiology has increased dramatically over the past 20 years, the appearance of novel targeted therapeutic agents has either failed or has only marginally improved survival. As such, supportive care with fluid resuscitation and broad spectrum antibiotics remains the first line of defense followed by treatment with more focused antibiotics once the causative infectious agent has been identified (3). Numerous trials were conducted on agents that block the inflammatory cascade — corticosteroids (4), antiendotoxin antibodies (5), tumor necrosis factor (TNF) antagonists (6, 7), interleukin-1–receptor antagonists (8), and ibuprofen (9), which all yielded equivocal results. This has led investigators to question whether death in patients with sepsis results from uncontrolled inflammation (10-13).

Recombinant human activated protein C (Xigris), an anticoagulant, is the only FDA approved agent that has proven effective in the treatment of sepsis (14, 15), and has resulted in an improvement in survival of 10 to 20 percent in a subset of patients, as the benefits have to be weighed out against the increased risk of bleeding in patients receiving the drug. Another adjunct therapy that has seen marginal efficacy is intensive glycemic control with insulin (16). Patients whose blood glucose levels were maintained between 80-110 mg per deciliter with insulin therapy demonstrated an improved survival of ~15% over patients whose blood glucose levels were maintained at 180-200 mg per deciliter, and the frequency of sepsis in bacteremic patients

was reduced by 46%. Still the magic bullet remains elusive as these therapies are only marginally effective and the frequency of sepsis continues to increase.

Consequences

The progression of sepsis to septic shock, disseminated intravascular coagulopathy, multiple organ dysfunction syndrome, or multiple organ failure can occur rapidly despite adequate fluid resuscitation and antibiotic treatment, is often irreversible, and is associated with a poor prognosis.

Interestingly, autopsy studies have not revealed exactly why patients with sepsis die. A patient with sepsis may die of refractory shock, but this is the exception rather than the rule. Although patients with sepsis present with profound myocardial depression and components of acute respiratory distress syndrome, cardiac output is usually maintained because of cardiac dilatation and tachycardia, and hypoxia or hypercapnia rarely cause death. Kidney failure is the most common organ to fail, but that alone is not fatal, because dialysis can be used. Liver dysfunction rarely progresses to its worst stages, failure or hepatic encephalopathy. Thus, the exact cause of death in patients with sepsis remains elusive (reviewed by Hotchkiss and Karl (3)).

An often underappreciated consequence of sepsis, and the focus of much of this report is the development of immunological dysfunction in septic patients. Originally, sepsis was thought to be caused by the infection itself, as the body failed to respond to the microorganism. However, Lewis Thomas developed a controversial theory in 1972 that challenged this notion proposing that the body's primary response to infection and injury is uncontrolled hyperinflammation, and that the pathophysiology of sepsis is actually the result of the robust host response to the pathogen, and not directly mediated by the pathogen (17). Although there is merit to this theory, it has become increasingly evident that patients with sepsis simultaneously have features

consistent with immunosuppression, including a loss of delayed hypersensitivity, an inability to clear infection, and a predisposition to nosocomial infections (1, 3, 18, 19). These observations have led others to develop a new theory for the immunological events that occur in sepsis. Many believe that the initial SIRS component of sepsis is transitory, and is followed by a compensatory anti-inflammatory response syndrome (CARS), where there is a prolonged state of hyporesponsiveness to the initial infection and to secondary infections in the patient (20).

Although attractive, this theory also suffers from a lack of evidence as it is known that there is no temporal lag between the presumed proximal SIRS and subsequent CARS components of the sepsis response (21, 22). Instead, the response in sepsis is more likely a concordant pro- and anti- inflammatory surge that may be better termed as a cytokine storm or mixed inflammatory/anti-inflammatory response (MARS) where the innate and adaptive immune systems are rendered hyporesponsive to further stimulation simultaneous with an early proinflammatory response (23). For these reasons, the current studies examined the roles of individual cell types of the adaptive immune system and analyzed whether they are required for an appropriate response or are components of the immunological dysfunction that occurs during sepsis.

Innate and Adaptive Immune System

The immune system can be simplistically divided into two components, the innate and the adaptive immune systems, although such distinctions minimize the close interactions between these two systems. The innate immune system is phylogenically older than the adaptive immune response and has been traditionally considered an immediate, but nonadaptive response to infection or tissue injury (24). The innate system relies heavily on cellular receptors and secreted proteins that recognize common molecular patterns associated with a microbial infection (1). Through recognition of microbes or microbial products, the innate immune system responds with

a generalized response pattern that is mediated in large part by the release of secreted proteins and cytokines. The overall goal of this response is to decrease the pathogen's ability to cause disease, to delay the microorganism's growth and invasion, and to permit host responses that will eventually eliminate the bacterial infection or that will protect the host until an adequate acquired immune response can be mounted. These responses include, but are not limited to, the production of acute phase reactants, induced host changes deleterious to microorganism growth (fever, increased iron sequestration, etc.), and induced local inflammation (1). This general response also leads to increased phagocyte activation (neutrophils, macrophages, and eosinophils), the production of free radicals (superoxide, hypochlorite, nitric oxide, and peroxynitrite), and increased capacity of cells to present antigen (DCs, B-cells, and macrophages) (25).

Recently, the belief that the innate immune system is completely nonspecific has been challenged. It is now recognized that specific danger signals induce cells to activate generalized immune responses. Pattern recognition receptors (PRRs) are present on cells which recognize common pathogen-associated molecular patterns (PAMPs) to initiate immune responses. Toll-like receptors (TLRs) are a specific type of PRR that were originally discovered as Toll receptors in Drosophila that can recognize molecules ranging from lipoproteins to nucleic acids, molecules commonly found in bacteria, viruses, parasites, or fungi that are underrepresented on mammalian cells. The nature and the duration of the innate immune response is responsible for directing specific effector responses after recognizing a particular group of pathogens (26). In addition, the ability of the host to mount a specific Th2 response against protozoan infections versus a predominantly Th1 response against intracellular bacterial or viral infections is determined by the nature of the early innate immune response to these different microbes. The specific pattern

of PRRs activated on antigen presenting cells (APCs) controls the activation of the specific type of adaptive immune response generated by driving APCs to upregulate the appropriate costimulatory molecules and to produce the appropriate cytokines to polarize the appropriate helper T cell response.

Once activated, helper T cells can be polarized into Th1, Th2, or Th17 responses. Th1 responses develop when T cells are activated in the presence of interferon (IFN) α/β, or IFN-γ, and predominantly drive the elimination of cancer and intracellular pathogens such as viruses, fungi, and some bacteria (27, 28). Th2 type reactions are driven in the presence of interleukin (IL)-4 or IL-10, and generally drive immunity to extracellular toxins or pathogens such as parasites, worms, and extracellular bacteria (29). A new type of T cell response that has been shown to be important in pathogenic autoimmune diseases is the Th17 response that depends on the factors TGF-β, IL-6 and possibly IL-23 (30, 31). Humoral immunity, or the production of antibodies by B cells, can be driven by either Th1 or Th2 immune responses, but the isotypes of antibody are different in each case. In mice, IgG1 and IgE predominate during a Th2 response, whereas IgG2a responses predominate during a Th1 response (27, 32).

Dendritic Cells in Immunity

As mentioned above, antigen presenting cells are important in polarizing T cell responses. In the 1970s, Ralph Steinman first described the dendritic cell (DC) as a novel APC population in the mouse spleen (33-35). These studies originated from research attempting to explain the requirement for nonlymphocyte cells in generating the T cell dependent antibody responses. Together, Steinman, Cohn, and Inaba isolated these previously undescribed stellate cells from the spleen and demonstrated their requirement for maximal adaptive immune responses (34-36). Subsequently, DCs were demonstrated to exist in tissues of the

reticuloendothelial system, lymphoid organs, and tissues such as skin that are exposed to the external environment where frequent antigenic exposure occurs (37). DCs from different tissues express varying cell membrane markers and functions, and many different classification schemes of DCs have been used to try to define the unique abilities of their various subpopulations. The predominant classification schemes that exist are based on maturity (immature versus mature DCs) and morphology (conventional versus plasmacytoid).

Conventional DCs

Despite phenotypic and functional differences, most conventional DCs have several features in common. First, DCs originate from bone marrow stem cells or circulating monocytes and migrate via the blood stream to almost all tissues, where they develop into immature DCs. In this immature state, DCs continuously sample the environment and readily take up antigen through receptor-and nonreceptor-mediated mechanisms to present this antigen to T-helper cells. The immature DC, in comparison to the mature DC, has a unique set of chemokine receptors that guides its migration to inflammatory tissues. In response to antigens and danger signals, DCs upregulate MHC Class II molecules and costimulatory (CD80 and CD86) molecules, and attain the capacity to migrate through the lymphatic system to the T cell-rich areas of lymphoid organs, where they stimulate $CD4^+$ T cells (37, 38). This response can be induced through activation of TLRs on DCs, but the maturation of DCs by bacteria and viruses is intimately regulated by the production of Type I IFN (IFN α/β) (39). Finally, factors in the DCs microenvironment, such as cytokines, can direct T cells into specific clones. For instance, DC production of the p70 subunit of IL-12 is necessary for the generation of a Th1 response (40).

In mice, all conventional DCs express CD11c and MHCII. At rest, conventional DCs express CD80 and express variable levels of CD86. A previous characterization scheme of DCs

within lymph nodes and spleen used the markers CD11b, CD8α, DEC205, and CD4 to classify conventional DCs as being either myeloid or lymphoid in origin. However, this classification scheme has been disputed, and it has been suggested that the expression of these markers may be more indicative of their state of activation rather than of their origin (reviewed by (25)). Probably more important is the fact that any of these populations can induce T cell chemotaxis and responsiveness through their ability to produce large amounts of CC and CXC chemokines, as well as IL-12 upon stimulation (25).

Plasmacytoid DCs

Plasmacytoid DCs (pDCs) were first identified in humans by their plasmacytoid morphology and their ability to produce large amounts of type I IFN in response to viral infection (41). In mice, pDCs can be classified by their unique cell surface phenotype, characterized by the expression of the granulocyte marker Gr-1, the B cell marker B220, the DC marker CD11c (42) and the pDC markers 120G8 (43)and PDCA-1 (44), both of which may be interferon inducible proteins. Their signature response to infection or engagement of TLR7/8 or TLR9 is their ability to produce large amounts of Type I IFN, and their lack of or low level IL-12 response (45, 46). However, their ability to produce Type I IFN has been shown to drive conventional DC maturation and IL-12 production, which can lead to the indirect stimulation of T cells by pDCs. Despite their propensity to produce IFN, pDCs have been regarded as toleragenic in that they have the capacity to generate regulatory or suppressive T cells (47).

$CD4^+$ Helper T Cells

Arguably, the architects of adaptive immune responses, $CD4^+$ Helper T cells (T_H cells) use cell-contact and soluble mediators such as cytokines to orchestrate humoral and cell mediated immunity. Without $CD4^+$ T cells, the host is susceptible to viral, fungal, and bacterial infections, and the incidence of sepsis increases in AIDS patients and patients who are on

immunosuppressive therapies that target T cells. All T cells undergo positive and negative selection in the thymus to delete autoreactive T cells. The majority of $CD4^+$ T cells that leave the thymus become effector T cells that patrol lymphoid organs waiting to be activated by their antigen specific receptor. Interestingly, there are many mechanisms to negatively regulate $CD4^+$ T cell activation, such as the deletion of self reactive T cells in the thymus, clonal anergy of T cells in the periphery that recognize antigen without costimulation, activation induced cell death, and the up-regulation of CTLA-4, an inhibitory signaling molecule that can bind to CD86 and prevent further costimulation, causing a self-limiting response (48). Further negative regulation of $CD4^+$ T cell function can be achieved by a subset of $CD4^+$ cells that also express CD25 and Foxp3 (49).

Regulatory T cells

T cells can be divided into several classes of regulatory T cells (Treg) that participate in immunoregulation, including Tr1 (IL-10 producing), Th3 (TGF-β producing), and naturally occurring $CD4^+CD25^+$ regulatory T cells that express the transcription factor forkhead box P3 (Foxp3) and suppress through direct cell-cell contact (50). However, T cells do not act alone in processes of immune regulation. Indeed, complex cellular networks coordinate at the immunological synapse to direct responses toward either immunity or tolerance. In fact the interaction of regulatory T cells with APCs in vivo may be as important as direct contact in influencing T cell reactivity (51).

Although many markers are associated with Treg, three markers have been highlighted in recent literature. Suppressive T cells were originally described in the 1970s (52), but due to lack of appropriate characterization, this research was largely abandoned until the mid 1990s when Sakaguchi found that a population of Treg expressing the marker CD25 was responsible for preventing autoimmune disease in thymectomized mice (53). More recently, Tregs were also

found to express the marker glucocorticoid induced TNF receptor (GITR), and stimulation through this receptor can abrogate Treg suppressor function (54). Subsequently, GITR has also been found to be expressed on recently activated $CD4^+$ effector T cells, and can act as a costimulatory molecule on these cells (55, 56). Perhaps the most promising marker on Tregs is Foxp3. Forced expression of Foxp3 in T cells can initiate a suppressive phenotype in those cells (57), and mice that lack Foxp3 expression develop a fatal autoimmune disease (58).

The importance of Treg in infection is beginning to be realized. Infections with pathogens that activate Treg can lead to pathogen persistence whereas the ablation of Treg can increase host injury due to unregulated host inflammation (59). Recently Treg have been found to express TLRs, and can increase their suppressive function upon TLR ligation (60). Furthermore, an increase in Treg has been shown in the blood of septic patients (61), and Treg have been shown to mediate control over inflammatory reactions (62), but the relative importance of these findings to sepsis pathophysiology has not been determined.

Immune System Dysfunction in Sepsis

Despite the dramatic early activation of the immune system during the SIRS component of sepsis, patients who survive this phase (which is most patients) have features consistent with immunosuppression, including a loss of delayed hypersensitivity, an inability to clear infection, and a predisposition to nosocomial infections (1, 18, 19, 63). The cytokine storm which may render cells refractory to further stimulation has been demonstrated by the reduced capacity of lipopolysaccharide-stimulated whole blood from septic patients to produce the inflammatory cytokines TNF-α and IL-1 than that of whole blood from control patients (64). Specific defects in monocyte activation of septic patients have been observed as a late consequence of sepsis as monocyte MHC class II expression was decreased, as was their ability to induce inflammatory

cytokine production. Furthermore, in patients with decreased monocyte function, treatment with IFN-γ restored monocyte function and resulted in clearance of sepsis in 8 of 9 patients. This study demonstrates that immunomodulation may be beneficial in septic patients (65).

Many more defects in the adaptive immune response have been observed in septic patients. In a study by Maclean et al, surgical patients who could not develop a delayed type hypersensitivity response (DTH, Th1 mediated), were more likely to develop sepsis (63). Furthermore, progressive non-responders all died from septic complications. This study demonstrated that a lack of T cell responses was likely detrimental in sepsis. In patients with bacterial peritonitis, Heidecke et al. demonstrated that T cells from patients produced less Th1 cytokines and did not produce more Th2 cytokines, hallmarks of T cell anergy (66). Defective T-cell proliferation and cytokine secretion in these patients correlated with mortality (66). Interestingly, in patients with trauma or burns, non-infectious causes of SIRS also have reduced levels of circulating T cells, and their surviving T cells are anergic, highlighting the potential connection of SIRS with T cell anergy (67).

Another important mechanism of immune dysfunction in sepsis is the massive apoptosis of lymphocytes in sepsis. These findings have been found in both animal models of sepsis and in autopsy findings of patients who died of sepsis. Surprisingly, immune cells that underwent apoptosis include $CD4^+$ T cells, B cells, and DCs at times when clonal expansion of the lymphocytes should have been maximal (68-70). Losses of B cells, $CD4^+$ T cells, and DCs should theoretically decrease antibody production, macrophage activation, and antigen presentation, respectively. The potential importance of the depletion of lymphocytes is illustrated by studies showing that prevention of lymphocyte apoptosis improves survival in rodent models of sepsis (71). These results were confirmed using multiple methods of inhibiting apoptosis from

the use of pan-caspase inhibitors to the transgenic expression of an anti-apoptotic protein specifically in lymphoid cells. These results not only prove that preventing lymphocyte apoptosis is a useful strategy in sepsis, but suggest that other methods that may improve lymphocyte function may be beneficial in preventing sepsis induced immune dysfunction.

CHAPTER 2
MATERIALS AND METHODS

A broad array of cellular, immunological, and molecular techniques were employed in the course of completing this dissertation project. This chapter outlines the techniques most commonly utilized from the major experiments conducted, and provides a sufficient degree of detail to facilitate their adaptation and/or replication by other investigators. Although several techniques that were performed are common among the various chapters, modifications were made in the specific chapters to facilitate their use to answer the specific questions that were being asked. This chapter highlights general protocols developed by the laboratory for experiments completed during this dissertation, as well as some procedures (i.e. genotyping) that were performed that are not discussed in detail in the subsequent chapters.

Note on Mouse Strains

Many strains of mice were utilized in these studies. The two most commonly used mouse strains were the inbred C57Bl/6 and BALB/c strain. Other strains include C.FVB-Tg(Itgax-DTR/EGFP)57Lan/JCD11c (or DCKO or CD11c-DTR on a BALB/c background), B6.129S4-Il2ratm1Dw/J (CD25 KO), B6.129P2-Il10tm1Cgn/J (IL-10 KO on a C57Bl/6 background), Foxp3-DTR (on a C57Bl/6 background), C.Cg-Tg(DO11.10)10Dlo/J T cell receptor transgenic (DO11.10 on a BALB/C background), and B6(129P2) Nos2tm1Lau-chtl/J (iNOS KO on a B6.129 background), B6.129S2-Il6tm1Kopf/J (IL-6 KO on a C57Bl/6 background), C3HeJ (TLR4 mutant) and C3HeOuJ, IFNAR KO mice (on an SvEv background) as well as the SvEv background controls. All mice were obtained from Jackson Laboratories except the Foxp3-DTR mice which were obtained from Dr. Daniel Littman, the DO11.10 mice which were bred by Dr. Clare-Salzler and Dr. Xia in the Department of Pathology SPF, and the IFNAR KO and SvEv mice which were obtained from Dr. Westley Reeves.

Genotyping of Transgenic Mice

Tail fragments from the mice were obtained under general anesthesia in 3 week old mice at the time of weaning with a razor blade and the mice were ear-tagged. Tails were cauterized using an electrocautery. DNA was isolated using the Qiagen DNEasy Blood and Tissue kit for animal tissue following manufacturer's guidelines. DNA concentrations were measured in samples diluted 1:100 using a spectrophotometer. 5 ng of tail DNA was then used in the PCR reaction recommended by Jackson Laboratories in a TC412 Thermal Cycler (Techne). Then the final product was loaded on a 2% agarose gel containing 0.01% ethidium bromide and electrophoresed under 100 V for 30-40 minutes. Gels were then visualized on an imager with an ultraviolet light and a photograph was taken.

Three strains of mice required genotyping: the DCKO mice, the CD25 KO mice and the Foxp3-DTR mice. The CD11c-DTR and Foxp3-DTR mice were both genotyped using the same primer sets: Primer set 1: Forward 5' CAA ATG TTG CTT GTC TGG TG 3' and Reverse 5' GTC AGT CGA GTG CAC AGT TT 3'. Primer set 1 amplified a 200 base pair fragment from the wild type allele and was an internal control present in all of the mice regardless of whether they express the diphtheria toxin receptor (DTR) allele or not. Primer set 2: Forward 5'- GGG ACC ATG AAG CTG CTG CCG -3' and Reverse 5'- TCA GTG GGA ATT AGT CAT GCC -3'. Primer set 2 amplified a DTR specific sequence 625 base pairs in length. One hemizygous mouse expressing the transgene was bred with one wild-type mouse that did not express the transgene. All progeny expressed one allele of the transgene or did not express the transgene and were used as the wild type controls.

For the CD25 KO mice, two other sets of primers were used. CD25 KO mice were genotyped using the following primer sets: Primer set 1: Forward 5' CTT GGG TGG AGA GGC TAT TC 3' and Reverse 5' AGG TGA GAT GAC AGG AGA TC 3'. Primer set 1 amplified a

280 base pair fragment from the wild type allele. It is the generic primer set used by Jackson Labs for mice that have a neomycin cassette insertion-based knockout strain, and is the gene expressed by heterozygote mice and the homozygous knockout mice that do not express CD25 protein. Primer set 2: Forward 5'- CTG TGT CTG TAT GAC CCA CC -3' and Reverse 5'- CAG GAG TTT CCT AAG CAA CG -3'. Primer set 2 amplified a 140 base pair sequence specific to the wild type gene and was expressed by heterozygous mice and homozygous wild type mice that express the CD25 gene. The colony was maintained as heterozygote-heterozygote crosses as homozygote recessive CD25 KO mice are infertile. Only homozygous wild type and homozygous CD25 KO mice were used for the studies.

Cecal Ligation and Puncture Model of Polymicrobial Sepsis

All of the procedures performed using animals were approved by the Institutional Animal Care & Use Committee (IACUC) of the University of Florida under protocols #B334-2004, #D880, and #E228. For the cecal ligation and puncture (CLP) procedure, mice were anesthetized using 2-3% isofluorane and the abdomen was shaven using rodent clippers. The abdomens were then scrubbed first with an ethanol wipe followed by betadine. Then, a laparotomy of approximately one centimeter was made with scissors, and the cecum was identified and exteriorized. A ligation approximately 0.5 to 1 cm was then made around the cecum with 3-0 silk, and the cecum was then punctured through and through with a needle (20 gauge to 25 gauge). The cecum was then replaced within the abdomen, and the skin and peritoneum was then closed with surgical clips. The mice were given 0.8 mls of sterile normal saline subcutaneously on the back and were placed back in their cage. For sham mice, controls for the surgery itself, mice underwent identical procedures, except once the cecum was exteriorized, it was replaced in the abdomen without ligation or puncture.

For the initiation of polymicrobial sepsis, the model we chose to employ was CLP. Although other models of rodent sepsis exist, such as endotoxemia, or a bolus injection of bacteria into the blood stream or a body cavity, we chose CLP because the laboratory has considerable expertise with this particular model and it is the most clinically relevant model of sepsis in duration, signs and symptoms, as well as outcome. One advantage of this model from a technical consideration is that the severity of the response can be varied by the location of the cecal ligation, and the size and number of enterotomies. This consideration allows the model to be tailored to perform the appropriate degree of severity depending on the biological question asked. For instance, to ascertain the effects of a biological response modifier (cell-based and pharmacologic) aimed at improving outcome to a CLP, we used an LD_{50}-LD_{90} CLP model. On the other hand, if a potential treatment is presumed to worsen outcome, or if we are examining immunological parameters that take 7 to 10 days to develop (such as antibody production) for the final outcome measure of the particular study, we would employ an LD_{10}-LD_{20} model so that most mice would survive. This would allow us to use the smallest group of mice to achieve statistical significance. Regardless of the severity of the model, the same general procedures are undertaken with the size of the enterotomy and the gauge of the needle being the only variables from experiment to experiment.

Additionally, other procedures were performed around the time of the CLP or sham procedure in some mice. These included intraperitoneal injections of antibody 30 minutes, 24 hours, or 72 hours prior to surgery, subcutaneous immunization with an antigen in alum or in phosphate-buffered saline (PBS) at the time of surgery, and/or subcutaneous injection of an antibody 24 hours after surgery.

Tissue Harvesting

At various indicated time points after surgery, mice were euthanized by CO_2 inhalation. Verification of euthanasia was performed by cardiac puncture and exsanguination, or cervical dislocation. Depending on the experiment, blood, spleen, peripheral lymph nodes (inguinal, axillary, and cervical), mesenteric lymph nodes, bone marrow or any combination of these tissues were harvested from the mice. If the tissue was going to be used in cell culture experiments, the tissue was kept as sterile as possible (harvest performed under a tissue culture hood using 70% ethanol to ensure surfaces are sterile) with sterile reagents (PBS; or NH_4Cl lysis buffer). If tissue to be analyzed contained red blood cells (RBCs), such as spleen or blood, RBCs were first lysed with NH_4Cl lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.4% EDTA); otherwise, the tissue was placed directly in PBS. To obtain single cell suspensions, cells were passed through cell strainers (Becton Dickinson, Piscataway, NJ), using the plunger of a sterile 1 cc syringe. Cells were then centrifuged at 1200-1300 rpm and counted using a hemacytometer.

Flow Cytometry

All cell culture reagents were from CellGro (Herndon, Va) unless otherwise stated. Cells were placed in 5 ml polystyrene flow cytometry tubes (BD Falcon, Piscataway, NJ) washed once with flow buffer (either Hank's Buffered Salt Solution without phenol red, Ca^{2+} or Mg^{2+}containing 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, Mo), 1 mM EDTA (Fisher Scientific, Atlanta, GA), and 0.1% sodium azide (Sigma Chemical Co.) or PBS) and then blocked with Fc antibodies (0.2% anti-CD16/CD32, BD Pharmingen, San Diego, CA) for 15 min. For live cells from lymph nodes or spleen, cells were stained using various fluorescein isothiocyanate (FITC), R-phycoerythrin (PE) or allophycocyanin (APC)-conjugated antibodies for 15 minutes at room temperature in the dark for acquisition on a FACSCalibur six parameter flow cytometer (BD Pharmingen). Cells were then washed with excess flow buffer and

centrifuged before being placed in a 1% 7-amino-actinomycin D (7-AAD; PharMingen) solution in PBS for at least 5 minutes before acquisition on the cytometer. For acquisition on the LSRII flow cytometer, cells may also have been stained with PE-cyanine 7 (Cy7), Peridinin chlorophyll protein (PerCP), PerCPCy5.5, APC-Cy7, or Pacific Blue conjugated antibodies and cells were washed and placed in a solution containing 0.1% Sytox Blue. At least $1x10^4$ non-debris (by forward/side scatter gating), live (7-AAD^- or Sytox Blue^-) events were acquired in each live acquisition.

For fixed cells (when intracellular cytokine or molecule staining), cells were fixed after staining with a 1% paraformaldehyde solution or a commercial reagent (eBioscience Fix/perm buffer, eBioscience, San Diego, Ca) for 30 minutes, followed by permeabilization by washing two to three times with flow buffer containing 0.5% saponin or another commercial reagent (eBioscience Permeabilization solution, eBioscience) and vortexing. Cells were then blocked with 1% normal rat serum for 15 minutes before adding the antibody for the intracellular stain for 30 minutes in the dark at 4°C. At least $1x10^4$ cells of interest were then acquired using the FACSCalibur or LSRII flow cytometers. For all fluorescent antibodies, at least initially, isotype control antibodies conjugated to the same fluorochrome were used to assess background fluorescence of the cell populations. For analysis, CellQuest (BD) was used for the FACSCaliber, and either FACSDiva (BD) or FCS Express (De Novo Software, version 2.200.0023, Thornhill, Ontario, CA) was used for analyzing data obtained from the LSRII.

This protocol is a general protocol for flow cytometry used in the laboratory. For more detailed descriptions of the antibodies used, the Methods sections of the subsequent chapters will contain more details.

Cell Purification

Unless otherwise stated, cell culture reagents were obtained from CellGro and separation reagents were obtained from Miltenyi Biotec (Auburn, Ca). Splenocyte or lymph node single cell suspensions were obtained as described above. The $CD4^+$ T cell population was purified from the total population by negative selection using the $CD4^+$ T cell enrichment cocktail or positive selection using CD4 (L3T4) microbeads. Following labeling with the microbeads (10 μl per $1x10^7$ cells) for 15 minutes at 4°C, cells were washed and run through LS magnetic columns in a QuadroMACS magnet. The $CD4^+$ fraction was typically 85-90% pure by negative selection, 90-95% pure by positive selection when examined by flow cytometry. If further separation was required, high speed cell sorting was performed on a FACSVantage cell sorting flow cytometer (BD). The $CD4^+$ fraction was incubated with CD3ε-FITC (1 μl per $1x10^6$ cells) and CD25-PE antibody (2 μl per $1x10^6$ cells) and cells were sorted into a $CD3^+CD4^+CD25^+$ fraction (~95% pure) and a $CD3^+CD4^+CD25^-$ fraction (~99% pure). An antigen presenting cell population (APC) was obtained either by incubating spleen cells from a control mouse with CD90 microbeads (10 μl per 10^7 cells) for 15 minutes on ice and obtaining the CD90- population (purity was never ascertained) by running them through an LS column on the QuadroMACS magnet, or by using the $CD4^-$ fraction from sham mice. Then, either population was irradiated (3,000-3,300 rads) before subsequent studies were performed. No difference was ever obtained using either population of APC in the proliferation experiments described below, however, if an APC population was not utilized in the co-culture experiments, proliferation was generally a fraction of the optimal response.

Generation of Bone Marrow-Derived Dendritic Cells

Dendritic cells were cultured using standard protocols. Bone marrow cells were harvested from tibiae and femurs of mice by first briefly dipping the bones in 70% ethanol to sterilize them. Then both ends of each bone were cut off and the inside marrow of the bones was flushed out with a 3 cc syringe and attached 30 gauge needle filled with PBS. Cells were collected, passed through a cell strainer, then depleted of red blood cells (RBC) with lysis buffer (150 mM NH_4Cl, 10 mM NaHCO3, and 0.4% EDTA). Cells ($2.0x10^6$) were cultured in Petri dishes (100 mm diameter, Falcon, Heidelberg, Germany) in 10 ml culture media (RPMI 1640, Cellgro, Herndon, VA) with 10% heat-inactivated fetal bovine serum, 0.000375% 2-mercaptoethanol (Sigma Chemical Co.), and 1% penicillin-streptomycin-neomycin (Gibco, Grand Island, NY), supplemented with 2000 U granulocyte macrophage-colony stimulating factor (GM-CSF; Peprotec, Frankfurt, Germany). On Day 3, 10 ml culture medium containing 2000 U GM-CSF was added to the dishes. On Day 6, 10 ml media were removed and centrifuged, and the pelleted cells were resuspended in 10 ml fresh culture medium containing 2000 U GM-CSF. On Day 8, nonadherent and loosely adherent cells were harvested, washed twice with PBS, and used for adoptive transfer experiments.

Cell Culture for Proliferation Experiments and Suppression Experiments

Cells were cultured in RPMI 1640 medium supplemented with 5 mM Hepes, 2 mM L-glutamine, 1% Penicillin/Streptomycin solution (Invitrogen, Carlsbad, CA), 0.000375%-mercaptoethanol, 10% fetal bovine serum in U-bottom 96-well plates (Costar, Cambridge, MA). For proliferation assays, $CD4^+$ T cells or $CD4^+CD25^-$ T cells (2.5×10^4 cells) obtained as described above were cultured with irradiated APCs (2.5×10^5 cells, 3,000 or 3,300 rads) and treated with either 2.5 μg of soluble anti-CD3ε antibody (clone 145-2C11 BD Pharmingen) or 5 μg soluble anti-CD3ε antibody and 2.5 μg/ml soluble anti-CD28 (clone 37.51; BD Pharmingen)

as the polyclonal stimulus. In some cases, antigen specific proliferation was assessed in DO11.10 T cell receptor (TCR) transgenic mice using 5 μg of the specific antigenic peptide corresponding to amino acids 323-339 from chicken ovalbumin ($Ova_{323-339}$ peptide; Genscript, Piscataway, NJ) over a 48-72 hour culture. For all proliferation assays, cells were incubated over a 48-72 hour culture period. One μCi of ^{3}H-Thymidine (Amersham Biosciences, Piscataway, NJ) was added during the final 16-18 hours of culture. Supernatants from at least three replicate wells were collected for each condition at the time of ^{3}H-Thymidine addition for the determination of cytokine release in the media. Proliferation was assessed by measuring ^{3}H-thymidine incorporation using a liquid scintillation counter.

For suppression assays, a modification of the standard proliferation assay was performed. Regulatory T cells ($CD4^{+}CD25^{+}$) were added in increasing ratios (0:1, 1:1, and 1:0) to a constant number of Teff cells (2.5×10^{4} cells/well). For these studies, a combination of 5 μg/ml soluble anti-CD3 and 2.5 μg/ml soluble anti-CD28 provided the polyclonal stimulus for proliferation over the three-day culture period and suppression was determined by the reduction of ^{3}H-Thymidine incorporation in the combination of cells and is calculated by the following equation: Percent suppression= (1 - (mean CPM Treg+Teff)/(mean CPM Teff) x 100%).

Adoptive Transfer Experiments

Cells from one mouse were collected aseptically and injected via syringe into the tail vein of another mouse under anesthesia. Generally, one to three days were given to the mice for the cells to rest after injection. These experiments were performed twice. In one experiment, 10^{7} wild type bone marrow derived DCs were injected into DCKO mice to determine if the presence of wild type DCs could improve survival of DCKO mice. On another occasion, 5×10^{6} DO11.10

$CD4^+$ T cells were injected into BALB/c mice to assess the in vivo expansion of antigen specific T cells following immunization.

CHAPTER 3
ASSESSMENT OF THE ROLE OF DENDRITIC CELLS IN SEPSIS

Introduction

Dendritic cells (DC) are components of the innate immune system that sense microbial products and initiate adaptive T-cell responses (25). Activation of DCs leads to their migration to peripheral lymph nodes, as well as their maturation. This maturation process is characterized by increased secreted cytokine production and surface expression of MHC I, II and costimulatory molecules which allows them to present antigen and activate T-cells. Engagement of pattern recognition receptors such as toll-like receptors (TLRs) by pathogen associated molecular patterns on DCs can induce their phenotypic maturation as well as their production of inflammatory cytokines and chemokines capable of polarizing T cell responses (25). Autocrine or paracrine Type I interferon (IFN) production alone or following TLR engagement has been shown to be necessary and sufficient for maturation of DCs following pathogen exposure (39). Defects in the DC maturation process may lead to either immune suppression or autoimmunity (72).

Despite continuing progress in understanding the pathogenesis of sepsis, interventional therapies have only been able to modestly decrease the associated high morbidity and mortality (6, 8, 14, 73). Trauma and sepsis are associated with profound immunological dysfunction, including anergy, and increased apoptosis of various immune cell populations, such as $CD4^+$ lymphocytes, which dramatically alters the host's adaptive response to an invading pathogen (70, 74). Interestingly, anti-apoptotic treatments, such as caspase-3 inhibition and over-expression of Bcl-2 in T cells, have been shown to improve outcome in animal models of sepsis (71, 75).

Recently, we reported a profound loss of $CD11c^+$ DCs from spleen and lymph nodes in murine sepsis induced by a CLP (76). Forty to fifty percent losses of both total ($CD11c^{high}$) and

lymphoid ($CD8\alpha^+$) DC populations were seen within 24 hours. Similar results have been observed by others (77, 78), and Hotchkiss et al. have reported an increased loss of DCs from the spleens of patients who had died from sepsis (68). Furthermore, the intrapulmonary installation of bone marrow-derived DCs prevented lethal *Aspergillus* infection in septic animals (79), suggesting a functional role for DCs in opportunistic fungal infections in the lung during sepsis.

The current study was undertaken to determine whether DCs play a determinant role in survival in a mouse model of polymicrobial sepsis. First, we showed that the relative and absolute numbers of conventional DC (cDC) were reduced for several days after sepsis, while pDC numbers were not reduced. The remaining DCs represented mature DCs, expressing high surface levels of MHCII and CD86, and deficiency or mutations of MyD88, TRIF, TLR4, Type I IFN receptor or IL-6 did not affect the ability of DC to mature after sepsis. Prior depletion of $CD11c^{high}MHCII^{high}$ DCs dramatically increased mortality to sepsis induced by a CLP. Because DC depletion may transiently affect other cell populations, we also performed a reconstitution study with the intravenous administration of wild-type myeloid DCs. We showed that adoptive transfer with wild-type bone marrow-derived DCs restored survival in this model of polymicrobial sepsis. The increased mortality to sepsis in this model did not appear to be associated with reduced anti-microbial activity or an altered inflammatory response. Furthermore, depletion of pDCs alone did not affect mortality in sepsis, suggesting that cDCs play a direct role in sepsis, and attempts to maintain cDC number or function during sepsis may lead to improved outcome.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine prior to their initiation. Specific, pathogen-free

B6.FVB-Tg.Itgax-DTR/EGFP.57Lan/J (referred to as DCKO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Type I IFN receptor deficient ($IFNAR^{-/-}$) and SvEv control mice were the kind gift of Westley H. Reeves. These mice were maintained in a breeding colony at the University of Florida College of Medicine on standard rodent food and water ad libitum. DCKO mice were genotyped from tail DNA using published primer sequences (80). Age and sex-matched littermates between 8-12 weeks of age and not expressing the transgene were used as controls. $MyD88^{-/-}$ and $TRIF^{-/-}$ mice on a B6.129 background as well as their background B6.129 mice were maintained in an SPF colony at Schering Plough Biopharma (Palo Alto, CA).

Depletion of Dendritic Cells

To deplete $CD11c^{+}$ dendritic cells, DCKO mice were treated with an intraperitoneal injection of 4 ng/g of diphtheria toxin (15). As controls, DCKO mice received an intraperitoneal injection of saline vehicle, and wild-type littermates were treated with diphtheria toxin. Following intraperitoneal injections, mice underwent CLP or were sacrificed for harvesting of spleen and blood. For the specific depletion of pDCs, C57Bl/6 mice were given an intraperitoneal injection of 500 μg rat anti-mouse 120g8 antibody the day before CLP and another dose of 250 μg of the same antibody 24 hours after the procedure. As a control, mice were given the same dosing scheme of an isotype control antibody (rat anti-human IL-4).

Cecal Ligation and Puncture

For induction of polymicrobial sepsis, mice (n=11 per treatment group) were subjected to cecal ligation and puncture (CLP) as previously described (81, 82). In brief, laparotomy was performed, and the cecum was isolated, ligated, and punctured through and through with a 22-gauge needle. When indicated, animals were either given an intraperitoneal injection of

diphtheria toxin (4 ng/g body weight) or an identical volume (200 μl) of sterile normal saline 24 hours prior to the CLP. Also, when indicated, mice received a tail vein injection of 10^7 bone marrow-derived DCs from wild-type animals, or 200 μl of normal saline at the time of diphtheria toxin injection. Depending on the experiment, mice were either euthanized at 6 hours after surgery to harvest blood, liver, lungs, and spleen, or animals were observed for up to seven days to determine survival.

Determination of Bacteremia

Blood bacteremia was determined by culturing 10 μl of whole blood diluted with ten volumes of sterile physiologic saline on sheep's blood agar plates (Fisher Scientific, Pittsburgh, PA) at 37ºC in 5% CO_2 for 48 hours.

Plasma Cytokine Concentrations

Plasma cytokine concentrations were determined using a multiplex approach based on antibody recognition of the individual cytokines, as previously described (83). Briefly, capture antibodies were spotted on individual nitrocellulose pads affixed to glass slides. Following blocking, the samples were incubated on the slides. Biotinylated secondary antibodies and Cy5-conjugated streptavidin were subsequently used for detection. Concentrations were calculated from standard curves.

Culture and Intravenous Injection of Bone Marrow-Derived DCs

Bone marrow-derived DCs were cultured using the procedures of Lutz et al (84). Briefly, mice were euthanized and femurs and tibiae of 6-12 week old wild-type littermates were removed and cleansed of tissue, and sterilized in 70% ethanol. Both ends of each bone were cut and marrow was flushed with phosphate-buffered saline (PBS). Red blood cells were lysed with an ammonium chloride lysis solution and cells were plated at 2 x 10^6 per 100 mm petri dish

(Falcon, BD Biosciences, San Jose, CA) in 10 ml RPMI-1640 medium, supplemented with penicillin, streptomycin, 2-mercaptoethanol, and 10% heat inactivated and filtered fetal calf serum. 2000 U rmGM-CSF (Peprotech, Rocky Hill, NJ) was added to the plates and again at three days of culture. On day 6, half of the cell-containing supernatant was collected, centrifuged, and resuspended in 10 ml fresh media containing GM-CSF. On day 8, nonadherent and loosely adherent cells were harvested, washed in PBS, and collected into a syringe at 5 x 10^6 cells per 100 μl. Then, 200 μl were injected intravenously to each mouse at the time of diphtheria toxin administration, 24 hours prior to the CLP.

Phenotypic Analysis of DC Populations

Spleens were harvested 12, 18, or 24 hours after diphtheria toxin injection and single cell suspensions were created by passing the cells through a 70-μm pore size cell strainer (Falcon). Contaminating erythrocytes were lysed with an ammonium chloride lysis solution. After washing twice with buffer (1% bovine serum albumin (BSA), 1 mM EDTA; (Fisher; Pittsburgh, PA) and 0.1% sodium azide (NaN_3; Sigma-Aldrich, St. Louis, MO) in Hanks Balanced Salt Solution (HBSS) without phenol red, calcium, and magnesium (Cellgro; Mediatech, Herndon, VA), cells were resuspended in 4% Hanks' azide buffer (HBSS without calcium, magnesium, or phenol red, with 4% BSA, 0.1% sodium azide, 0.2% anti-CD16/32, and 1 mM EDTA), and then stained. All antibodies were purchased from BD Pharmingen except the anti-Plasmacytoid Dendritic Cell Antigen-1 (PDCA-1) antibody which was purchased from Miltenyi Biotech (Auburn, CA). DCs were characterized using anti-I/A-I/E (MHCII)-conjugated to fluorescein isothiocyanate (FITC), anti-CD11c-conjugated to allophycocyanin (APC), and anti-CD8-conjugated to phycoerythrin (PE) (clone 53-6.7), anti-CD11b-PE, or anti-PDCA-1-PE, and anti-B220-FITC antibodies. The maturation status of the $CD11c^+$ DCs was assessed by the level of MHC class II expression.

Samples were acquired and analyzed on a FACSCalibur™ or an LSRII flow cytometer (BD Biosciences, San Jose, CA). Myeloid DCs (mDCs) were characterized as $CD11c^{+}MHCII^{+}CD11b^{high}CD8\alpha^{-}$, lymphoid DCs (lDCs) were characterized as $CD11c^{+}MHCII^{+}CD8\alpha^{high}$, and plasmacytoid DCs (pDCs) were characterized as PDCA-$1^{+}B220^{+}CD11c^{low}$ or PDCA-$1^{+}B220^{+}CD11b^{-}$. Precursor DCs were characterized as $CD11c^{+}MHCII^{-}$ cells. At least 3 x 10^{4} non-debris, live cells ($7AAD^{-}$ on the FACSCaliber or Sytox $Blue^{-}$ on the LSRII) were used for analysis.

Statistics

Differences in survival were determined by Fisher Exact Test. Continuous variables were first tested for normality and equality of variances. Differences among groups were evaluated by analysis of variance, with post-hoc comparisons performed using Dunn's multiple range test. Significance was determined at the 95% confidence level.

Results

Sepsis Decreases cDC Numbers but Increases cDC Activation Status in the Spleens of Septic Mice

Although studies have shown that DCs are lost in sepsis, the long-term effects of sepsis on cDC and pDC subsets are not known. We therefore examined the long-term consequences of sepsis in mice surviving sepsis for up to 10 days after the septic insult (Figure 3-1). We found that both the relative and absolute numbers of cDC were dramatically reduced for the first 5 days of sepsis. By day 7, the absolute number of cDCs began to rebound even though relative number was still dramatically reduced, most likely due to the dramatic increase in spleen size that occurs beginning at around day 7 in mice surviving sepsis. During this same period, the relative and absolute numbers of pDCs did not change as much as the numbers of cDCs (Figure 3-1). There was a relative increase in pDC numbers at day 1 after sepsis, and an absolute increase at day 3

after sepsis, but these responses were transient and indicated that pDCs were not as susceptible to losses during the septic response.

The $CD11c^{+}MHCII^{+}$ Cells Remaining in Sepsis Display a Mature Phenotype

Since sepsis often induces a state of immunoparalysis where monocyte MHC Class II expression is often decreased, but DC maturation following pathogen exposure occurs rapidly, we wished to assess whether the $CD11c^{+}$ cell population left in the spleen following sepsis were either mature or may be contributing to the hyporesponsive period with a failure to induce maturation markers. We assessed DC maturation as assessed by an increase in MHCII and CD86 expression. We found that as early as 24 hours after sepsis, splenocyte DC maturation was dramatically increased (Figure 3-2). These data likely point to the fact that decreased numbers of DCs, and not a failure to mature may contribute to the immunosuppressive environment that occurs in sepsis.

Sepsis Induced DC Maturation is Unaffected by IFNAR, MyD88, TRIF, or IL-6 Deficiency or TLR4 Mutation

Since many pathways lead to DC maturation ex vivo and in vivo, we wished to determine whether any pathway is responsible for DC maturation in sepsis. The first pathway we chose to examine was the Type I IFN pathway, as Type I IFN production is the most potent inducer of DC maturation in many experimental systems (39). We performed CLP or sham surgery on mice that were genetically deficient in the sole receptor (IFN α/β receptor; IFNAR) for all Type I IFNs, and their background SvEv controls. Surprisingly, unlike most other models, we found that deficiency in IFNAR did not affect the maturation of DCs as there was over a 2-fold increase in mature splenic DCs ($MHCII^{high}CD86^{high}$) in CLP versus sham treatment in the IFNAR mice, whereas there was just under a 2 fold increase in the same population in the CLP versus the sham mice in the SvEv mice (Figure 3-3. There was also an increase in coexpression of CD86 and

MHCII in the $B220^+$ or PDCA-1^+ subset of CD11c-expressing DCs, indicating that pDCs were also being activated in both strains of mice (data not shown) independent of IFNAR signaling.

We next examined whether deficiency in either of the TLR proximal signaling molecules, MyD88 or TRIF, affected the ability of DCs to mature in response to sepsis. However, similarly to deficient Type I IFN signaling, deficiency in either MyD88 or TRIF did not affect maturation of DCs during sepsis (Figure 3-4). Similar results were obtained in C3H/HeJ mice (TLR4 mutant mice that cannot respond specifically to endotoxin) as well as IL-6 KO mice (data not shown). These data indicate that maturation of splenic DCs in response to sepsis is a well-conserved phenomenon, and deficiency in one signaling pathway may either lead to compensation by the other signaling pathways, or activation of another signaling pathway (such as activation through CD1d restricted NK-T cells responding to glycolipids, activation of the inflammasome pathway by bacterial products, NOD/NOD-like receptors, or bowel ischemia induced cytokine production) may be sufficient to cause DC maturation in sepsis.

Diphtheria Toxin Depletes $CD11c^{high}MHCII^{high}$ DCs in DCKO Mice

Since sepsis induces dramatic alterations in both DC numbers and DC maturation, we wished to examine their importance in sepsis. The transgenic B6.FVB-Tg.Itgax-DTR/EGFP.57Lan/J mice express the diphtheria toxin receptor on the CD11c promoter which allows for selective depletion of DCs following administration of diphtheria toxin. In these mice, a single injection of diphtheria toxin did not produce significant morbidity or mortality. We first verified that diphtheria toxin causes depletion of DCs from the spleen of DCKO mice. As shown in Figure 3-5A, 24 hours after diphtheria toxin administration, spleens were depleted of predominantly $CD11c^{high}MHCII^{high}$ DCs and to a lesser extent $CD11c^{low}$ DCs. The loss began by 12 hours and was sustained for at least 24 hours (Fig 3-5B). Next, we determined the kinetics and subtypes of $CD11c^+$ DC loss following diphtheria toxin administration. We found that the

nadir in $CD11c^{high}MHCII^{high}$ DCs was 24 hours after diphtheria toxin administration, regardless of DC subtype. The $CD11c^{high}$ lymphoid DC (lDCs, $CD8\alpha^{+}$) subtype was depleted most by diphtheria toxin, and demonstrated a 95% loss by 24 hours (Fig 3-5d). $CD11c^{high}$ myeloid DCs (mDCs, $CD11b^{high}CD8\alpha^{-}$) were also dramatically lost (88-90%) by 24 hours, whereas plasmacytoid DCs (pDCs, $PDCA\text{-}1^{+}B220^{+}CD11c^{low/int}CD11b^{-}$), which are intermediate expressers of CD11c, demonstrated only a 75% loss by 24 hours (Figure 3-5c, 3-5d). Interestingly, by 24 hours after diphtheria toxin administration, a $CD11c^{low}MHCII^{-}$ DC (preDCs) population began to appear in the spleen (Figure 3-5c). These cells could be recent bone marrow emigrants that are DC precursors, but may become susceptible to diphtheria toxin as they begin to express increased levels of CD11c.

Increased Susceptibility to Sepsis Mortality in DCKO Mice

To determine whether loss of $CD11c^{+}$ DCs increases mortality after CLP, DCKO mice underwent CLP 24 hours after diphtheria toxin or saline administration. As shown in Figure 3-6A, DCKO mice pre-treated with diphtheria toxin displayed increased mortality after CLP compared to DCKO mice pre-treated with saline (100% vs 45%; $p<0.05$). Although wild-type mice do not express the diphtheria toxin receptor, we wanted to assure that diphtheria toxin had no direct effect on survival after CLP in littermates not expressing the $CD11c^{+}$-diptheria toxin receptor transgene. Figure 3-6B shows that additional DCKO mice treated with diphtheria toxin displayed increased mortality compared to their littermates not expressing the diphtheria toxin receptor, but also treated with diphtheria toxin, following CLP, indicating that diphtheria toxin has no survival effect on wild-type littermates (100% vs 45%; $p<0.05$).

Unaltered Bacteremia and Cytokine Profiles in Septic DCKO Mice

Since DCKO mice treated with diphtheria toxin begin to display significant mortality earlier than 12 hours following the CLP, we examined whether the increased mortality was associated with an increased blood bacteremia, or an exaggerated inflammatory response with associated organ injury. If the reduced immune surveillance was associated with the loss of $CD11c^+$ DCs, it might present as increased systemic bacteremia derived from the release of luminal contents. We found that blood from sham-treated healthy mice did not yield positive bacterial culture (0/3 vs 0/3), and that there was no difference between the numbers of mice which developed bacteremia 6 hours after CLP in the DCKO and wild-type littermates treated with diphtheria toxin (3/7 versus 4/8, respectively; $p>0.05$). In those mice that exhibited bacteremia, there was also no gross difference in the numbers of aerobic bacteria recovered from the blood (Figure 3-7).

We next assessed whether the increased mortality after CLP in DCKO mice was associated with an exaggerated inflammatory response. Our concern in this case was that the necrotic loss of these DCs might prime these animals for a subsequent inflammatory response to the CLP. As shown in Table 3-1, there were dramatic increases in the plasma concentrations of a number of inflammatory cytokines six hours after the CLP. These included sTNFR1, sTNFR2, IL-1ra, IL-6, MCP-1, and MIP-2, as well as IL-1β, LIX, eotaxin and MIP-1α (all $p<0.05$ by ANOVA). No differences in the plasma concentrations of IL-4, IL-5, IL-10, IL12p70, IL-13, IFN-γ, RANTES, or TNF-α were found between any groups ($p>0.05$ by ANOVA). Also, there were no differences in the concentration of any individual cytokine in DCKO pretreated with diphtheria toxin versus wild-type littermates pretreated with diphtheria toxin after sham or CLP, with the exception that IL-18 concentrations, which were markedly higher in the DCKO mice ($p<0.05$ by ANOVA).

Adoptive Transfer with Wild-Type BMDCs Protects DCKO Mice from Septic Mortality

We next examined whether the loss of DCs is directly responsible for the increased mortality in the DCKO mice. We have previously characterized the DCs generated for use in this experiment and found that they are a mixture of $CD11c^{high}MHCII^{high}$ (37-39%) and $CD11c^{low}MHCII^{low}$ DCs (85). We found that intravenous injection of 10^7 bone marrow-derived DCs (BMDCs) obtained from wild-type animals at the time of diphtheria toxin injection protected DCKO mice from mortality. Mortality declined from 100% to 65% in DCKO mice reconstituted by the intravenous administration of DCs ($p<0.05$) (Figure 3-8).

Depletion of pDCs Does Not Increase Susceptibility to Mortality from Sepsis

Since the DCKO transgenic mouse model causes depletion of both conventional and plasmacytoid dendritic cells, the relative contribution of each DC subset to mortality cannot be assessed using this model. However, a monoclonal antibody, 120g8 has been developed that can be used to specifically deplete pDCs (43). In the literature, this antibody was shown to deplete 75-80% of pDCs from the spleen, blood, and peripheral lymphoid organs of mice, which is the approximate level of pDC depletion in the DCKO mice. We found that C57Bl/6 mice treated with this antibody are depleted of approximately 80% of their pDC population compared to mice treated with an isotype control antibody (Figure 3-9A). We next assessed whether mice specifically depleted of pDCs were more susceptible to sepsis mortality. However, we did not find a significant survival advantage or disadvantage in mice treated with the pDC-depleting antibody versus mice treated with the isotype control (Figure 3-9B, $p=0.45$). These data suggest that conventional DCs, and not pDCs confer a survival advantage to polymicrobial sepsis in mice.

Discussion

The treatment of sepsis remains a clinical conundrum, in part because our understanding of the immunological basis for the progression of sepsis to organ injury and mortality remains incomplete (1). The alterations in the innate and acquired immune system produced by severe sepsis are dramatic, and include profound losses of $CD4^+$ T-cells, $CD11c^+$ DCs, and B-cells, but not NK cells or macrophages from peripheral lymph nodes and spleen (70, 74, 76, 78, 86).

The loss of DCs in sepsis has been well-documented, but as new DC subtypes appear, the effects of sepsis on them need to be assessed. In this study, we found that conventional DCs are lost for extended periods of time, whereas plasmacytoid DC numbers are maintained throughout the course of sepsis. We also found that the remaining DCs in the spleen 24 hours after sepsis are dramatically mature. This is also the case in mice that are deficient in various signaling components of common inflammatory (MyD88, TRIF, TLR4, or IL-6) or immunomodulatory (Type I IFN) pathways. This demonstrates that either redundancy exists to cause DC maturation during a systemic bacterial infection, or other signaling pathways that we have not tested are completely capable of causing DC maturation in the absence of the tested pathways.

The functional significance of this loss of T-cells and DCs from secondary lymphoid organs during the sepsis response has been the subject of considerable controversy. The loss of both $CD4^+$ T-cells and DCs appears to be associated with an increase in their cellular apoptosis, which has proven to be an attractive but unproven therapeutic target in sepsis (87). Treatment of septic mice with caspase-3 inhibitors prevents mortality, although these studies could not identify the cellular target for the inhibition of apoptosis (71). We and others have shown that improved survival to severe sepsis induced by CLP could be seen in transgenic mice whose lymphocytes or myeloid cells were overexpressing the anti-apoptotic protein, Bcl-2 (71, 88, 89).

In these animals, the increased loss of apoptotic T- or myeloid cells was prevented, and mortality was subsequently reduced.

Unfortunately, similar findings have not been previously seen with $CD11c^+$ DC populations in bacterial sepsis. There is, however, a body of circumstantial evidence to suggest that $CD11c^+$ DC function is required for a successful outcome to sepsis. For example, depletion of $CD11c^+$ DCs has a significant impact on the natural course of infections or inflammation. In the original description of these DCKO mice, Jung and colleagues observed that $CD11c^+$ DC depletion resulted in the abrogation of cytotoxic T-lymphocyte responses to Listeria and Plasmodium infections (80). In a more recent finding, van Rijt observed that DCKO mice could not respond with an appropriate Th2 response to airway inflammation (90).

Alternatively, we have demonstrated that modifying DC phenotype can also have a substantial impact on outcome from bacterial sepsis. In two previous reports, we observed that the in vivo transfection of DCs with an adenoviral recombinant expressing IL-10 generated a regulatory-like DC population that protected mice from CLP-induced mortality (82, 88). Interestingly, similar improvements in outcome could be obtained when the animals received adoptive transfer of DCs transduced ex vivo to produce IL-10 (91). These modified regulatory DCs, when readministered into the footpads of mice, were shown to traffic to the draining lymph nodes, but not to the spleen. Such findings suggest that DC function can play a central role in determining outcome to severe sepsis, and modifying either the number or phenotype of these cells can impact outcome from severe sepsis.

The question that naturally arises from these studies is: what are the mechanisms by which the loss of $CD11c^+$ DCs results in such dramatic reductions in survival? The one-time administration of diphtheria toxin to these transgenic mice results in the necrotic and apoptotic

cell death of DCs in organs of the reticuloendothelial system with no direct effect on mortality by itself. We were concerned that the presence of necrotic cells might serve as an endogenous "danger signal" for the innate immune system (92), and induce an exaggerated inflammatory response by tissue macrophages or other inflammatory cells that moderate the inflammatory response to sepsis. To address this concern, we examined the inflammatory cytokine responses in these mice to CLP with the expectation that the magnitude of the proinflammatory response would be heightened in DCKO mice, if their innate immune system had been primed by the presence of dying or necrotic $CD11c^+$ cells. As shown in Table 1, CLP was associated with dramatic changes in the plasma concentrations of a number of proinflammatory cytokines in the septic mice 6 hours after the induction of CLP. Although there were changes in the concentrations of several proinflammatory cytokines, the differences in concentrations between wild-type littermates and the DCKO were very modest, with only IL-18 concentrations differing. Thus, it appears unlikely that the increased mortality seen in the DCKO mice was due to an overall exaggeration in the proinflammatory response.

An alternative explanation is that the loss of DCs in these animals reduced the antimicrobial capacity of the host that resulted in a more rapid and complete dissemination of the microbial pathogens. To assess the overall antimicrobial response in the DCKO mice, we examined blood bacteremia at 6 hours following CLP. There was no gross difference in either the frequency or magnitude of aerobic bacteremia in the DCKO mice when compared to their wild type littermates. Although examining a larger group may have yielded significant differences between the groups, it is unlikely that the small differences in bacterial clearance would explain the dramatic discrepancies in mortality.

The question of underlying mechanism(s) remains unresolved, although it is clear that the reductions in survival can be directly attributed to the loss of these CD11c expressing cell populations. Alterations in innate and acquired immunity produced by the cell death of the CD11c expressing cells cannot explain the differences in outcome, since adoptive transfer with bone-marrow derived DCs obtained from wild-type mice protected animals from lethality. Since these DCKO mice lose significant numbers of their myeloid, lymphoid, and to a lesser extent, their plasmacytoid DCs simultaneously, the contribution that these individual cell populations to outcome cannot be defined with these mice. However, using a pDC specific depleting antibody, we concluded that conventional DCs play a more important role in survival of mice as depletion of pDCs did not significantly affect mortality. These studies have unequivocally demonstrated for the first time that normal numbers of $CD11c^+$ conventional DCs are required for a successful outcome from severe sepsis.

Table 3-1 – Select plasma cytokine concentrations following CLP.

	sTNF-R1 (pg/ml)	IL-1ra (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-13 (pg/ml)	IL-18 (pg/ml)	IL-12 (pg/ml)	IFN-γ (pg/ml)	MCP-1 (pg/ml)	MIP-2 (pg/ml)
Sham, wild-type littermates + DT	222 ± 192	2684 ± 2705	59 ± 6	12 ± 0	147 ± 213	12 ± 0	12 ± 0	12 ± 0	188 ± 76	24 ± 0
Sham, DCKO mice + DT	479 ± 217	1854 ± 741	1875 ± 2598	12 ± 0	83 ± 71	67 ± 95†	12 ± 0	12 ± 0	291 ± 211	225 ± 348
Wild-type littermates + DT following CLP	999 ± 178 *	55089 ± 52935*	15806 ± 7956 *	664 ± 830	244 ± 239	19 ± 19	12 ± 0.8	72 ± 158	1949 ±1779 *	4818 ± 4258 *
DCKO mice + DT following CLP	1082 ± 356 *	31547 ± 27092 *	18181 ± 12607 *	365 ± 280	328 ± 285	267 ± 185†	25 ± 22	21 ± 18	1602 ± 1020*	3027 ± 2403 *

Values represent the mean ± S.D. * $p<0.05$ CLP vs sham; † $p<0.05$ DCKO vs wild-type littermates

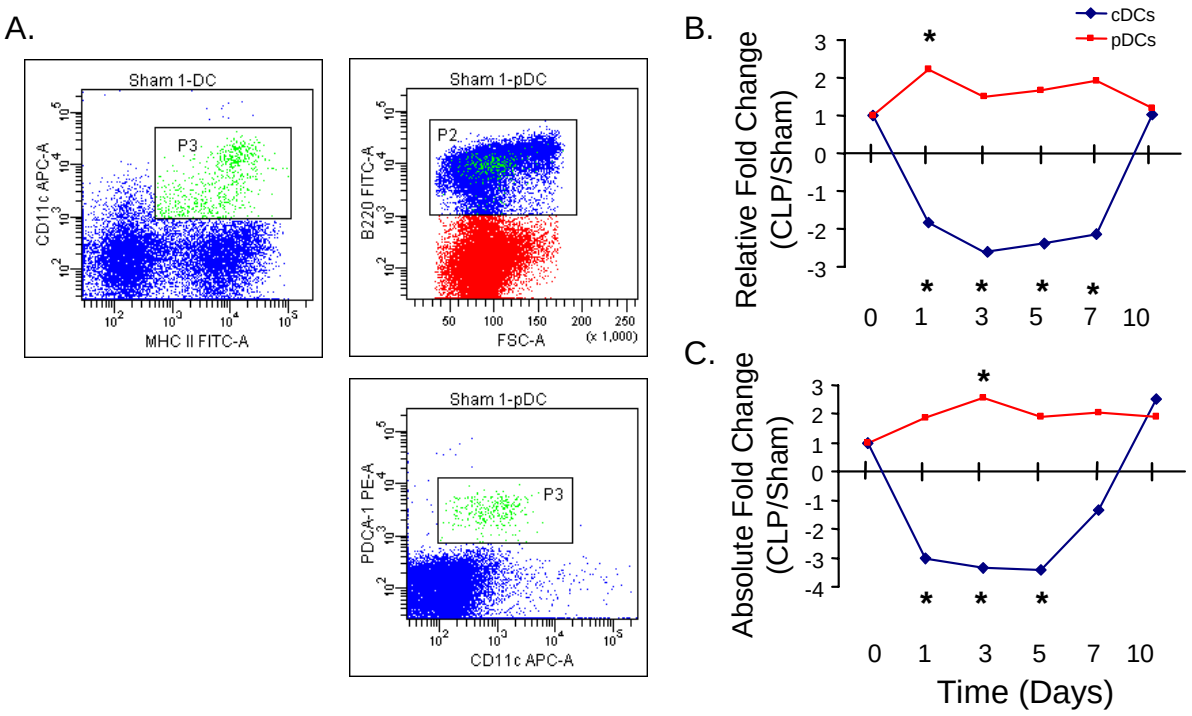

Figure 3-1. Changes in conventional and plasmacytoid dendritic cells in the spleen during sepsis. Mice underwent CLP or sham treatment and were euthanised at set intervals. A) The left panel shows the gating of conventional DCs (cDCs) defined as $CD11c^{+}MHCII^{+}$ cells. The right panels show the gating of plasmacytoid DCs (pDCs). First $B220^{+}$ cells (upper right) were gated followed by gating on the $PDCA\text{-}1^{+}CD11c^{+}$ cells. Debris and dead cells were first gated out of the analysis of both cell types by forward and side scatter gating and 7-AAD positivity from all panels. The percentage B) and absolute number C) of cDCs and pDCs were determined from sham (n=4) or septic (n=5) mice at all time points. The total cellularity of each mouse was determined by counting on a hemacytometer.

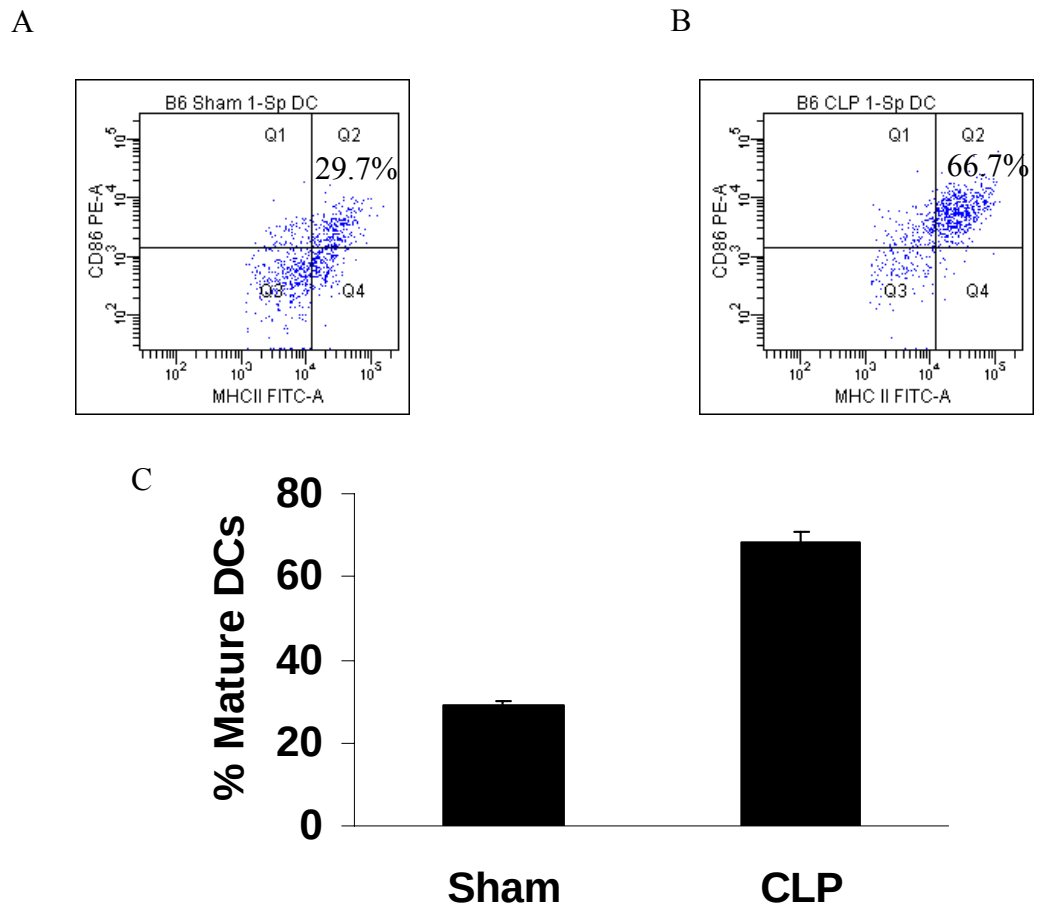

Figure 3-2. Sepsis causes the remaining splenic DCs to mature. 24 hours after CLP (CLP) or sham treatment, splenocytes were harvested and stained for CD11c, MHCII, and CD86. A representative example showing the expression of CD86 and MHCII on DCs defined as $CD11c^{+}MHCII^{+}$ cells (including cDCs and pDCs) from a sham mouse (A) and a septic mouse (B). Tabulated results are shown in C) from one experiment showing an n=3 mice in each group. This experiment was performed at least 12 times using 5 different strains of mice with similar results. $p<0.001$ for CLP vs sham mice.

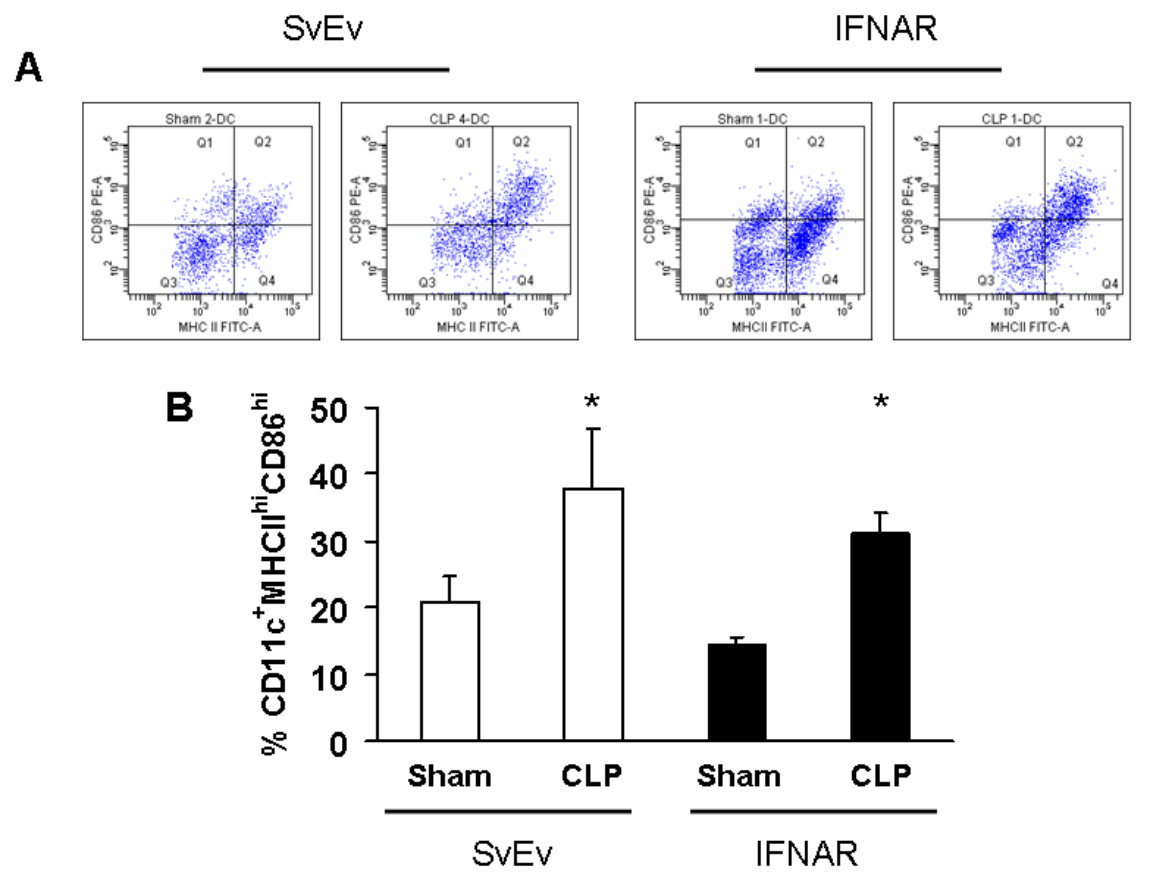

Figure 3-3. Type I IFN does not participate in sepsis-induced DC maturation. IFNAR-/- or SvEv mice were subjected to CLP (CLP) or sham surgery and 24 hours later, splenocytes were harvested for flow cytometry. Cells were stained as described in the Methods section. At least 5x104 non-debris, living (Sytox Blue-) cells were acquired from each sample on an LSRII flow cytometer. A) Representative dot plots showing CD86-PE staining on the y-axis and MHCII-FITC on the x-axis from left to right showing Sham and CLP mice from the SvEv background, then Sham and CLP mice from the IFNAR-/- mice. Mice were first gated for living, non debris, $CD11c^{+}MHCII^{+}$ cells. B) Tabulated results of the % of MHCIIhighCD86high cells in the Q2 (upper right) quadrant of n=3 mice in each Sham group and n=5 in each CLP group.

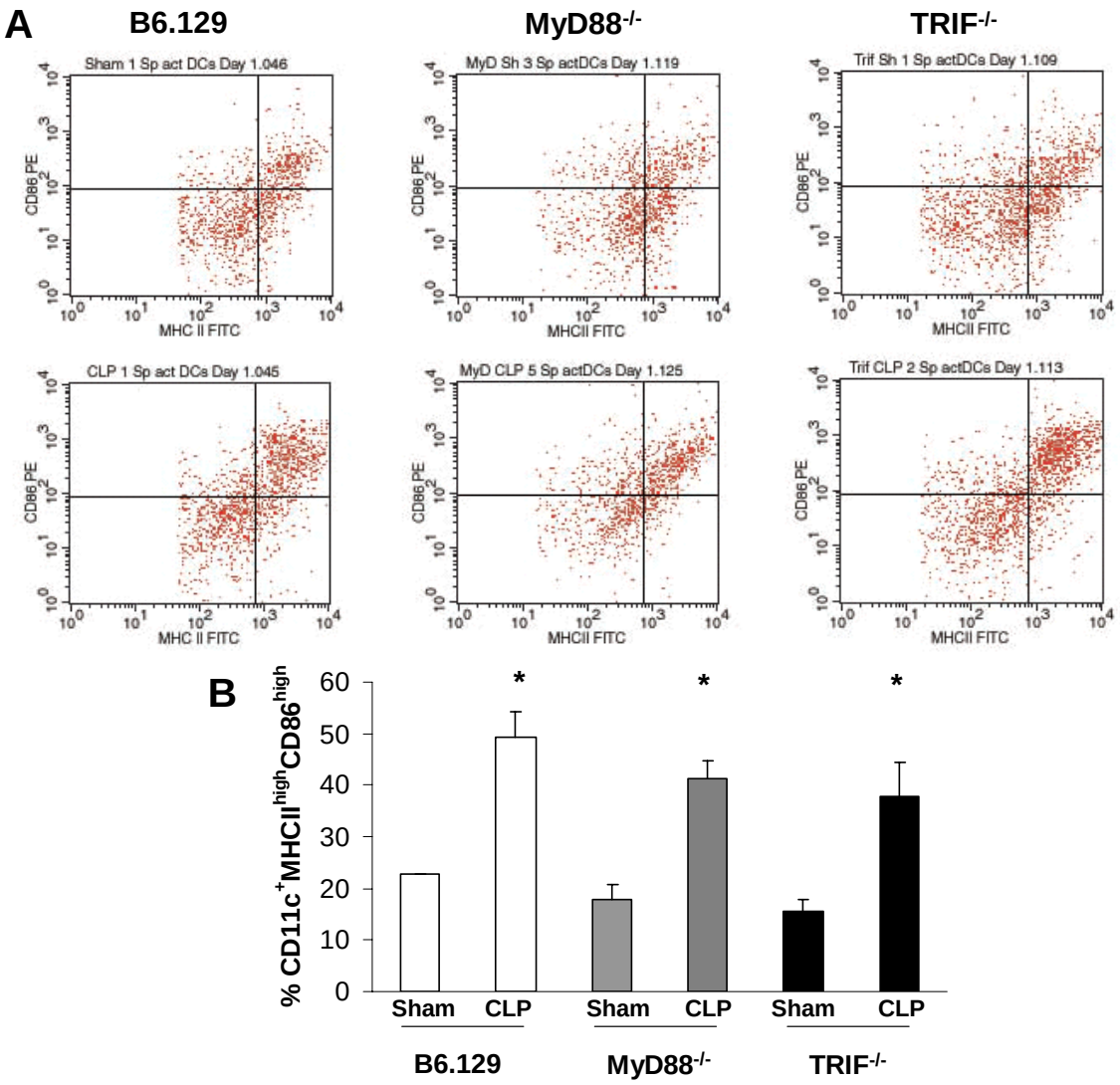

Figure 3-4. MyD88 or TRIF deficiency does not affect DC maturation in sepsis. B6.129 control, MyD88-/-, or TRIF-/- mice were subjected to CLP (CLP) or sham surgery and 24 hours later, splenocytes were harvested for flow cytometry. Cells were stained as described in the Methods section. At least 1x105 non-debris, living (7-AAD-) cells were acquired from each sample on a FACSCaliber flow cytometer. A) Representative dot plots showing CD86-PE staining on the y-axis and MHCII-FITC on the x-axis showing plots from Sham mice on top and plots from CLP mice on bottom from the B6.129 background mice (left panels), MyD88-/- mice (middle), and from the TRIF-/- mice (right panels). Mice were first gated for living, non debris, $CD11c^{+}MHCII^{+}$ cells. B) Tabulated results of the % of MHCIIhighCD86high cells in the upper right quadrant of n=3 mice in each Sham group and n=5 in each CLP group.

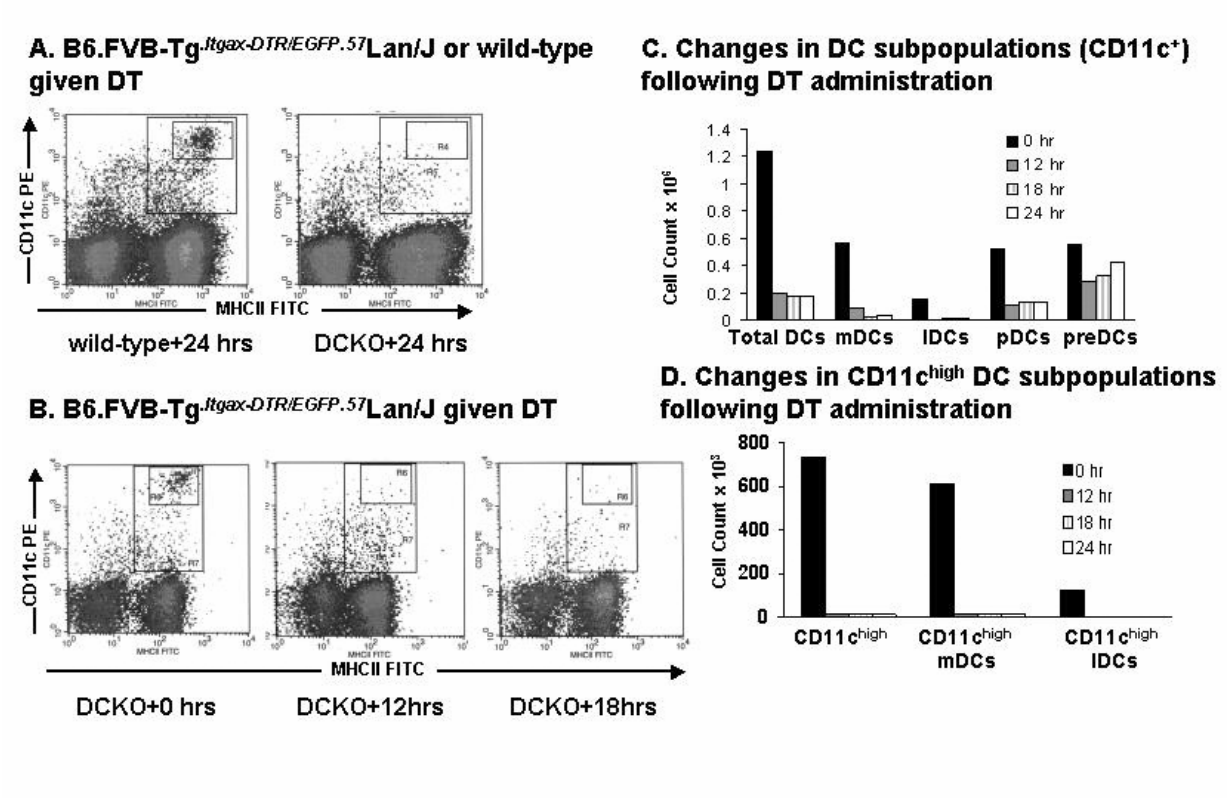

Figure 3-5. Loss of $CD11c^+$ DCs following diphtheria toxin administration. B6.FVB-Tg.Itgax-DTR/EGFP.57Lan/J mice either expressing the diphtheria toxin receptor on a CD11c promoter (DCKO) or wild-type littermates were administered 4 ng/g body weight of diphtheria toxin (DT), and splenocytes were obtained at intervals thereafter. After gating out for the presence of debris and dead cells ($7\text{-}AAD^+$), living cells were gated for CD11c, MHCII and specific DC subpopulations, as described in the Materials and Methods. Panel A. Depletion of $CD11c^+$, $MHCII^+$ DCs in DCKO and wild type littermates 24 hours after the administration of DT. Panel B. Depletion of $CD11c^+$, $MHCII^+$ DCs in DCKO mice 0, 12 and 18 hours after the administration of DT. Panel C. Changes in DC subpopulations following DT administration. Total DCs were identified in panel 1A from the R5 population of $CD11c^+$,$MHCII^+$ cells. Myeloid DCs (mDCs) were identified as $CD11c^+$,$CD11b^{high}CD8\alpha^-$, lymphoid (lDCs) as $CD11c^+$,$CD8\alpha^+$, plasmacytoid (pDCs) as $PDCA1^+$, $B220^+$, $CD11c^{low}$ and $CD11b^-$, and precursor DCs (preDCs) as $CD11c^{low}$,$MHCII^-$. DT treatment of DCKO produced a dramatic loss of all DC populations, with a rebound in precursor DCs at 18 and 24 hours. Panel D. Changes in $CD11c^{high}$ DC populations. $CD11c^{high}$ DC populations were identified in Panel B from region R6. DT treatment resulted in the near complete elimination of both myeloid and lymphoid DC populations expressing high levels of CD11c.

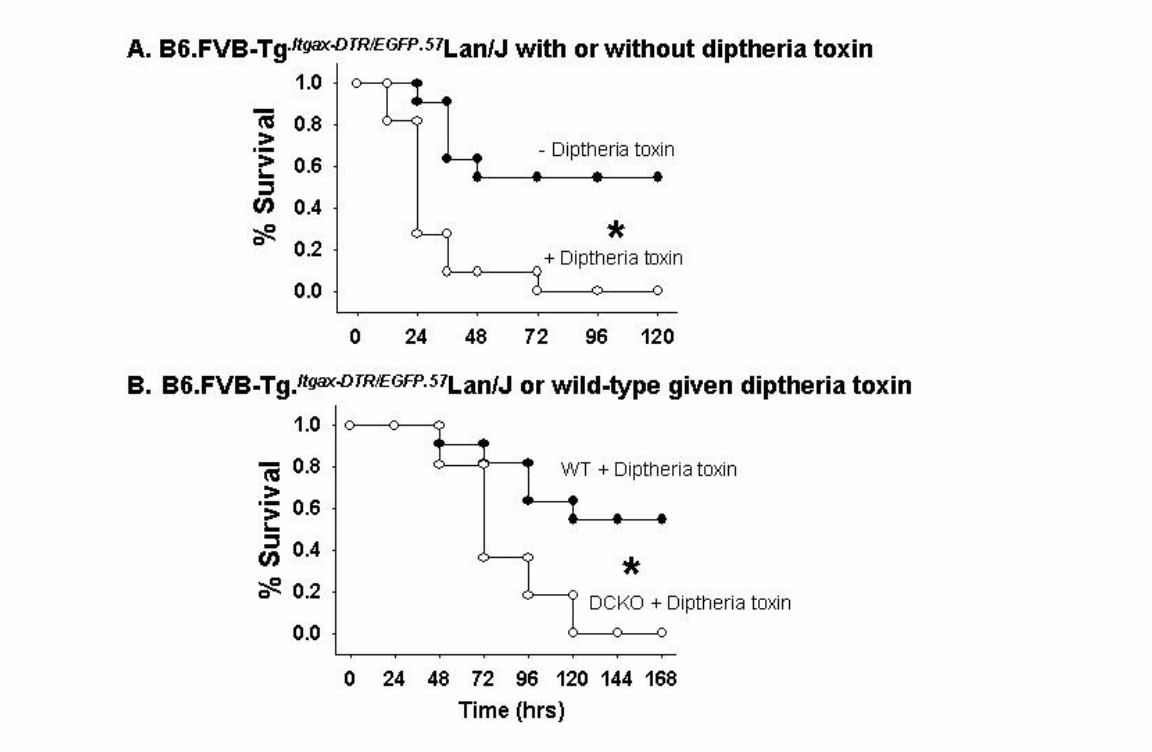

Figure 3-6. DCKO, but not wild-type, mice are more susceptible to lethality following CLP after diphtheria toxin (DT) administration. Panel A. Survival was determined in DCKO (n=11) versus wild-type littermates (n=11) following the administration of 4 ng/g of DT 24 hours prior to the induction of CLP (CLP). Survival was improved in wild-type littermates versus the DCKO (* $p<0.05$). Panel B. Survival following CLP was markedly reduced in DCKO mice (n=11) pretreated with DT, compared to wild-type mice also receiving DT (* $p<0.05$).

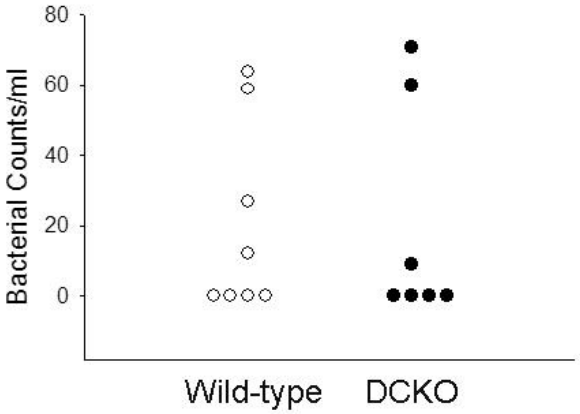

Figure 3-7. CLP induces blood bacteremia in DCKO and wild-type mice treated with diphtheria toxin. DCKO (n=7) and wild-type mice (n=8) were subjected to CLP (CLP) 24 hours after administration of 4 ng/g diphtheria toxin. 6 hours following CLP, 100 μl of whole blood was aseptically plated on sheep blood agar plates, and placed in an incubator (37º C, 5% CO_2). Total aerobic bacterial colonies were determined 24 hours after plating. Blood from sham-treated DCKO and wild-type mice (0/3 and 0/3) did not yield positive bacterial culture (data not shown).

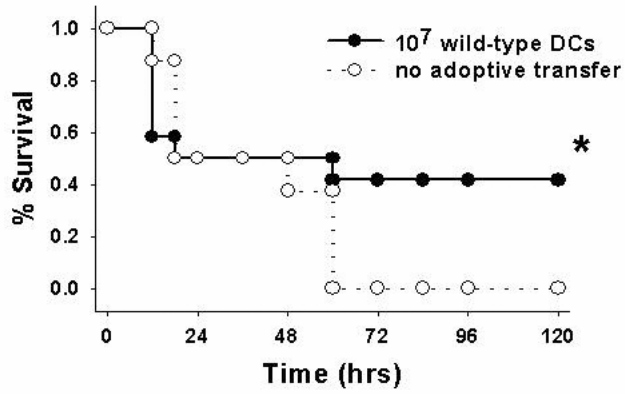

Figure 3-8. Adoptive transfer of 10^7 bone marrow-derived wild type DCs prevents CLP-induced mortality in DCKO mice pretreated with DT. DCKO mice were pretreated with 4 ng/g of DT 24 hours prior to CLP. At the time of pretreatment, animals either received the intravenous administration of 10^7 DCs obtained from wild-type littermates (n=12) or an identical volume of normal saline (n=8). Mice that received the intravenous adoptive transfer with wild-type DCs had improved outcome after CLP (* $p<0.05$ by Fisher exact test).

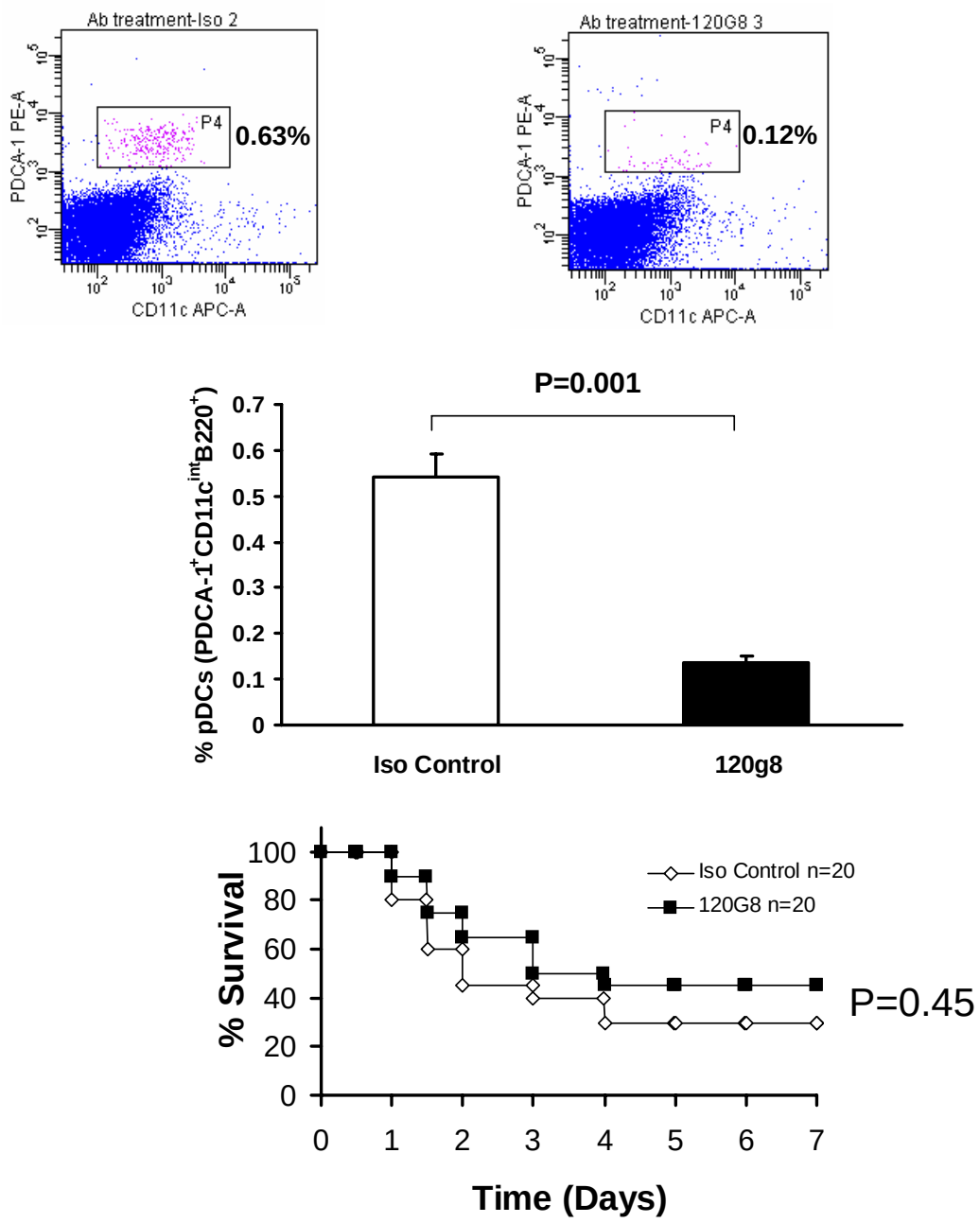

Figure 3-9. Loss of pDCs does not affect sepsis mortality. A) Representative example of a flow cytometry plot examining the expression of PDCA-1 and CD11c after previous gating of live ($7AAD^-$), non-debris, $B220^+$ cells from C57Bl/6 mice receiving an intraperitoneal injection of 500 μg 120g8 or isotype control antibody. B) Depletion of pDCs was approximately 80% in mice treated with 120g8 antibody. C) Mice receiving 120g8 or isotype control antibody were subjected to CLP or sham surgery 24 hours later. 24 hours after surgery mice received another intraperitoneal injection of 250 μg of antibody. Survival of mice was monitored for 7 days.

CHAPTER 4
THE CONTRIBUTION OF $CD4^+CD25^+$ REGULATORY T CELLS TO SEPSIS

Introduction

Despite progress in antibiotic and other supportive therapies over the past 20 years, sepsis remains the leading cause of death in the intensive care unit affecting over 750,000 patients in the United States and causing over 200,000 deaths annually (2). Significant advancements in our understanding of sepsis pathophysiology and immune dysfunction have occurred during this time period, predominantly focusing on the roles of inflammation and activation of the innate immune system. Unfortunately, mortality from severe sepsis has only declined modestly (2, 93) and several anti-inflammatory approaches have failed in clinical trials. There is a growing recognition that defects in the acquired immune response may also contribute to this adverse clinical outcome and may represent an appropriate target for therapeutic intervention.

New insights into specific immunomodulatory cells that contribute to immune tolerance, such as T regulatory (Treg) cells, may yield valuable insights into immune perturbations in sepsis syndromes. Despite their initial description over 30 years ago by Gershon and Kondo (52), "suppressor T cell" research was largely abandoned until Sakaguchi and colleagues (53) suggested a small population of $CD4^+CD25^+$ T cells, referred to as Tregs, was responsible for the prevention of organ-specific autoimmunity. Since their original description, a growing body of studies have suggested that naturally occurring Tregs play a major role in suppressing immune reactivity, ranging from autoimmunity to infectious disease (94) and to injury (62). Other studies have discovered inducible Treg subpopulations possessing immunoregulatory functions, including TGF-β producing T-helper type 3 (T_H3) cells having a role in oral tolerance to ingested antigens (95), and interleukin (IL)-10 producing T regulatory type 1 (T_R1) cells (96, 97). Many cell-surface molecules serve as markers co-expressed on Tregs, including glucocorticoid-induced

tumor necrosis factor receptor (GITR) (54) and intracellular cytotoxic T lymphocyte antigen 4 (CTLA-4) (98, 99), while still other factors contribute to the development and activity of regulatory cells, such as the forkhead box transcription factor, Foxp3 (100, 101) and toll like receptors (60, 102, 103), which recognize pathogen associated molecular patterns or can augment Treg function or proliferation.

Monneret et al. first observed that sepsis increases $CD4^+CD25^+$ T cells in the peripheral blood of septic patients (61). This was subsequently found to be a relative increase in Tregs, due to a decrease in the $CD4^+CD25^-$ T effector cell populations (104). However, whether these Tregs suppress T effector cell proliferation was not examined. Interestingly, expanding the endogenous population of activated Tregs by adoptive transfer prior to or following the initiation of polymicrobial sepsis improved outcome by enhancing peritoneal and mast cell TNF-α production, and bacterial clearance (105). At the same time, whether endogenous Tregs modulate outcomes in sepsis is unknown.

In this report, we used the murine CLP model to examine the effects of polymicrobial sepsis on endogenous Treg number, phenotype, and suppressor activity. We also ascertained whether antibody mediated depletion of Treg, genetic deficiency of CD25, or the T_R1-produced cytokine IL-10 would affect outcome to sepsis. Understanding the immunomodulatory effects of endogenous Tregs may lead to novel therapeutic approaches to ameliorate the mortality observed in severe sepsis.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine before their initiation. Specific pathogen-free 6 week old C57Bl/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME)

and were utilized between 8-12 weeks of age. Heterozygous CD25 null (B6.129S4-Il2ra$^{tm1Dw/}$J) mice backcrossed to a C57Bl/6 background and C57Bl/6 IL-10 null mice (B6.IL-10$^{-/-}$) breeding pairs were also purchased from The Jackson Laboratory and were maintained in a breeding colony at the University of Florida College of Medicine. Homozygous recessive mice (referred to as CD25 null) from the B6.129S4-Il2ra$^{tm1Dw/}$J colony and wild-type littermates were genotyped from tail DNA using primer sequences provided by Jackson Laboratory. Transgenic mice expressing the simian diphtheria toxin receptor on the Foxp3 promoter (Foxp3-DTR) were a kind gift of Dr. Daniel Littman from the Skirball Institute at New York University and were genotyped using tail vein DNA. Experiments using the CD25 null and age- and sex-matched littermates were performed at 5 weeks of age since the mice readily developed symptoms of inflammatory bowel disease shortly after 6 weeks of age. Similarly, IL-10 null mice were used prior to 6 weeks of age as they also developed symptoms of colitis at ~ 8 weeks of age.

CLP, and Other Animal Procedures

For induction of polymicrobial sepsis, mice were subjected to a CLP as previously described (82, 106). In brief, a laparotomy was performed, and the cecum was isolated, ligated below the ileocecal valve, and punctured through and through with a 23-gauge needle. Sham operation was performed by isolating the cecum without ligation or puncture. When indicated, animals were given either an i.p. injection of 500 μg of affinity purified CD4 (GK1.5 hybridoma) or CD25 (PC61 hybridoma) depleting antibody (BioExpress, Inc West Lebanon, NH) or an identical volume (200-250 μl) of sterile normal saline 72 h before the CLP. Administration of the antibody (250 μg) was repeated one hour before the procedure. Depletion of cell populations was confirmed by flow cytometry and lasted up to 8 days following the injection. Depending on the

experiment, mice were either euthanized at 24 h after surgery to harvest splenocytes, or animals were observed for up to 7 days to determine survival.

For the attempted depletion of Foxp3 expressing cells from Foxp3-DTR mice, mice were injected intraperitoneally with 1 μg of diphtheria toxin then were injected again 24 hours later. 18 hours after the second injection, mice were euthanized and spleens were processed and stained extra- and intracellularly as described below to determine depletion of Foxp3 expressing cells.

Flow Cytometry

Spleens were harvested 24 h after CLP or sham-treatment, and processing and flow cytometry was performed as previously described (106). Total cell counts were obtained using a hemacytometer. All antibodies were purchased from BD Pharmingen except the anti-GITR antibody and the anti- Foxp3 staining set, which were purchased from eBioscience. Antibodies used include anti-CD4 (RM4.4, conjugated to FITC, or GK1.5 conjugated to APC), anti-CD25 (3C7 conjugated to PE or PC61 conjugated to APC), anti-Foxp3 (FJK-16s) conjugated to FITC or allophycocyanin, and anti-GITR (DTA-1) or anti-CTLA-4 (UC10-4F10-11) conjugated to PE. For Foxp3 staining, extracellular staining was performed followed by intracellular staining using the manufacturer's recommendations.

Cell Purification

All magnetic bead kits were obtained from Miltenyi Biotec. Erythrocyte-depleted splenocytes were collected as described previously (106). APCs were obtained from control mice by incubating splenocytes with anti-CD90 microbeads then running them through LD columns (~97% T-cell depleted) using manufacturer's instructions. These cells were then irradiated (3,000 rads). The $CD4^+$ T-cell population from septic or sham-treated mice were purified by negative selection (>90% purity) for intracellular IL-10 analysis or positive selection using CD4 (L3T4)

microbeads (>98% purity) followed by high-speed cell sorting with CD3-FITC and CD25-PE on a FACSVantage (BD Pharmingen) for suppression assays. The purity of $CD3^+CD4^+CD25^{high}$ cells (Tregs) was >95% and the purity of $CD3^+CD4^+CD25^-$ cells (T effector cells) was >99%.

Foxp3 Real Time RT-PCR

Quantitative Real Time RT-PCR was performed as described previously, with some modifications (107). To measure quantities of Foxp3 mRNA, total RNA was extracted from an identical number (2.5×10^5) of cells using TriReagent (Molecular Research Center). Samples were treated with DNAse (RNase free DNase, Invitrogen) and total RNA was reverse transcribed into complementary DNA using Superscript II cDNA kit (Invitrogen). Foxp3 mRNA was quantified using qRT- PCR and SYBR Green real time PCR kit (Applied Biosystems) on the ABI PRISM using previously published murine Foxp3-specific primers (98). Foxp3 mRNA quantity in $CD4^+CD25^+$ or $CD4^+CD25^-$ cells was compared to 18S rRNA (Ambion) as the internal control and the ratio of Foxp3 to 18S rRNA was calculated using the $2^{-\Delta\Delta CT}$ method with the ratio in total splenocytes set as baseline.

Intracellular IL-10 Staining

Following isolation of $CD4^+$ cells by negative selection, 2×10^6 cells were placed into 24-well plates (Costar) coated with anti-CD3 (5 μg, clone 145-2C11) in RPMI 1640 complete media (CellGro) supplemented with 5 mM HEPES, 2 mM L-glutamine, penicillin (50 μg/ml)/streptomycin (50 μg/ml) (Invitrogen, Carlsbad, CA), 50 μM 2-mercaptoethanol, and 10% FS. Recombinant murine IL-2 (100 units/ml; BD Pharmingen) and anti-CD28 (1 μg; clone 37.51; BD Pharmingen) were also added to activate the T cells. Cells were activated for 6 hours in the presence of GolgiStop (BD Pharmingen), harvested, and stained extracellularly as described above. The cells were then fixed in buffer containing 1% formaldehyde for 30 min,

permeabilized by washing 3x in flow buffer containing 0.5% saponin, and stained with anti-IL-10 (JES5-16E3) conjugated to APC.

Suppression Assay

After purification and washing, a suppression assay was developed to test the capacity of $CD4^+CD25^+$ regulatory T-cells to suppress the proliferation of co-cultured T effector cells. Tregs were added in decreasing ratios (1:0, 1:1, and 0:1) to a constant number of T effector cells (2.5 x 10^4 cells/well) in at least quadruplicate wells. A combination of 5 μg/ml soluble anti-CD3 and 1 μg/ml soluble anti-CD28 provided the polyclonal stimulus for proliferation over a 72 hr culture period. The 2.5 x 10^5 irradiated (3000 rads) T-cell-depleted antigen presenting cells were added to each well in a total volume of 200 μl of complete RPMI media. Then, 1 μCi ^{3}H-thymidine (Amersham Biosciences, Piscataway, NJ) was added for the final 16 hours of culture to assess proliferation. Suppression was determined by the reduction of ^{3}H-thymidine incorporation in the combination of cells, and is calculated by the following equation: percent suppression = [1 – (mean cpm Treg + T effector)/(mean cpm T effector) x 100%].

Statistics

Differences in survival were determined by the Fisher exact test. Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric analyses were evaluated by Student's t-test. Differences in suppressor activity of septic versus sham-treated Tregs were determined by comparing differences between septic and sham-treated Treg mediated suppression from separate experiments by paired t test. Significance was set at $p=0.05$.

Results

Increased Proportion of Splenic $CD4^+CD25^+$ T cells Expressing Treg Markers in Sepsis

We first examined whether Treg numbers were increased following polymicrobial sepsis. Since no differences in Treg proportion or total number (by any marker used) were found between normal control mice and sham-operated mice at the time intervals examined, we included only the sham versus CLP comparisons in this study. The percentage of CD25 expressing $CD4^+$ T cells in the spleens of sham or control mice differed from experiment to experiment but was consistent with the literature as it ranged from 4-10% of the total $CD4^+$ T cell population. We observed a 2 to 2.5-fold increase in the percentage of $CD4^+CD25^+$ T cells in the spleen 24 hours following induction of generalized peritonitis from a CLP (Figure 4-1A). Since CD25 is also a marker of activated T cells, we examined a more specific marker of T cell activation, CD69, and more specific Treg- associated markers, such as GITR, intracellular CTLA-4, and Foxp3. We found that there was an approximate 75% increase in the percentage of $CD4^+CD25^+CD69^-$ T cells, indicating that a nonactivated $CD4^+CD25^+$ T cell subset was increasing following sepsis (Figure 4-1B; $p<0.001$). However, we did not find an increase in the absolute number of these cells in the spleen. Interestingly, there was also an approximate 4-fold elevation in the percentage of $CD4^+CD69^+CD25^-$ cells, indicating a dramatic increase in the activation of non-regulatory or effector splenic T cells 24 hours after a CLP as well. We subsequently examined whether the $CD4^+CD25^+$ T cells expressed other known markers of endogenous Tregs. To this question, we saw a similar increase in the percentage of $CD4^+CD25^+GITR^+$ T cells, but not the absolute number of these cells in the spleen of septic compared to sham-treated mice (Figure 4-1D; $p<0.03$).

Expression of Foxp3 Confirms that Tregs Are Proportionally Increased in Sepsis

Because GITR may also be expressed on activated T cells, we examined the expression of the transcription factor Foxp3 as a more definitive marker of Tregs. The relative level of Foxp3 mRNA was assessed by quantitative real-time reverse transcriptase–PCR (RT-PCR) from 2.5 x 10^5 $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells isolated by cell sorting of splenocytes from septic or sham-treated mice. Consistent with previous reports, Foxp3 mRNA expression was limited to the $CD4^+CD25^+$ population in control (Sham) mice (Figure 4-2A). $CD4^+CD25^+$ cells from sham-treated mice expressed approximately 30-fold the amount of Foxp3 mRNA over control splenocytes, whereas $CD4^+CD25^+$ cells from sham-treated mice expressed near baseline values. $CD4^+CD25^+$ T cells from septic mice, on the other hand, expressed 140-fold the amount of Foxp3 mRNA over control splenocytes, and 3.5-fold greater expression of Foxp3 mRNA than the $CD4^+CD25^+$ population from sham-treated mice. Interestingly, there was about a 3-fold increase in Foxp3 mRNA levels over baseline (and over expression from $CD4^+CD25^-$ T cells from sham-treated animals) in the $CD4^+CD25^-$ T cells from septic animals (Figure 4-2A). Similar data were obtained from three independent experiments, and one representative example is shown. These data confirm that there is an increase in the expression of Foxp3 per $CD4^+CD25^+$ T cell, indicating perhaps these cells may possess increased suppressor cell activity.

Since the expression of Foxp3 mRNA may not necessarily translate to the expression of Foxp3 protein, we further examined whether the augmented $CD4^+CD25^+$ T cell population from septic mice expressed Foxp3 protein. We found that spleens from septic mice had almost a 2-fold increase in the percentage of $CD4^+CD25^+Foxp3^+$ T cells (Figure 4-2B and 4-2C; $p<0.01$) by 24 hours, but again there was no significant increase in the absolute number of this cell population at 24 hours. We also observed that the relative increase in $CD4^+CD25^+Foxp3^+$ cells was maintained up to 3 days following the septic challenge, but levels soon diminished to baseline in

surviving animals thereafter (Figure 4-2C, left panel) and remained that way for up to 10 days after CLP. Interestingly, there was an increase in the absolute number of $CD4^+CD25^+Foxp3^+$ cells at Day 3 when the number of total splenocytes rebounded following the septic insult (Figure 4-2C, right panel). When examined, over 75 to 85% of the $CD4^+Foxp3^+$ population from septic or control mice also expressed intracellular CTLA-4, indicating that the phenotype of these cells ($CD4^+CD25^+Foxp3^+GITR^+CTLA\text{-}4^+CD69^-$) was more likely to be Tregs than activated T cells. Also, these data demonstrate that a relative increase in the percentages of Tregs during sepsis is probably due to persistence of these cells (possibly because they are more resistance to apoptosis) instead of proliferation during sepsis, whereas a loss of many cells such as T cells, B cells, and DCs (most likely by apoptosis) is known to occur (71, 76).

$CD4^+CD25^+$ Tregs from Septic Mice Are Better Suppressors of T Effector Cell Proliferation

Tregs are best characterized as being poor responders to mitogenic stimuli and suppressors of T effector cell proliferation. Since the $CD4^+CD25^+$ T cells from septic mice expressed more Foxp3 than $CD4^+CD25^+$ T cells from sham-treated mice, we examined whether they possessed increased suppressor cell activity. Indeed, we found that the $CD4^+CD25^+$ T cells from both septic and sham-treated mice were hyporesponsive to CD3/CD28 stimulation, and suppressed syngeneic T effector cell proliferation (Figure 4-3A). Interestingly, the CD^+CD25^+ T cells from septic mice displayed increased suppressive properties over $CD4^+CD25^+$ T cells from sham-treated animals (Figure 4-3B; $p=0.032$). However, T effector cells from septic mice only proliferated to approximately 1/2 the magnitude of cells from sham-treated mice, indicating some dysfunction exists with regards to T effector cell proliferation during the early sepsis period (Figure 4-3A). This finding will be further explored in the next chapter as it generated a new arm of research off of this project.

Antibody-Mediated Depletion of Tregs Does Not Alter Sepsis-Induced Mortality

Elimination of $CD4^+CD25^+$ Tregs by administration of a monoclonal antibody to CD25 is one of the most utilized methods to determine the role of Tregs in immunological reactions or various disease processes. In this study, we used monoclonal antibodies against CD4 and CD25 to deplete both of these cell types prior to sepsis. First, we confirmed that injection of the antibody depleted the cell of interest. We found that our treatment regimen depleted >90% of the cell population of interest for at least 8 days following the second dose with >95% depletion on the day of CLP (Figure 4-4A and 4-4B). Surprisingly, depletion of $CD4^+$ or $CD4^+CD25^+$ cells did not alter mortality to sepsis (Figure 4-4C; $p>0.05$). Furthermore, when we varied the baseline mortality produced by the CLP by altering the size of the enterotomy or the ligation, mortality rates between the antibody-treated and non-treated mice remained similar.

To confirm that $CD4^+CD25^+$ cells do not influence survival outcome in this model of sepsis, we performed CLP in CD25 null mice. Again, we found no survival difference in mice deficient in Tregs versus control mice (Figure 4-5D; $p>0.05$). Taken together with the antibody-mediated depletion experiments, we conclude that $CD4^+CD25^+$ Tregs do not influence survival in sepsis.

CLP Increases IL-10 Expressing T Cells but IL-10 Null Mice Are Not More Susceptible to CLP

Although $CD4^+CD25^+$ Tregs did not influence survival in sepsis, other Treg populations exist, including the inducible T_R1 type Tregs. These T_R1 Tregs are known to produce copious amounts of IL-10 and the generation of TR1 cells by contact with natural Treg may actually mediate some of the in vivo suppressive effects of natural Tregs. We therefore examined whether IL-10 expressing T cells were increased in response to sepsis. We found that stimulation of $CD4^+$ T cells from the spleens of septic mice with CD3/CD28 stimulation in the presence of IL-2

caused a 4 fold increase in the number of cells producing IL-10 when compared to cells from sham-treated mice (Figure 4-5A and 4-5B; $p<0.001$). Since IL-10 is important in the suppressive activity of T_R1 cells, we next wished to determine whether IL-10 null mice displayed increased mortality to sepsis, and whether this was due to a lack of T_R1 cells. In contrast to previous reports demonstrating that mortality progressed more rapidly in IL-10 null mice (108), we did not identify any role for IL-10 in sepsis mortality using this strain of mice (Figure 4-5C). Therefore, the production of this cytokine by a subset of cells that produce it (T_R1 cells) most likely did not have an effect on sepsis mortality.

Injection of Foxp3-DTR Mice with Diphtheria Toxin Does Not Deplete Foxp3-Expressing Cells

Although CD25 is a good marker for Tregs, depletion of $CD25^+$ cells also depletes recently activated $CD4^+$ and $CD8^+$ T cells. To determine whether depletion of Foxp3-expressing cells, the lineage marker of Treg, alters outcome in sepsis, we obtained transgenic mice expressing the diphtheria toxin receptor (DTR) on the Foxp3 promoter (Foxp3-DTR mice). According to the literature, two daily injections of 1 μg diphtheria toxin (DT) should eliminate Foxp3 expressing cells in the mice expressing the Foxp3-DTR transgene, but not their wild-type littermates. Mice were genotyped using published primers for the DTR gene and subjected to DT. When Foxp3 expression was examined on Foxp3-DTR expressing mice and their wild-type littermates, no difference in Foxp3 expressing cells was found (Figure 6). Because the donating investigator found that his mice also lost the ability to deplete Foxp3 expressing cells, the colony was abandoned and we are currently awaiting their rederivation of the mouse colony. Therefore, the determination of the specific contribution of Foxp3-expressing Tregs to sepsis outcome could not be determined.

Discussion

Studies ongoing for more than a decade have provided firm evidence for the existence of a unique $CD4^+CD25^+$ population of regulatory/suppressor T cells that prevent the activation and effector function of auto-reactive T cells that have escaped other mechanisms of tolerance (53). Subsequently, these regulatory T cells have been found to play major roles in a wide assortment of biological processes including transplantation tolerance (109), infection (59), inflammation/injury (62, 110), and tumor persistence/progression (111). $CD4^+CD25^+$ T cells have unique immunologic characteristics compared with other immune cells. For example, they do not proliferate in response to antigenic stimulation in vitro (naturally anergic), and can potently suppress the activation and proliferation of $CD4^+CD25^-$ T cells through either an antigen-nonspecific (112) or antigen-specific (57, 112) manner, through cell-to-cell contact.

In this study, we found that polymicrobial sepsis increases the proportion, but not the absolute number, of splenic $CD4^+CD25^+$ Tregs and these increases are transient, lasting only a few days. The findings are consistent with those of Venet et al. who observed that sepsis in humans results in the relative increase of Tregs in peripheral blood (104). In contrast to our findings, they found no increase in Foxp3 mRNA expression in the blood of septic patients. They collected their blood in PAXgene™ tubes, however, in which lymphocyte RNA is significantly diluted with RNA from erythroid precursor cells as well as other leukocyte populations, such as neutrophils, which make up the majority of leukocytes in the blood of septic patients. When examining highly purified T cell subpopulations, we not only found that sepsis increases Foxp3 mRNA expression in $CD4^+CD25^+$ cells, but also causes a similar increase in Foxp3 expression in $CD4^+CD25^-$ cells (albeit at a much lower absolute expression level). Similarly, we observed a low number of $Foxp3^+$ cells in the $CD4^+CD25^-$ fraction of sham-treated mice that was increased

in septic mice (*data not shown*). However, these cells were not included in our analysis as our suppressor assay was performed using the $CD4^+CD25^+$ fraction as Tregs.

Interestingly, we found that Tregs from septic mice had increased suppressor cell activity ex vivo, when compared to Tregs from sham-treated mice. It is known that activation of Treg by anti-CD3 engagement can increase their suppressive properties (105). Recently engagement of Toll like receptors by pathogen associated molecular patterns on Tregs has also been shown to induce their proliferation and activation, while causing an increase in their suppressor cell function (60, 102). This is a likely explanation for the increase in the suppressive function of Tregs from septic mice in our experiments, as bacteremia and organ colonization in the spleen by enteric microorganisms is a natural component of the sepsis response to a CLP.

However, despite their increased proportion and suppressor cell activity ex vivo, genetic deletion of endogenous Tregs or antibody-mediated elimination of Tregs prior to the onset of sepsis did not alter survival to the septic challenge. Furthermore, elimination of the total T helper cell population with anti-CD4 antibodies also did not affect survival to sepsis. These data are not fully consistent with reports by Hotchkiss et al. that apoptotic loss of T helper cells is detrimental to sepsis outcome (71, 113). This indicates that the endogenous $CD4^+$ population during sepsis does not play a major role in outcome, likely due to an already inherent dysfunction in the $CD4^+$ T cell population produced by sepsis. It is known that an increase in apoptotic death occurs in the $CD4^+$ T cell population during sepsis (69, 114), and contributes to the anergy in the T cell compartment (63, 115). Similar to this T cell anergy, we observed decreased effector cell function (proliferation to a mitogenic stimulus) in the $CD4^+CD25^-$ cell population isolated from spleens of septic animals compared to sham-treated animals. Because the T cell dysfunction that occurs normally during sepsis yields a similar outcome to when the $CD4^+$ population is

completely depleted from the animals prior to the onset of sepsis, these findings suggest that preserving or stimulating $CD4^+CD25^-$ numbers and function during sepsis might be appropriate therapeutic targets (87).

Recently, Heuer et al. demonstrated that treatment with ex vivo activated Tregs actually improves sepsis mortality (105). In contrast, we found that endogenous Tregs do not play a role in sepsis mortality. Although a recent report showed that CD25-mediated antibody "depletion" of Tregs, does not deplete Tregs (116), but instead deactivates them, we used two methods (antibody mediated depletion/ deactivation along with genetic depletion in CD25 null mice) to determine the role of Tregs in sepsis mortality. Several reasons may explain why endogenous Tregs do not alter outcome to sepsis, whereas adoptive transfer of activated Tregs does. First, the adoptive transfer of Tregs likely results in an expanded number of activated Tregs present in more organs. Furthermore, recent evidence suggests that IL-6 and TNF-α, cytokines commonly induced by sepsis both locally and systemically, can inhibit the function of endogenous Tregs (117, 118). Because these cytokines are increased during sepsis, endogenous Treg activity may actually be inhibited in vivo.

The role of IL-10 in sepsis remains controversial (21). Since certain pathogens can induce T_R1 type regulatory T cells that may contribute to sepsis pathology, we examined whether the inducible $CD4^+$ T cell population producing IL-10 (corresponding to a T_R1 regulatory cell subtype) was increased in sepsis, and whether these cells played a functional role in sepsis mortality. We observed over a 4-fold increase in the percentage of splenic IL-10 producing $CD4^+$ T cells from septic versus sham-treated mice. However, when sepsis was induced in IL-10 null mice, we found no appreciable difference in survival. Since IL-10 deficiency yielded no difference in outcome, we could not fully ascertain the role of IL-10 producing T_R1 cells in vivo.

These data suggest that compensatory mechanisms in addition to IL-10 or IL-10 producing T_R1 may exist in the IL-10 deficient mice to limit the inflammatory sequelae in sepsis.

Although the role of Tregs can be insinuated by these studies using markers of Tregs that are also found on other cell types, the true role of "natural" Tregs can only be achieved later, with the use of Foxp3-DTR mice.

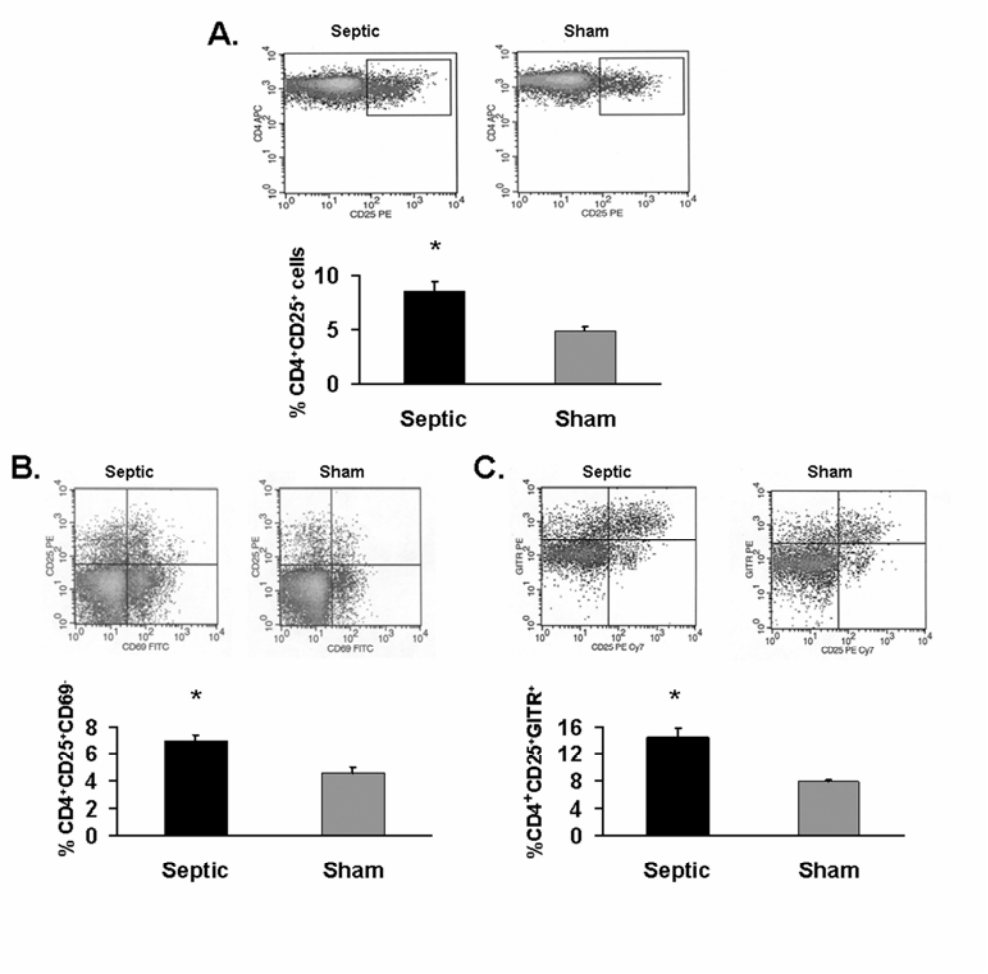

Figure 4-1. Sepsis increases the percentage of regulatory T cells. Sepsis was induced by CLP. 24 hours later, splenocytes were stained with fluorescently-labeled antibodies. A) Representative examples of CD25-PE versus CD4 APC on flow plots from one septic (top left panel) or one sham-treated (top right panel) spleen gated on 2 x 10^4 non debris, living ($7AAD^-$) $CD4^+$ T cells. The gate represents cells that were considered $CD4^+CD25^+$ in the calculations for the bottom panel (n=3 in each group). These results are consistent with results obtained from 4 different experiments. B) Representative examples of CD25 PE versus CD69 FITC from one septic (top left panel) or one sham-treated spleen gated on 2 x 10^4 non debris, living ($7AAD^-$) $CD4^+$ T cells. The upper left quadrant represents $CD4^+CD25^+CD69^-$ non-activated Tregs while the upper right panel represents $CD4^+CD69^+CD25^-$ activated T cells. The average percentage of $CD4^+CD25^+CD69^-$ T cells (n=3 per group) was increased in septic mice (lower panel). C) Representative examples of CD25 APC versus GITR PE from one septic (upper left panel) versus one sham-treated (upper right panel) mice gated on 1 x10^4 non-debris, live gated $CD4^+$ T cells. The average proportion (upper panel) of $CD4^+CD25^+GITR^+$ T cells (n=4 per group) was increased in septic mice (lower panel). * Indicates $p<0.05$ versus sham-treated.

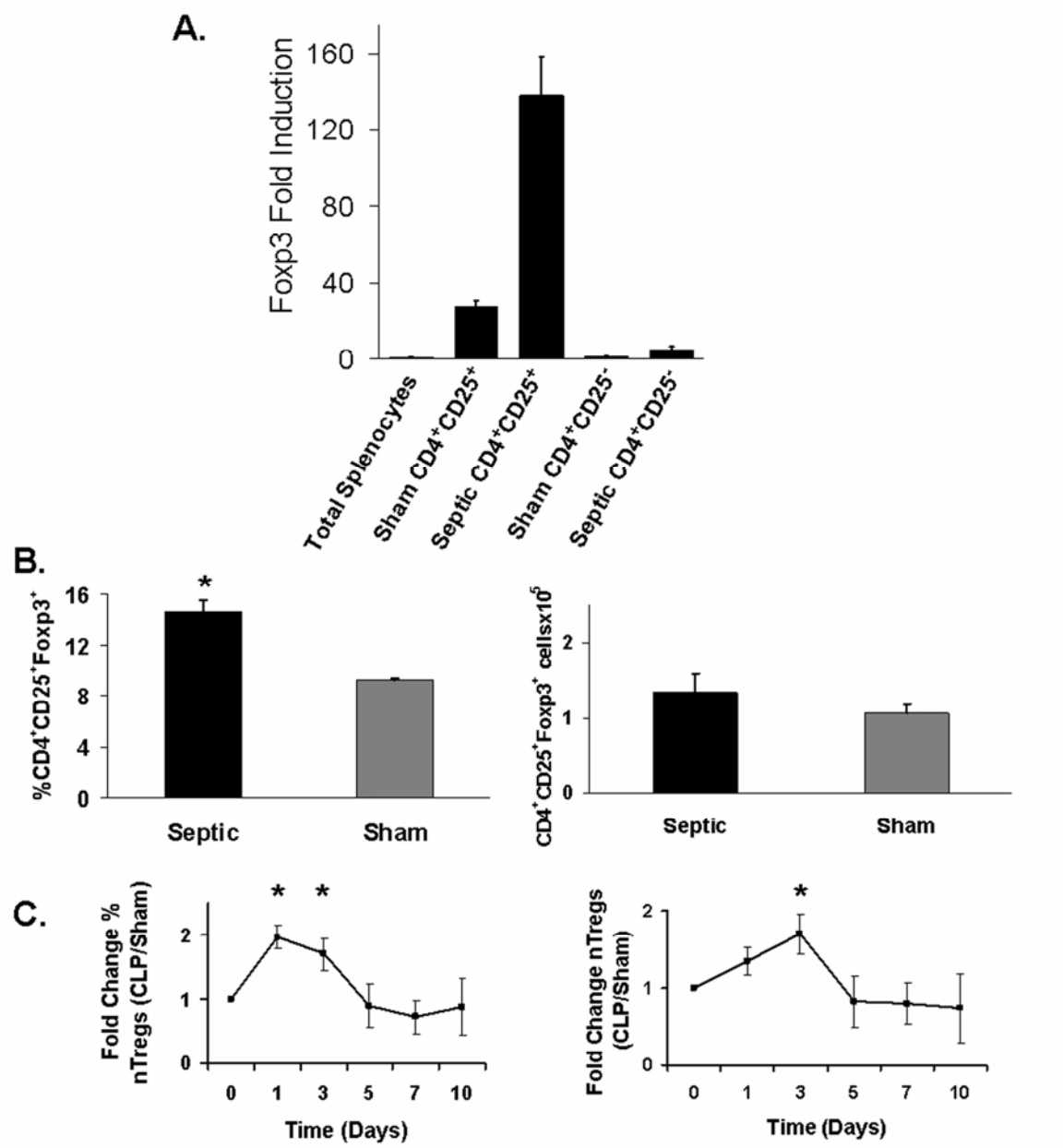

Figure 4-2. Increase in Foxp3 expressing T cells in septic spleens. A) 2.5 x 10^5 highly purified $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from two pooled septic or sham-treated mice (over 95% purity) were obtained. qRT-PCR for Foxp3 mRNA was performed in triplicate for each sample. Experiments were repeated a total of three times. Shown is a representative example and error bars represent the difference between triplicate runs for each sample. B) The percentage of $CD25^+Foxp3^+$ expressing $CD4^+$ T cells was increased in sepsis (n=3) versus sham-treated (n=3), but not C) the absolute number of cells per spleen at 24 hours. D) CLP or sham treatment was performed on C57Bl/6 mice and mice were euthanized and spleens were harvested at 1, 3 ,5 7, and 10 days after treatment. The ratio of the percentage (left panel) or total number (right panel) of $CD4^+CD25^+Foxp3^+$ cells in septic mice to $CD4^+CD25^+Foxp3^+$ cells in sham mice was calculated at each time point. * Indicates p=0.05 versus sham-treated mice.

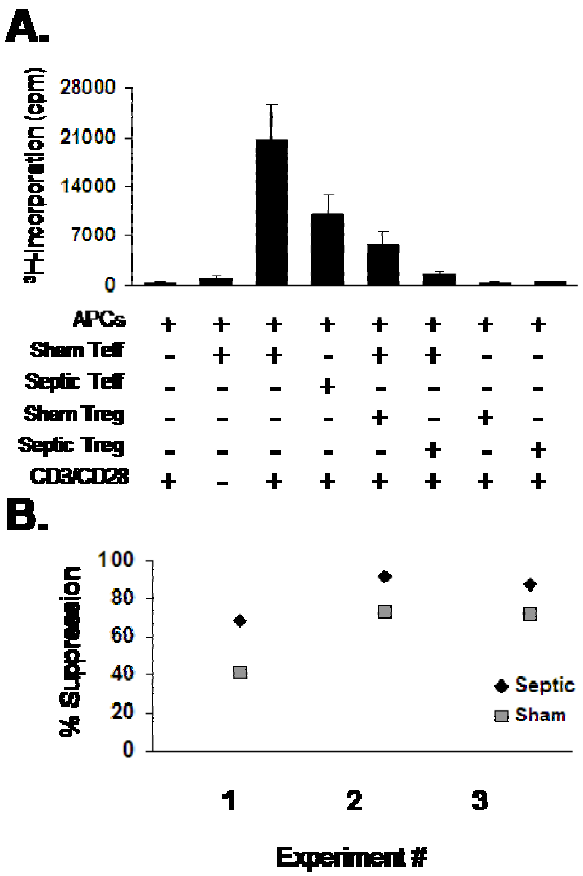

Figure 4-3. Treg suppression and anergy. A) Proliferation assays were undertaken to determine whether $CD4^+CD25^+$ T cells from septic mice were suppressive and anergic. Equal numbers of $CD3^+CD4^+CD25^-$ or $CD3^+CD4^+CD25^+$ T cells (2.5×10^4) from septic or sham-treated mice were cultured alone or together for 3 days with irradiated antigen presenting cells (2.5×10^5; 3000 rads), with or without anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) stimulation. Cell proliferation was measured using 3H-thymidine uptake B) Suppression ratios of $CD4^+CD25^+$ T cells from septic versus sham-treated T cells from 3 different experiments. Experiments 1 and 2 used T cells from three pooled septic or sham-treated mice, respectively, and Experiment 3 used T cells from one mouse each.

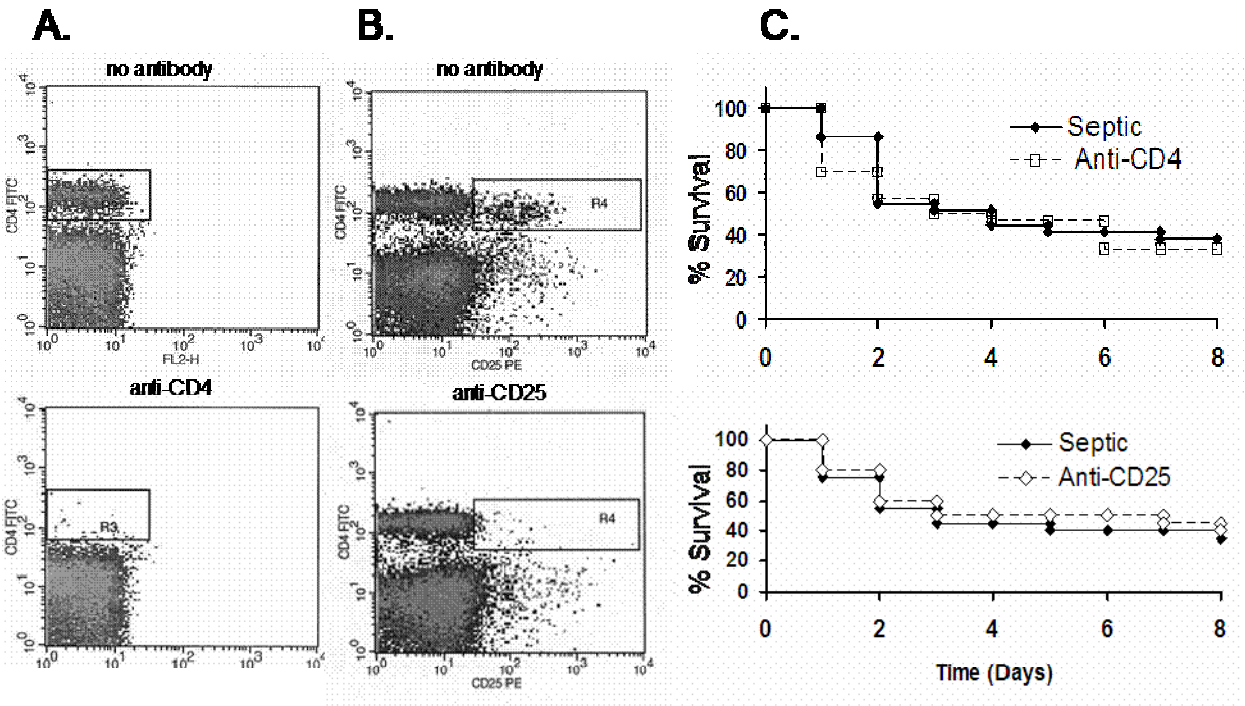

Figure 4-4. Antibody mediated depletion of $CD4^+$ or $CD25^+$ T cells does not alter survival after sepsis. A) Representative example of sham-treated, non-depleted (top panel) versus anti-CD4 depleted (GK1.5 clone) (bottom panel) T cells. B) Representative example of sham-treated, non-depleted (top panel) versus anti-CD25 depleted (PC61 clone) (bottom panel) T cells. C) Survival of mice depleted of $CD4^+$ cells versus sham-treated controls after CLP (Top panel; n=30 each group; $p>0.05$). The survival of mice depleted of $CD25^+$ cells versus sham-treated controls after CLP (Bottom panel; n=25 each group; $p>0.05$).

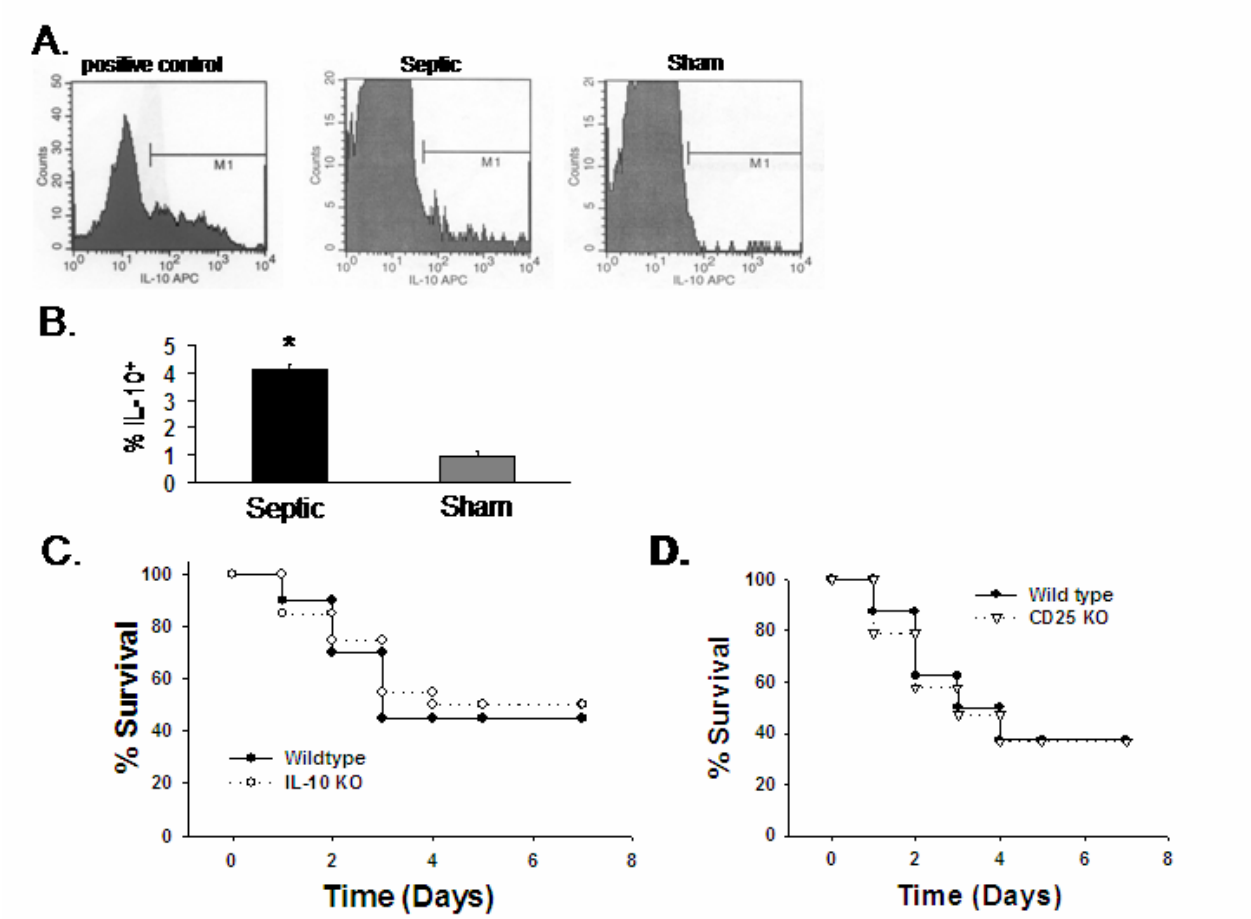

Figure 4-5. IL-10 and CD25 null mice are not more susceptible to sepsis than wild-type mice. A) $CD4^+$ T cells were isolated from septic or sham-treated mice and were treated for 6 hours with anti-CD3, anti-CD28, and recombinant murine IL-2 in the presence of GolgiStop™. Shown is a representative example of cells gated on $CD4^+CD25^+$ T cells. B) The total percentage of cells expressing intracellular IL-10 from septic versus sham-treated mice is shown (n=4). C) Survival to CLP in IL-10 knockout mice versus wild type mice (n=20 each group; $p>0.05$). D) Survival was determined in CD25 null mice (n = 19) versus wild-type littermates (n = 24) following the induction of CLP. Survival was monitored for 7 days. * Indicates $p<0.001$.

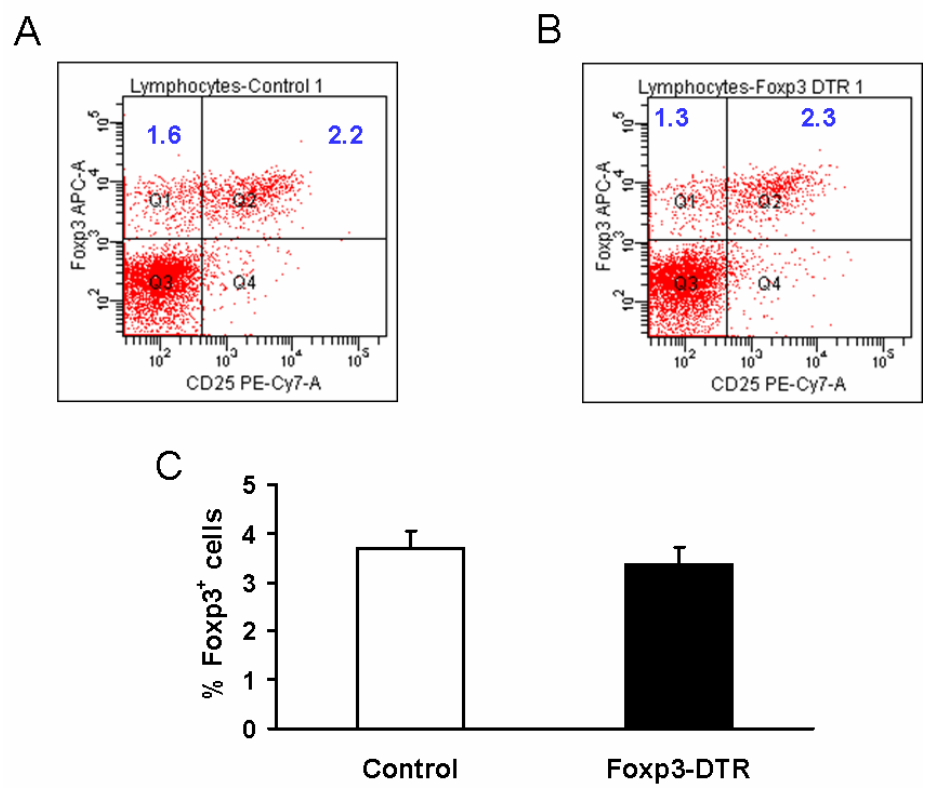

Figure 4-6. Diphtheria toxin injection of Foxp3-DTR mice did not deplete Foxp3 expressing cells. Foxp3-DTR mice and their wild-type controls were injected twice daily with 1 μg diphtheria toxin. 18 hours the final injection, mice were euthanized and spleen was harvested for flow cytometry. No cell population was altered by diphtheria toxin injection in the wild-type or Foxp3-DTR mice, including Foxp3-expressing cells.

CHAPTER 5
ROLE OF ADAPTIVE IMMUNE SYSTEM DYSFUNCTION IN OUTCOME TO SEPSIS

Introduction

Despite numerous studies showing that prevention of lymphocyte apoptosis improves survival in animal models of sepsis (70, 74, 86, 119), only a few studies using cytokine/anti-cytokine therapies have examined whether improving leukocyte function may improve outcome during sepsis (65, 120, 121). In a previous study, we found that despite increased regulatory T cell (Treg) activity in sepsis, depletion of regulatory T cells, and even depletion of all $CD4^+$ T cells, did not affect mortality in sepsis (122). We did find that even in the absence of Tregs, T effector cells from septic mice did not proliferate as well as T effector cells from sham mice. This indicates that the endogenous $CD4^+$ population during sepsis does not play a major role in outcome, likely due to an already inherent dysfunction in the $CD4^+$ T cell population produced by sepsis and augmentation of their responsiveness, either by inhibition of apoptosis or by direct stimulation may be necessary to overcome this dysfunction.

Glucocorticoid induced tumour necrosis factor (TNF) receptor family related gene (GITR) is a member of the TNF family that is expressed normally at high levels on $CD4^+CD25^+$ Treg cells and expressed at low levels on resting $CD4^+CD25^-$ and $CD8^+$ $CD25^-$ T effector cells, but the levels of GITR increase upon activation. Initially, GITR was found to abrogate Treg-mediated suppression (54), but it was later found to act as a costimulatory molecule on effector T cells (55, 56).

We hypothesized that stimulating CD4 effector T cell function during sepsis might be an appropriate therapeutic target. We analyzed lymphocyte function in sepsis, and determined whether anti-GITR treatment could prevent the adaptive immune dysfunction and improve survival in sepsis. We found that despite increased B cell activation and function, T cell

dependent IgM and IgG2a antibody production are reduced in septic mice. Furthermore, we find that $CD4^+$ T cells isolated from septic mice exhibit decreased CD3ζ chain expression, proliferate poorly to antigen nonspecifically and specifically, and produce less IL-2 and IFN-γ than $CD4^+$ T cells from sham mice. We demonstrate that pretreatment with a GITR agonistic antibody to mice increases T cell dependent class switching to both IgG2a and IgG1, increases CD3ζ expression and restores antigen nonspecific and specific proliferation in T cells leading to an improvement in survival in normal mice, but not mice depleted of $CD4^+$ T cells. These data indicate that augmenting T cell function may be an appropriate target in sepsis.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine before their initiation. All mice were maintained on standard rodent food and water ad libitum. Specific pathogen-free 6 week old C57Bl/6 and BALB/cJ female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were utilized between 8-12 weeks of age. T cell transgenic C.Cg-Tg(DO11.10)10Dlo/J (DO11.10) breeding pairs were purchased from the Jackson Laboratory and maintained in specific pathogen free conditions at a breeding colony at the University of Florida Health Science Center.

Reagents

4-hydroxy-3-nitrophenyl (NP) acetyl–keyhole limpet hemocyanin (KLH), and NP-Ficoll were purchased from Biosearch Technologies (Novato, CA). NP-bovine serum albumin (BSA) was purchased from Sigma Aldrich (St. Louis, MO). An antigenic peptide corresponding to amino acids 323-339 of chicken ovalbumin was purchased from Genscript Corporation (Piscataway, NJ). Phorbol-12-myristate-13-acetate and ionomycin were purchased from EMD

Biosciences (Darmstadt, Germany). Rat anti-mouse GITR agonistic (DTA-1) and anti-CD4 (GK1.5) antibodies were purchased from Bio Express Inc (West Lebanon, NH). Rat anti-human IL-4 used as an IgG2b isotype control antibody was obtained from Schering Plough Biopharma, Palo Alto, CA. CD4, CD90, and anti-APC microbeads were purchased from Miltenyi Biotech (Auburn, CA).

CLP

For induction of polymicrobial sepsis, mice were subjected to CLP as previously described, with a few modifications. In brief, a laparotomy was performed, the cecum was isolated and approximately 0.75 cm to 1 cm of cecum was ligated below the ileocecal valve and punctured through and through with a 23-gauge needle. Sham operation was performed by isolating the cecum without ligation or puncture. When indicated, animals were given either an intraperitoneal injection of 300 μg of GITR or isotype control antibody 30 minutes before surgery. Also when indicated, mice were immunized at the time of surgery with nitrophenyl-keyhole limpet hemocyanin (NP-KLH; 100 μg) or 100 μg $Ova_{323-339}$ mixed with alum (100 μl), or NP-Ficoll (30 μg) dissolved in PBS. In one experiment, depletion of $CD4^+$ T cells was achieved by intraperitoneal injection of anti-CD4 antibody (500 μg) 3 days prior to surgery and again at the time of anti-GITR treatment (250 μg). Depletion was confirmed by flow cytometry and lasted up to 8 days following the injection ((122) and data not shown). Depending on the experiment, mice were either euthanized at 24 h or 5 days after surgery to harvest splenocytes, bone marrow, and/or lymph nodes, or animals were observed for up to 10 days to determine survival or humoral antibody responses.

Flow Cytometry

Spleens, peripheral lymph nodes (inguinal and axillary), mesenteric lymph nodes, and/or bone marrow were harvested after CLP or sham surgery, and single-cell suspensions were created by passing the cells through a 70-μm pore size cell strainer (Falcon). Contaminating erythrocytes were lysed with an ammonium chloride lysis solution. After washing twice with phosphate buffered saline (PBS) without phenol red, calcium, and magnesium (Cellgro; Mediatech), cells were resuspended in PBS containing 0.2% anti-CD16/32 for Fc blocking, and then stained. Antibodies used include anti-B220 (RA3-6B2, conjugated to FITC), anti-CD19 (1D3, conjugated to allophycocyanin) anti-CD4 (RM4.4, conjugated to FITC, or GK1.5 conjugated to APC), anti-CD69 (H1.2F3, conjugated to PE) anti-MOUSE DO11.10 TCR (KJI-26, conjugated to allophycocyanin), anti-CD3ε (500A2, conjugated to Pacific Blue), anti-CD3ζ (H146-968, conjugated to FITC; Cedarlane Labs, Ontario, Canada or 6B10.2, conjugated to PE; Santa Cruz Biotechnologies, Santa Cruz, Ca). For intracellular staining for CD3ζ, extracellular staining was performed followed by intracellular staining using commercially available fixation and permeabilization solutions (eBioscience, San Diego, Ca) and their recommended directions. Samples were acquired and analyzed on either a FACSCalibur or an LSRII flow cytometer (BD Biosciences). At least 1 x 10^4 nondebris, live (7-aminoactinomycin D; $7AAD^-$ or Sytox $Blue^-$) cells were used for analysis except for intracellular staining where $4x10^4$ total events were used for analysis.

Immunization with NP-KLH and NP-Specific ELISA

T cell dependent humoral immune responses were quantified using an NP-KLH immunization model as previously described by Hurov and colleagues (123). T cell independent immune responses were performed similarly from mice immunized with NP-Ficoll. Briefly, tail

vein bleeds were completed on naïve C57BL/6 mice prior to treatment (control), sham, or CLP procedure. When indicated, animals were given either an injection of anti-GITR or isotype control antibody 30 min before CLP. At the time of surgery, mice were immunized with 100 μg of NP-KLH in alum subcutaneously. Ten days post immunization; mice were euthanized and bled by cardiac puncture. Experiments using NP-Ficoll were performed similarly except 30 μg NP-Ficoll was administered in sterile normal saline without alum and terminal bleeds were performed seven days after immunization. Titers of NP specific antibodies from serum samples were determined by ELISA. Immulon 4HBX 96 well plates (Dynex Tech) were coated with 1 mg of NP-bovine serum albumin (Biosearch Tech) per ml. Serum samples were bound to the plates in dilutions from 1:100 to 1:5 X 10^7. NP-specific antibodies were then bound to biotin-conjugated goat anti-mouse Ig isotype antibodies, anti-IgM, anti-IgG1, anti-IgG2a, and anti-IgG3 (CalTag). Streptavidin-conjugated horseradish peroxidase was incorporated to detect the biotin-Ig with 2,2'-azino-di(3-ethylbenzthiazolinesulfonate) (ABTS) substrate. Titers of Ig from individual mice were obtained by determining the dilution at which serum samples gave optical density readings at 414 nm of 0.2 Units above background. No significant levels of NP-specific IgG existed prior to initiation of either sham or CLP procedure.

Cell Purification

All magnetic beads were obtained from Miltenyi Biotec. Splenocytes were collected as described above. To obtain B cells, splenocytes from septic or sham mice were stained with anti-CD19 allophycocyanin, followed by anti-allophycocyannin microbeads and running through LS columns to obtain the positive fraction. The purity of the population was >90%. The $CD4^+$ T-cell population from septic or sham mice was purified by positive selection using CD4 (L3T4) microbeads (>95% purity). To deplete $CD25^+$ cells from the total $CD4^+$ population, high speed

cell sorting with CD3-FITC and CD25-PE on a FACSVantage (BD Pharmingen) was performed to obtain $CD3^+CD4^+CD25^-$ cells (Teffs) at >98% purity. APCs were obtained from control mice by either incubating splenocytes with CD90 microbeads, then running them through LD columns (~97% T-cell depleted) using the manufacturer's instructions or using the $CD4^-$ fraction from the L3T4 microbeads. These cells were then irradiated (3,000 rads). All cell counts were performed on a hemacytometer using trypan blue to exclude dead cells from the counts before culture.

B Cell Ex Vivo Antibody Production

Naïve B cells were isolated using anti-CD19-PE and CD138-PE (BD). B cells were then enriched by anti-PE micro beads (Milyenyi Biotech). Purity was verified by FACS and found to be >95% B cells. 5×10^4 isolated B cells from sham or septic mice were cultured for 5 days in RPMI media containing 10% FBS and 1% Penicillin/streptomycin with CpG1829 (Coley Pharmaceuticals) (1 μg/ml), anti-CD40L (1 μg/ml), and IL-4 (10 ng/ml) (BD). At the end of the culture, the cell free supernatant was obtained and an ELISA was performed to determine total IgM and IgG.

Proliferation Assay

After purification and washing, a proliferation assay was developed to test the capacity of total $CD4^+$ T cells or $CD4^+CD25^-$ T effector cells (Teff) to proliferate to anti-CD3 or specific peptide stimulation. T cells (2.5×10^4 cells/well) were cultured with irradiated APCs (2.5×10^5 cells/well) in at least triplicate. 2.5 μg/ml soluble anti-CD3 or 5 μg/ml $Ova_{323-339}$ provided the stimulus for proliferation over a 48-72 hr culture period in a total volume of 200 μl of complete RPMI media. 20 μl of media was harvested from each well for cytokine determination after 24 hours. Then, 1 μCi ^{3}H-thymidine (Amersham Biosciences, Piscataway, NJ) was added for the final 14-18 h of culture to assess proliferation. In preliminary studies, a Treg:Teff ratio of at least

1:2 was necessary to develop suppression of Teff cell responses, so depletion of $CD25^+$ T cells (~7-15% of all CD4 cells) from the total CD4 pool did not affect to a significant extent proliferation of either sham or septic mice. For this reason, the first proliferation experiment contains CD25-depleted $CD4^+$ T cells and the remaining experiments use total $CD4^+$ T cells in the proliferation studies.

In Vivo Expansion of Antigen Specific T Cells

5×10^6 $CD4^+$ T cells isolated by positive selection from the spleens of DO11.10 Tg mice were transferred via tail vein into BALB/c mice 3 days before sham or CLP surgery. Immediately following surgery, mice were immunized subcutaneously with Ova peptide (100 μg) immersed in alum, and five days later were euthanized and peripheral lymph nodes were harvested for flow cytometry to determine the expansion of KJI-26^+CD4^+ T cells in the lymph nodes.

Multiplex Cytokine Analysis

Assessments of cytokine profiles from the proliferation assay were performed using a commercially available multiplexed kit (Beadlyte Mouse Multi-Cytokine Detection System 2; Upstate Biotechnology, Waltham, VA) and the Luminex (100) LabMAP System (Austin, TX). Simultaneous measurement of 10 cytokines was performed: specifically IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), (tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte moncyte-colony stimulating factor (GM-CSF). All assays were performed according to the manufacturer's protocols. Samples from at least three replicate wells were then diluted 1:2 in tissue culture media before analysis. Cytokine concentrations were determined utilizing SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) with four-parameter data analysis.

Statistics

Differences in survival were determined by the Fisher exact test. Continuous variables were first tested for normality and equality of variances. Differences among groups were evaluated by Student's t-test, or one way ANOVA comparing more than two groups. If differences were found between multiple groups, t-tests were performed between groups groups of interest. Significance was determined at the 95% confidence level.

Results

Sepsis Diminishes T Cell Dependent Antibody Responses

In a study by Maclean et al, it was found that septic patients and trauma patients that progressed to sepsis displayed an inability to develop delayed type hypersensitivity responses (63). We initially used a standard model system whereby we can test whether an adaptive immune responses can be developed in septic mice by immunizing mice subcutaneously with the T cell dependent antigen NP-KLH mixed with alum, and examining antigen specific antibody production. Not surprisingly, septic mice displayed a decreased ability to produce NP-specific IgM and IgG2a antibodies, whereas IgG1 antibody responses (the predominant isotype of IgG antibodies formed when alum is used as an adjuvant) were largely intact (Figure 1). Total IgG was more variable where, in some experiments, total IgG was decreased significantly (Figure 1), whereas in other experiments total IgG was not diminished significantly (data not shown). For this reason, we chose to not use total IgG as an endpoint in the remainder of the studies.

B cells Are Not Dysfunctional in Sepsis

Since antibody responses were diminished in sepsis, we first wished to assess whether B cell functionality was altered in sepsis. We found that B cells express increased CD69 expression in multiple tissues, including spleen and bone marrow (Figure 5-2A and 5-2B). We next wished to see whether this increased B cell activation translated into increased antibody production. B

cells were isolated from sham or septic mice and stimulated with CpG, anti-CD40L, and IL-4, and polyclonal antibody secretion was determined in the media. We found that B cells isolated from septic mice produced about 3.5-fold more IgM than B cells isolated from sham mice, indicating that B cell function may not be hindered in sepsis (Figure 5-2C). However, since B cell function may still be hindered in the *in vivo* septic environment, we next assessed B cell function *in vivo* by immunizing mice with the T cell independent antigen NP-Ficoll at the time of CLP or sham surgery. Similar to the in vitro findings, we found that septic mice produced the highest levels of NP-specific antibodies, including increased levels of anti-NP total IgG and IgG3 (Figure 5-3). Taken together, these data suggest that B cells are fully functional, and in fact, may be more activated in sepsis and are likely not the cause of the decreased T cell dependent antibody responses.

Impaired $CD4^+$ T Cell Function in Sepsis

We first examined whether septic mice similarly displayed diminished adaptive immune responses. Similar to Unsinger et al. we found that T cells (both $CD4^+$ and total $CD3^+$ T cells) in spleens of septic mice display increased CD69 expression (Figure 5-4)(124). However, unlike B cells from septic mice, despite their increased activation, $CD4^+CD25^-$ T cells from septic mice proliferated poorly in response to polyclonal anti-CD3 stimulation 24 hours after surgery (Figure 5-5A). When stimulated with PMA and ionomycin, however their proliferative response was largely intact (Figure 5-5B).

Next we determined whether antigen specific $CD4^+$ T cell responses were decreased in sepsis using DO11.10 TCR transgenic mice. In preliminary studies, we found that immunization with Ova immersed in alum causes a more pronounced immunization effect in peripheral lymph nodes of DO11.10 mice than in spleen. For this reason, it was easier to assess the effects of immunization on septic versus sham mice using lymph node T cells rather than spleen cells. We

found that five days after surgery/immunization, DO11.10 lymph node cells $CD4^+$ T cells from septic mice proliferated less (Figure 5-6A), and produced profoundly less IL-2 and IFN-γ than cells from sham mice upon ex vivo restimulation with specific Ova peptide (Figure 5-6B and 5-6C), indicating that both antigen specific and non-specific $CD4^+$ T cell dysfunction occurs in sepsis.

CD3ζ Chain is Down-Regulated in Acute Sepsis

Since TCR mediated signaling is diminished, but responses to PMA/ionomycin were generally unaffected, these findings suggested that downstream signaling pathways are intact, but the likely cause of the T cell dysfunction is anergy. Since CD3ζ chain is the limiting factor in TCR assembly and expression (125), and down-regulation of the CD3ζ chain has been implicated in T cell dysfunction and anergy in many chronic inflammatory diseases (126-128), we wished to assess whether acute inflammation and free radical production associated with the sepsis induced inflammatory response could cause down-regulation of CD3ζ. Indeed, we found using two different clones of anti-CD3ζ antibody that 24 hours after sepsis initiation, there was a decrease in CD3ζ expression, with no decrease in CD3ε expression in both spleen and peripheral lymph nodes (Figure 5-7, and data not shown).

In a recent study, treatment with N-acetyl L-cysteine (NAC) was able to prevent oxidant stress induced down-regulation of CD3ζ (129).We assessed whether free radicals participate in the decreased CD3ζ expression caused by acute sepsis by treating septic mice with three doses of the antioxidant NAC (100 mg/kg) in normal saline. The first was administered intraperitoneally 30 minutes before surgery, followed by two doses given subcutaneously; one was given immediately after surgery and the final dose was given 4 hours after surgery. Sham and CLP control mice received the same volumes of normal saline at the indicated time points.

Importantly, NAC treatment increased CD3ζ chain expression in the spleen by 60% and in the peripheral lymph nodes by 80% (Figure 5-8A and 5-8B). However, NAC treatment did not improve CD3ζ expression in the mesenteric lymph nodes adjacent to the septic process (Figure 5-8C). These data suggest that the sepsis induced CD3ζ expression is partially dependent on free radical production.

Anti-GITR Treatment Restores T Cell Function in Sepsis

Signaling through GITR not only neutralizes the suppressive effect of Treg cells, but also augments activation, proliferation and cytokine production of effector T cells (130). We recently found that $GITR^{high}$ expressing T cells increase in septic compared to sham mice (122). In this study, we examined whether increased GITR signaling on T cells may augment adaptive immune function and prevent the immune suppression that occurs in sepsis. C57Bl/6 mice were injected with 300 μg of anti-GITR agonistic antibody (DTA-1) or a control antibody intraperitoneally 30 minutes before the initiation of sepsis. The mice were immunized with NP-KLH and alum at the time of sepsis and humoral immune function was determined by meausuring NP-specific antibody production. We found that anti-GITR antibody treatment did not improve anti-NP IgM production (Figure 5-9A), but caused a dramatic improvement in IgG2a (Figure 5-9B), and also increased IgG1 (Figure 5-9C), indicating that anti-GITR treatment improves class-switching to both Th1 and Th2 type immune responses.

Since T cell dependent antibody production was improved in septic mice treated with anti-GITR, we next assessed the effects of anti-GITR treatment on early T cell proliferation. Interestingly, 24 hours after sepsis, there was a marked improvement of septic T cells to proliferate to polyclonal stimulus when the mice were treated with an anti-GITR antibody but not the control antibody (Figure 5-10). In fact, early $CD4^+$ proliferation was improved over sham

levels (Figure 5-10). in mice treated with anti-GITR. To test whether antigen specific $CD4^+$ T cell function was also improved, two experiments were performed. First, DO11.10 mice treated with anti-GITR or control antibody were immunized at the time of sepsis with 100 μg NP-Ova in alum. Five days later, lymph nodes were harvested and a proliferation assay was performed. We found that $CD4^+$ T cells from anti-GITR treated mice demonstrated an increase in proliferation comparable to the levels seen in sham mice (Figure 5-11A). Since we also found that the number of $CD4^+$ T cells in the lymph nodes were higher in anti-GITR treated septic DO11.10 mice, we wished to examine whether in vivo expansion of antigen specific T cells was likewise improved by anti-GITR treatment. DO11.10 T cells were transferred into BALB/c mice 3 days prior to CLP or sham surgery and immunization with NP-Ova. Following 7 days, lymph nodes were harvested and the numbers of $CD3^+CD4^+$ T cells bearing the transgenic KJI-26 receptor were analyzed by flow cytometry. We found that anti-GITR treatment partially increased the percentage (Figure 5-11B and C) and almost restored the total number (Figure 5-11D) of KJI-26^+CD4^+ T cells to sham levels. This improvement in antigen-specific expansion confirms that anti-GITR can improve antigen specific $CD4^+$ T cell function in vivo, and indicates that the improvement of T cell helper function was likely the reason for the improvement in humoral immunity following immunization in anti-GITR treated septic mice.

Since it appears that anti-GITR treatment can break sepsis-induced anergy, we next assessed whether CD3ζ chain expression was increased by anti-GITR treatment. However, we found an expansion of immature myeloid cells in septic peripheral lymphoid organs beginning at day 5, which likely could affect the expression of CD3ζ chain (submitted, and (131)). We therefore chose to analyze the effects of anti-GITR treatment on CD3ζ chain expression in mice early after the septic insult and not employ the antigen specific T cell model. We found that anti-

GITR treatment restored CD3ζ chain expression on $CD4^+$ T cells in the spleen (~50%) and peripheral lymph nodes (~90%), but not in mesenteric lymph nodes (Figure 5-11). These data suggest that anti-GITR reversal of T cell anergy is associated with a preservation of CD3ζ chain expression.

Anti-GITR Treatment Improves Survival in Sepsis

To determine whether anti-GITR enhancement of T cell function results in improved outcome during sepsis, mice were again injected intraperitoneally with anti-GITR or control antibody 30 minutes before CLP procedure and survival was monitored for 10 days. Agonistic GITR antibody improved survival to CLP-induced sepsis by 26% ($p = 0.002$ by Fisher Exact; Fig. 5-11A), indicating that augmenting adaptive immune system function can improve outcome in sepsis.

Although predominantly T cell subsets including $CD4^+$, $CD8^+$, and NK T cells are activated by GITR signaling, other cells such as peritoneal and lung macrophages can also express GITR (130). To determine whether $CD4^+$ T cells are the source of the improvement in survival by anti-GITR treatment, we depleted $CD4^+$ T cells by an intraperitoneal injection of 500 μg anti-CD4 depleting antibody (GK1.5) 3 days before sepsis initiation followed by another dose of 250 μg of the same antibody at the time of anti-GITR treatment. We have previously shown that this treatment regimen depletes $CD4^+$ T cells to greater than 95% and that it does not affect mortality in the CLP model. We found that although anti-GITR treatment again improved survival in mice by 25% over control antibody, mice depleted of $CD4^+$ T cells showed no improvement in survival ($p=0.0407$ by Fisher Exact anti-GITR vs CD4-depleted anti-GITR; Figure 5-11B). This demonstrates that modulation of $CD4^+$ cells, and not another cell type, is the mechanism of anti-GITR improvement in survival.

Discussion

An important goal in sepsis research is to identify novel therapeutic targets, which will be critically dependent on an improved understanding of sepsis pathology.

Using the CLP model of polymicrobial sepsis, we found that sepsis-induced humoral immune dysfunction is not caused by B cell dysfunction, as these cells were activated and fully capable of mounting a T cell-independent antibody response. Not surprisingly, as T cell dysfunction in sepsis has been known for several decades, we found that sepsis-induced humoral immune dysfunction is contingent on dysfunctional $CD4^+$ T cells, as antigen specific $CD4^+$ T cells in immunized septic mice demonstrate impaired *in vivo* expansion when compared to immunized sham mice, produce less IL-2 and IFN-γ, and proliferate poorly upon antigenic restimulation 5 days after immunization.

Although T cell dysfunction has been known in sepsis, much of the attention has been focused on the inability to produce Th1 cytokines and the propensity to produce Th2 type cytokines, and the mechanism of T cell dysfunction has not been fully elucidated. In this study, we demonstrate that as early as 24 hours after sepsis initiation, T cells from septic mice have an anergic phenotype, similar to that seen in patients suffering from intraabdominal sepsis (66), characterized by the inability to proliferate to antigen specific or non-specific TCR mediated stimulation and a failure to produce IL-2 and IFN-γ. We also found that septic T cells were able to proliferate to mitogenic stimulation with PMA/Ionomycin treatment, indicating that the defect is most likely with TCR stimulation.

Importantly, T cells from both the spleen and lymph nodes demonstrated a considerable reduction in CD3ζ chain expression, demonstrating a possible mechanism of TCR mediated dysfunction in acute sepsis. CD3ζ is a master regulator of TCR signaling. Loss of CD3ζ

expression has been documented in infectious, inflammatory, autoimmune, and malignant diseases, suggesting that it may serve to limit T cell reactivity and effector responses at sites of tissue damage (132). Under conditions of chronic inflammation or stress, many factors have been attributed to the down-regulation of CD3ζ chain including TNF-α (133), oxidative stress (129), arginine depletion (134), and myeloid suppressor cells (135). Interestingly, acute exposure to agents that cause oxidative stress can also decrease CD3ζ chain expression in T cells (129), as can neutrophilic cells that are acutely recruited into the spleen by traumautic injury (136). This led us to believe that the "cytokine storm" and the myeloid derived oxidative burst that is a common response to acute sepsis or the systemic inflammatory response may also cause T cell anergy, and CD3ζ chain down-regulation possibly as a self-regulating immunomodulatory mechanism.

Two major types of immune dysfunction in sepsis are the loss of lymphocytes and DCs (by both apoptotic and non apoptotic means) and anergy/hyporesponsiveness of the cells that are not lost (3). Although many studies have found that inhibition of apoptotic loss of lymphocytes improves outcome in sepsis, not many studies have concentrated on preventing anergy as a mechanism of improving outcome. It was previously found that treating a subset of septic patients with poor monocyte function with IFN-γ reversed the monocyte dysfunction and improved outcome (65). However, cytokine and anti-cytokine therapies have been proven limited in efficacy in sepsis (3, 21). This study addressed whether improving T cell function by increasing signaling through GITR could alter outcome in sepsis. We recently found that the expression of $CD4^{+}CD25^{+}GITR^{high}$ increases on T cells of septic mice, as do other markers of T cell activation. In this study we found that pretreatment of mice with a GITR agonistic antibody, was able to restore CD3ζ expression, T cell proliferation, and humoral immune responses in

septic mice, thus breaking T cell anergy. Although GITR is found at high levels on $CD4^+CD25^+$ regulatory T cells, GITR expression is also found at lower levels on T effector cells, and also increases on T cells following activation. GITR signaling has a dual effect on T cells whereby it can abrogate Treg mediated suppression as well as costimulate T effector cell activity (54, 55). The beneficial effects of anti-GITR treatment on T cell function also led to a $CD4^+$ T cell dependent improvement in survival, as the improvement was abolished in mice that were previously depleted of $CD4^+$ T cells. The implications of these results are two-fold. It suggests that T helper cell dysfunction in sepsis is detrimental for host survival, and correction of this dysfunction with T cell specific therapies may lead to improved outcome in sepsis.

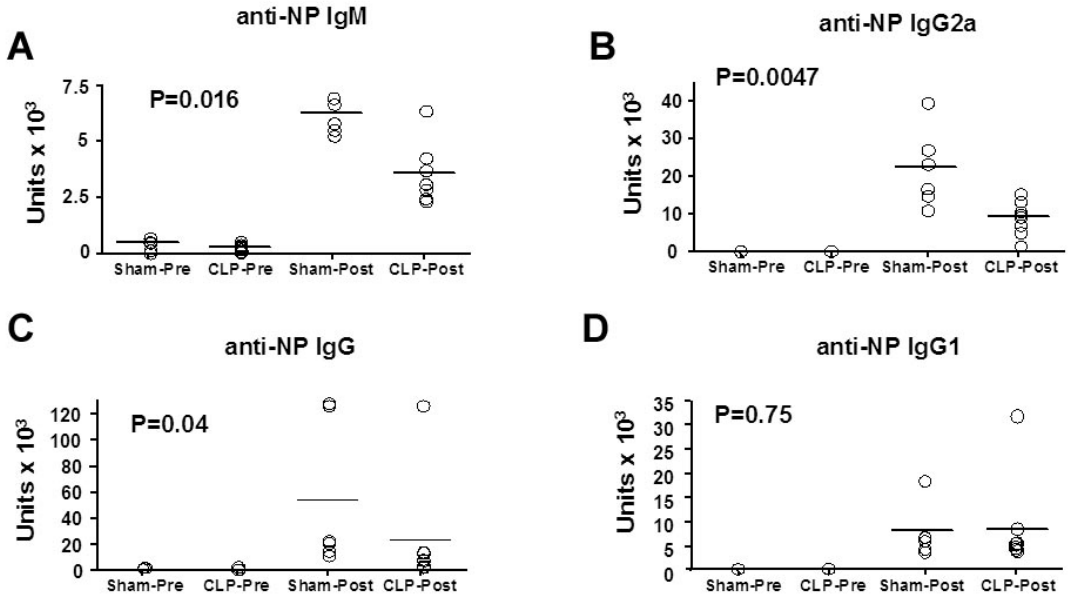

Figure 5-1. T cell dependent antibody responses are decreased in septic mice. C57Bl/6 mice were prebled, underwent sham or CLP (CLP) surgery, and were immunized subcutaneously with the T cell dependent antigen NP-KLH and alum. 10 days later, A) IgM, B) IgG2a, C) total IgG, and D) IgG1 anti-NP antibody responses NP were measured by ELISA. P values indicate the difference between sham and CLP mice groups post immunization by students T test.

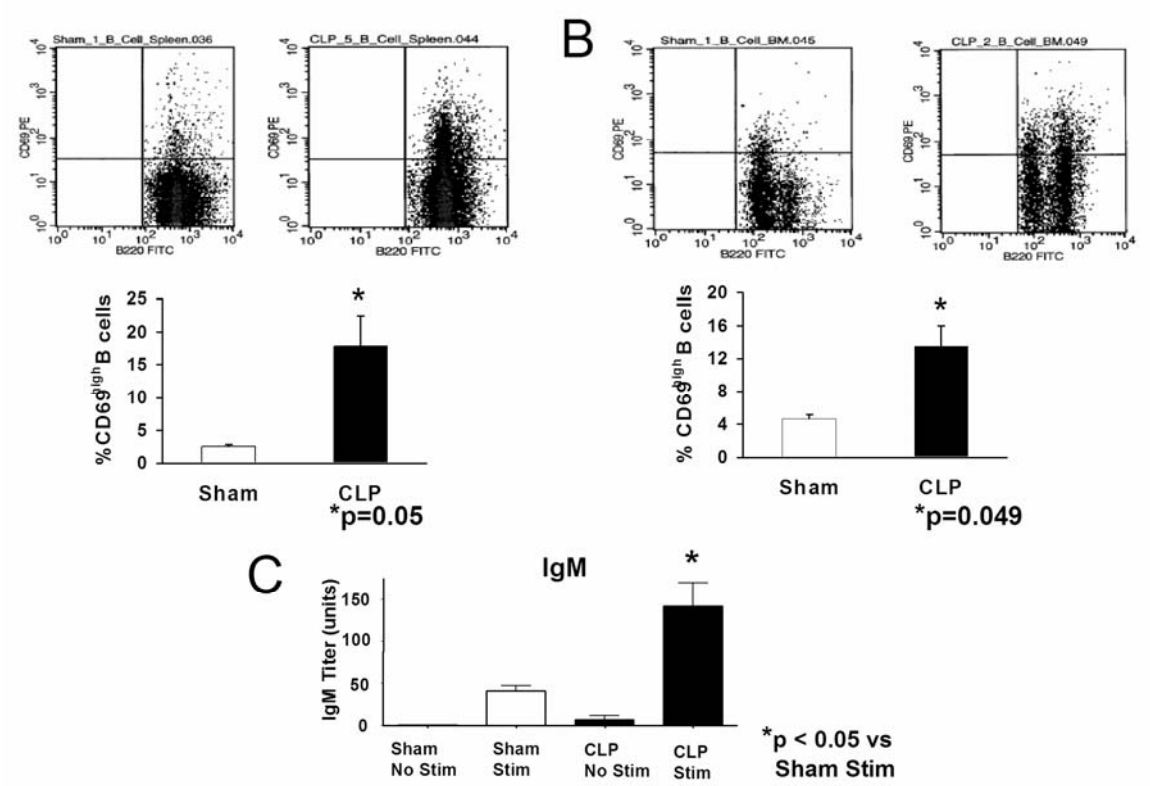

Figure 5-2. B cells display an activated phenotype in sepsis. CLP (CLP) or sham surgery was performed on C57Bl/6 mice and 24 hours later splenocytes were harvested for flow cytometry and cell culture and bone marrow cells were harvested for flow cytometry. A) Top panels show a representative example of B220 (x-axis) and CD69 (y-axis) from splenocytes from a sham (left) and a CLP (right) mouse. The percentage of B cells in the upper right quadrant was used for calculations in the bottom panel (n=3 mice). B) Top panels show a representative example of B220 (x-axis) and CD69 (y-axis) from bone marrow cells from a sham (left) and a CLP (right) mouse. The percentage of B cells in the upper right quadrant was used for calculations in the bottom panel (n=3 mice). C) $1x10^4$ B cells from sham and CLP mice separated using anti-CD19 APC antibody and anti-APC microbeads were cultured in complete RPMI media containing CpG, anti-CD40L, and IL-4 for 5 days. Media was collected and assayed for total IgM by ELISA. Results from one experiment (n=3 per each group) are shown. * $p<0.05$ by student t-test sham versus CLP.

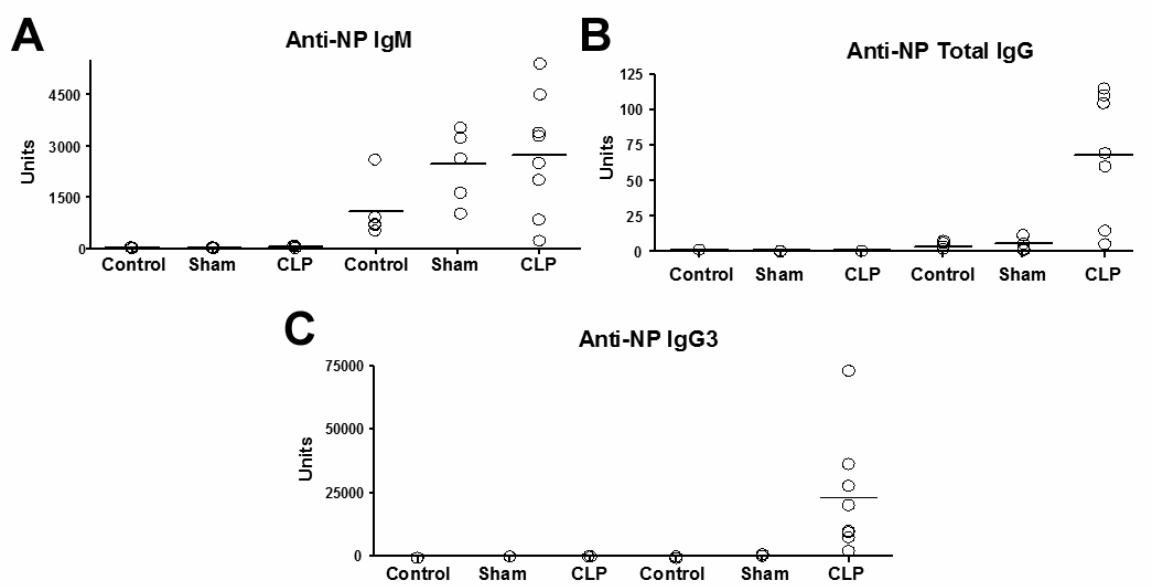

Figure 5-3. T cell independent antibody response is intact in septic mice. C57Bl/6 mice underwent sham or CLP (CLP) surgery and immediately after were immunized subcutaneously with the T cell independent antigen NP-Ficoll in normal saline. Seven days later mice were bled and anti-NP IgM (A), total IgG (B), and IgG3 (C) were measured by ELISA.

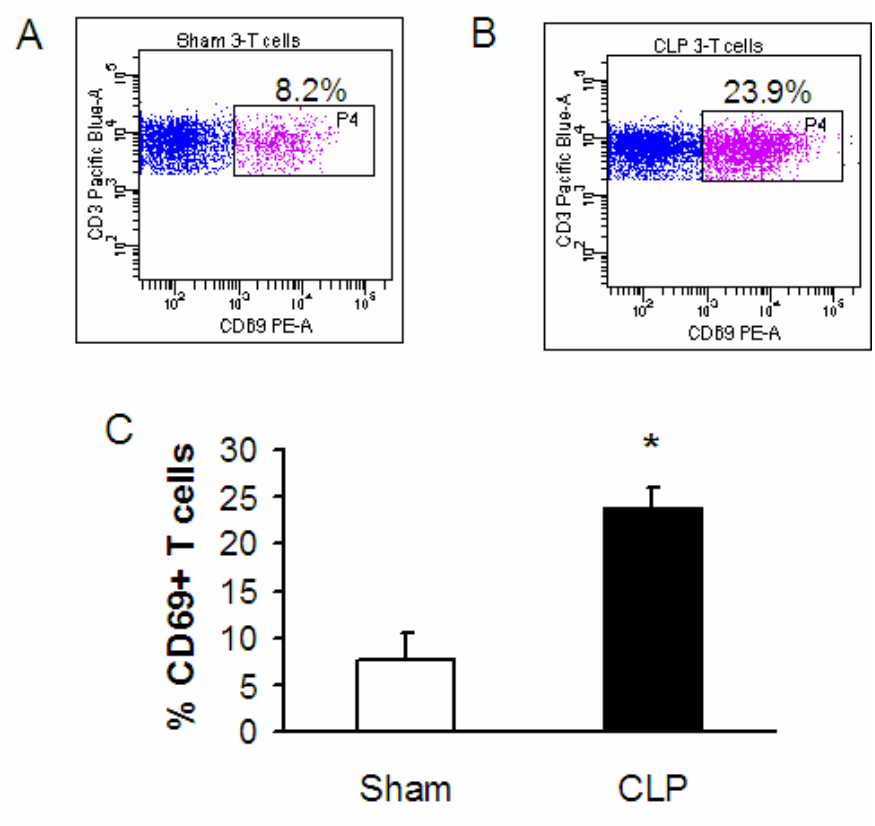

Figure 5-4. T cells from septic mice display increased CD69 expression. 24 hours after CLP or sham surgery, splenocytes were harvested and flow cytometry was performed. Representative examples of non-debris, living (7-AAD$^-$) cells examining CD69 on the x-axis and CD3 on the y-axis from one sham (A) and one CLP (B) mouse. C) The average percentage of CD69 expressing CD3+ T cells in P4 were calculated from sham (n=3) and CLP (n=5) mice. * $p < 0.05$ by student t test.

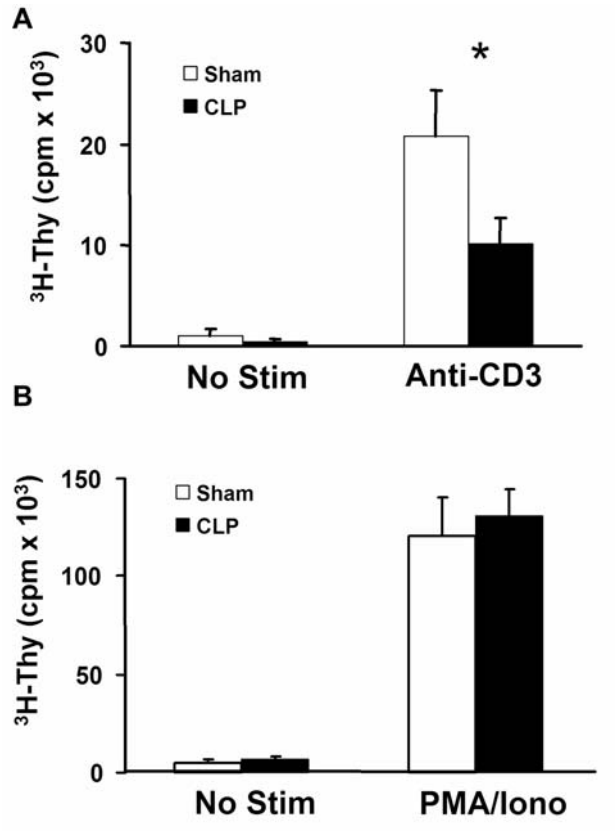

Figure 5-5. T cells from septic mice proliferate poorly to TCR stimulation. 24 hours after CLP (CLP) or sham treatment, CD25-depleted $CD4^+$ T effector cells were harvested as described in the Materials and Methods. $2.5x10^4$ T effector cells were cultured for 72 hours with either anti-CD3 (A) or PMA and ionomycin (B). ^{3}H-Thymidine was added during the last 14-18 hrs of culture and incorporation was measured using a liquid scintillation counter. *$p<0.05$ by student's t test.

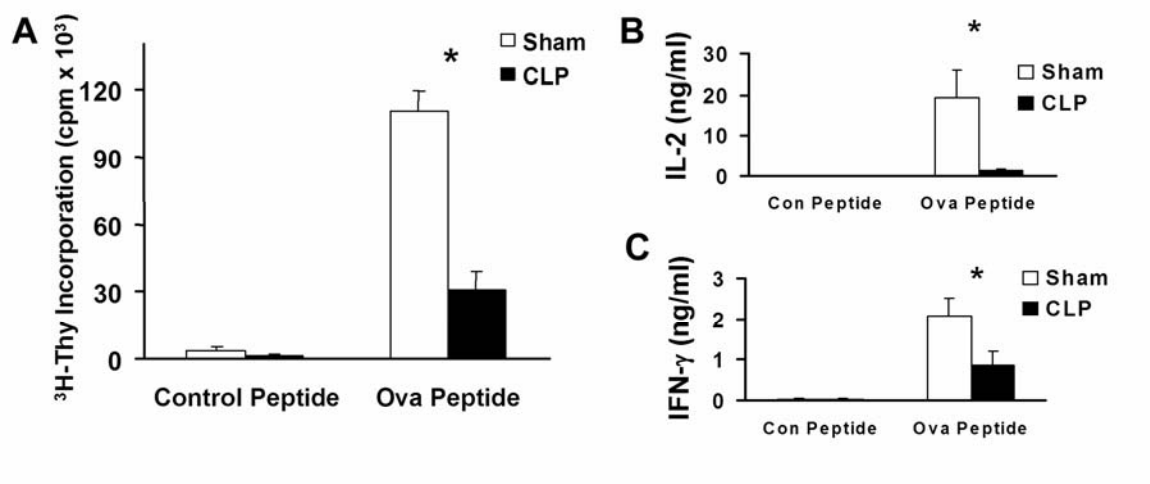

Figure 5-6. Sepsis reduces antigen specific T cell function. DO11.10 T cell receptor transgenic mice underwent CLP or sham surgery and were immunized with $Ova_{323-339}$ peptide subcutaneously in alum. 5 days later, peripheral lymph nodes were harvested and $CD4^+$ T cells and $CD4^-$ APCs were collected for culture. Proliferation assays were undertaken to determine whether $CD4^+$ T cells from septic mice able to proliferate to antigen specific restimulation. A) Equal numbers of $CD4^+$ T cells (2.5×10^4) from septic or sham-treated mice were cultured alone or together for 48 hours with irradiated antigen presenting cells (2.5×10^5; 3000 rads), with or without Ova-peptide (5 μg/ml) for stimulation and proliferation was measured using ^{3}H-thymidine uptake. At 30 hours, 25 μl of media was harvested to perform Luminex-based multiplex cytokine analysis. Only IL-2 (B) and IFN-γ (C) were different between the two groups. * $p<0.05$ by student's t test between sham and CLP mice post-stimulation.

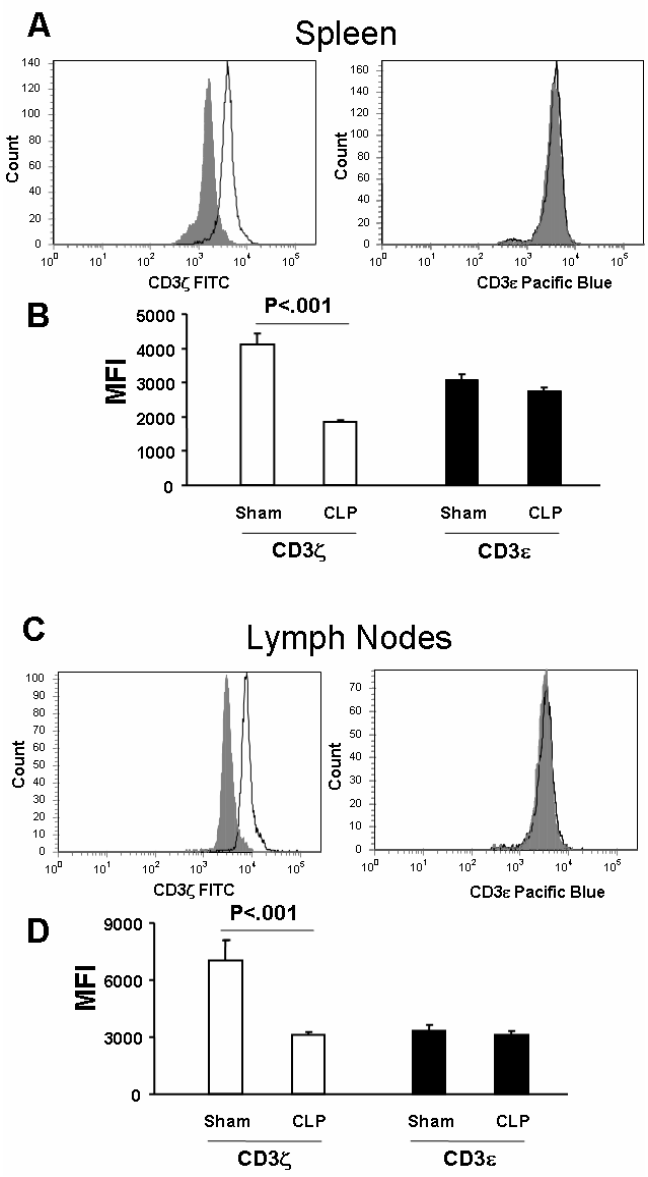

Figure 5-7. Decreased CD3ζ chain expression but not CD3ε expression in septic T cells. C57Bl/6 mice were subjected to CLP (CLP) or sham surgery and spleens and lymph nodes were harvested 24 hours later. Cells were stained for flow cytometry extracellularly using anti-CD3ε Pacific Blue, anti-CD4 APC, anti-CD8 APC-Cy7, and anti-CD69 PE. Cells were then fixed and permeabilized as described in Materials and Methods and stained for anti-CD3ζ FITC. Histogram overlays showing A) CD3ζ (left panel) and CD3ε (right panel) expression from splenic $CD4^+$ T cells of one sham (no fill) versus one CLP (gray fill), and B) shows the calculated mean fluorescent intensity (MFI) from n=4 mice per group. Similarly C) shows CD3ζ (left panel) and CD3ε (right panel) expression from lymph node $CD4^+$ T cells of one sham (no fill) versus one CLP (gray fill), and D) shows the calculated mean fluorescent intensity (MFI) from n=4 mice per group. Histogram overlays were made using FCS Express software.

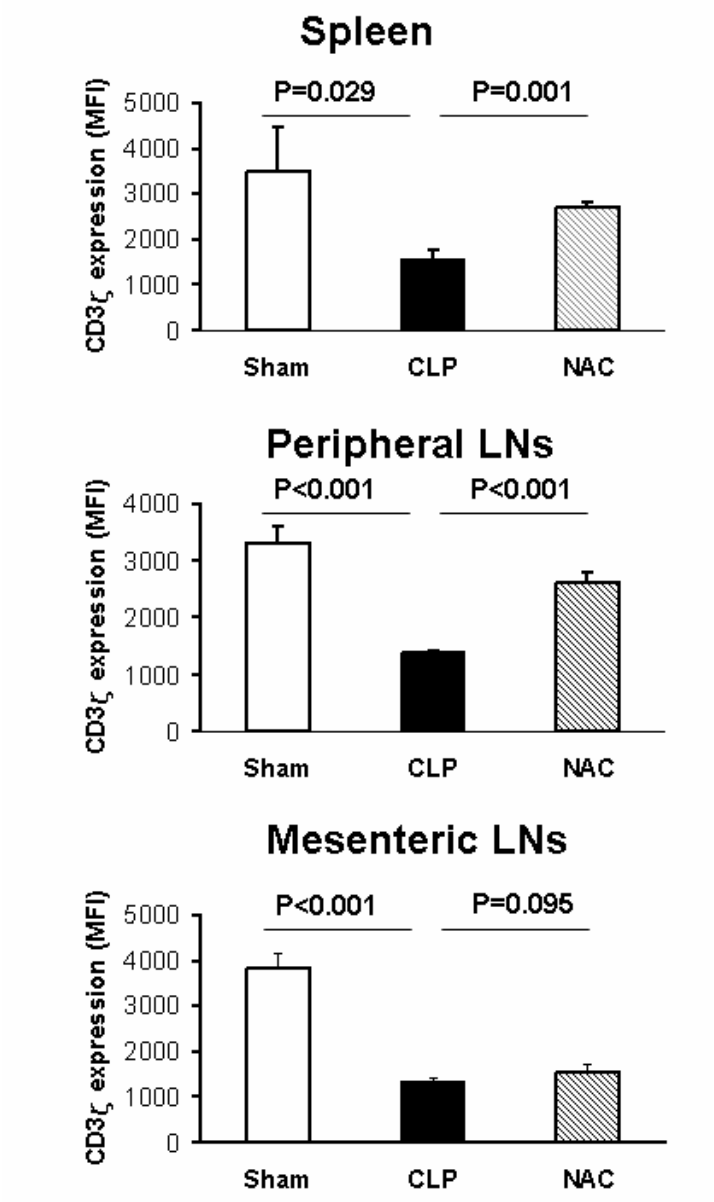

Figure 5-8. Decreased CD3ζ chain expression in sepsis is partially dependent on free radicals. C57Bl/6 mice were subjected to CLP (CLP) or sham surgery. One group of CLP mice received 3 doses of 100mg/kg N-acetyl L-cysteine (NAC). Spleens and lymph nodes were harvested 24 hours later. Cells were stained for flow cytometry extracellularly using anti-CD3ε Pacific Blue, anti-CD4 APC, anti-CD8 PerCP. Cells were then fixed and permeabilized as described in Materials and Methods and stained for anti-CD3ζ FITC. The top panel shows the calculated mean fluorescent intensity (MFI) for CD3ζ-FITC from spleens n=3 mice per group. Similarly the middle panel shows the calculated MFI for CD3ζ-FITC expression from peripheral lymph node (axillary and inguinal) $CD4^+$ T cells while the bottom panel shows the calculated MFI for CD3ζ-FITC expression from mesenteric lymph node $CD4^+$ T cells from the same mice.

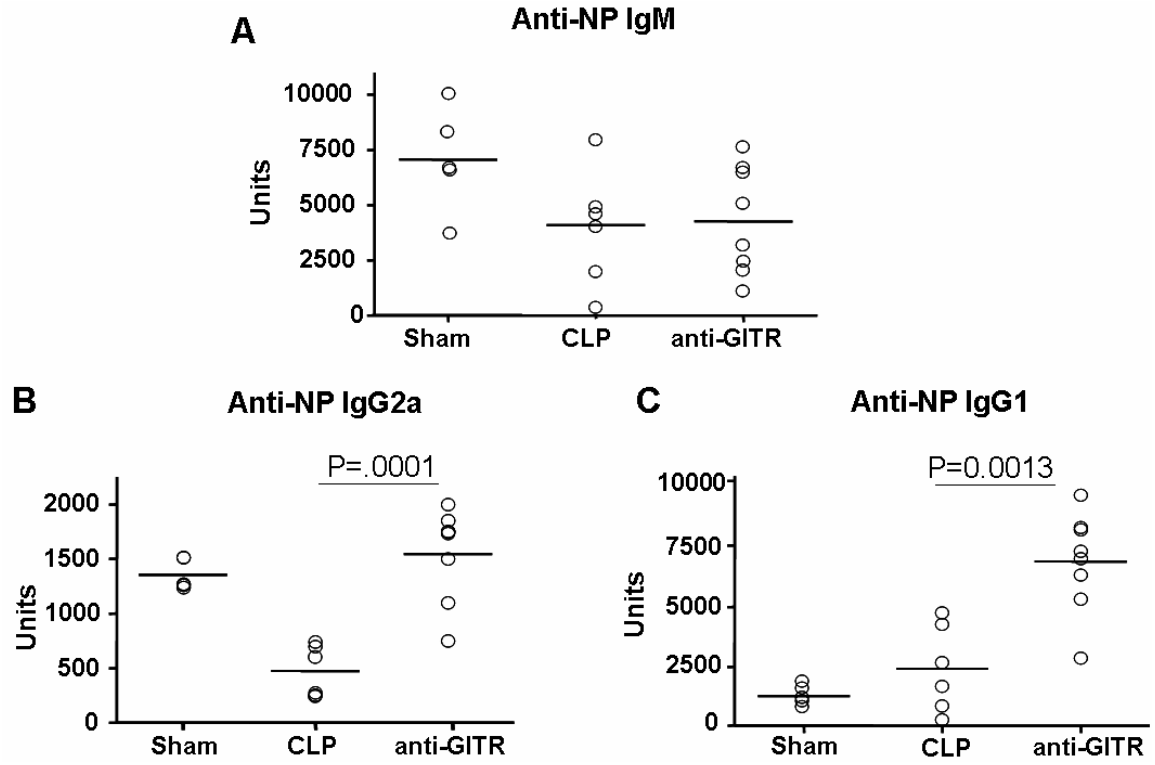

Figure 5-9. Anti-GITR agonistic antibody improves NP-specific class switching in septic mice. C57Bl/6 mice were given an intraperitoneal injection of 300 μg control antibody (Sham and CLP groups) or anti-GITR antibody and underwent CLP or sham surgery 30 minutes later. Immediately following, mice were immunized with NP-KLH and alum. 10 days later mice were bled and anti-NP specific IgM (A), IgG2a (B), and IgG1 (C) were measured from the serum.

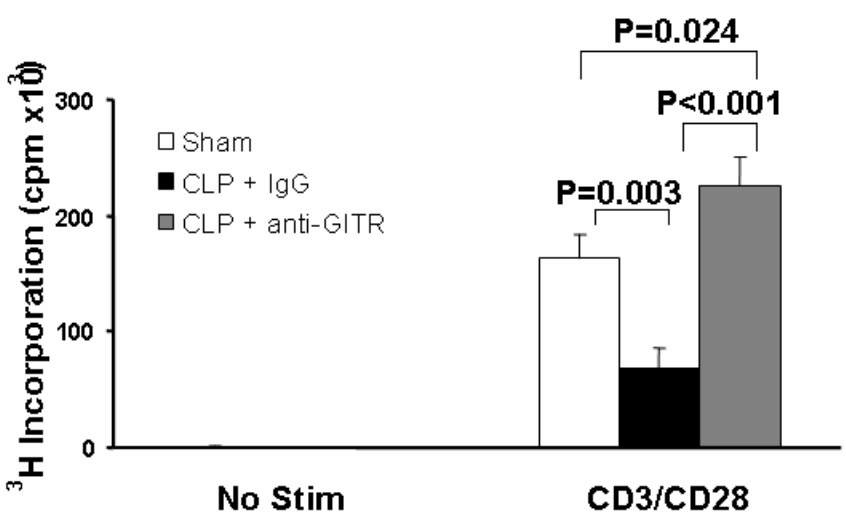

Figure 5-10. Treatment with anti-GITR improves early $CD4^+$ T cell proliferation to a polyclonal stimulus A) C57Bl/6 mice were given 300 μg anti-GITR or control antibody at the time of CLP or sham surgery. 24 hours later spleen cells were harvested and 2.5 x 10^4 $CD4^+$ cells were cultured with 2.5 x 10^5 irradiated APCs with anti-CD3 (2.5 μg/ml) peptide for 72 hours. Proliferation of $CD4^+$ T cells was measured as the incorporation of ^{3}H-Thymidine during the last 18 hours of culture.

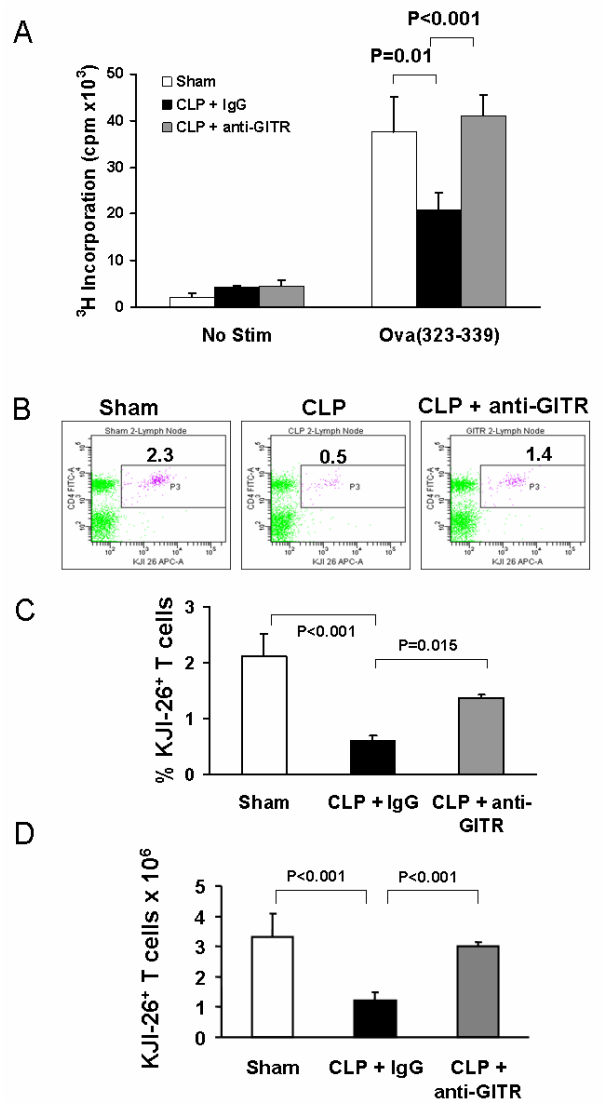

Figure 5-11. Treatment with anti-GITR improves antigen specific $CD4^+$ T cell function in vitro and in vivo. A) DO11.10 mice were given 300 µg anti-GITR or control antibody and were immunized 30 minutes later with $Ova_{323-339}$ in alum at the time of CLP or sham surgery. 5 days later lymph node cells were harvested and 2.5 x 10^4 $CD4^+$ cells were cultured with 2.5 x 10^5 irradiated APCs with Ova peptide for 72 hours. Proliferation of $CD4^+$ T cells was measured as the incorporation of ^{3}H-Thymidine during the last 18 hours of culture. B) Representative examples of flow plots demonstrating the expansion of DO11.10 T cells ($KJI\text{-}26^+CD4^+$) from lymph nodes of BALB/c mice that had 5 x 10^6 DO11.10 $CD4^+$ T cells injected intravenously 3 days before sham (left), CLP with control antibody (middle), or CLP with anti-GITR antibody (right) treatment at the time of immunization with $Ova_{323-337}$ peptide in alum. C) The calculated percentage of living ($Sytox\ Blue^-$), non-debris DO11.10 T cells (KJI^+CD4^+) expanded in the peripheral lymph nodes of the BALB/c mice. D) The calculated total number of live ($Sytox\ Blue^-$), non debris DO11.10 T cells after cell counts were performed with a hemacytometer.

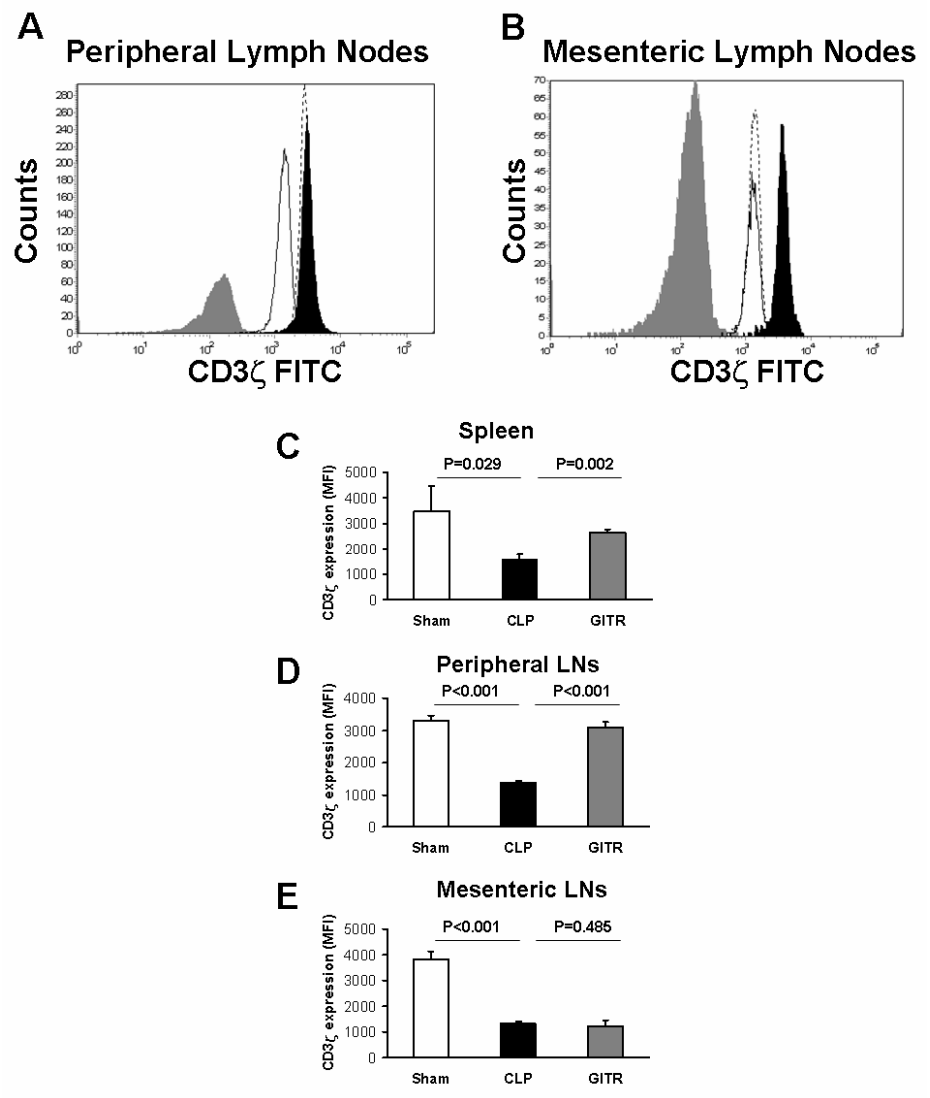

Figure 5-12. Anti-GITR treatment restores CD3z expression in septic mice. C57Bl/6 mice were subjected to CLP (CLP) or sham surgery. One group of CLP mice received 300 μg anti-GITR antibody while the other received 300 μg of control antibody. Spleens and lymph nodes were harvested 24 hours later. Cells were stained for flow cytometry extracellularly using anti-CD3ε Pacific Blue, anti-CD4 APC, and anti-CD8 PerCP. Cells were then fixed and permeabilized as described in Materials and Methods and stained for anti-CD3ζ FITC. Histogram overlays showing CD3ζ from $CD4^+$ T cells of one sham mouse (filled black) versus one control antibody treated CLP mouse (solid black line, no fill) versus one anti-GITR treated CLP (dotted line, no fill) and an isotype control antibody (filled grey) from A) peripheral lymph nodes or B) mesenteric lymph nodes. The calculated mean fluorescent intensity (MFI) from C) spleen, D) peripheral lymph nodes, and E) mesenteric lymph nodes from n=3 mice per group. Histogram overlays were made using FCS Express software.

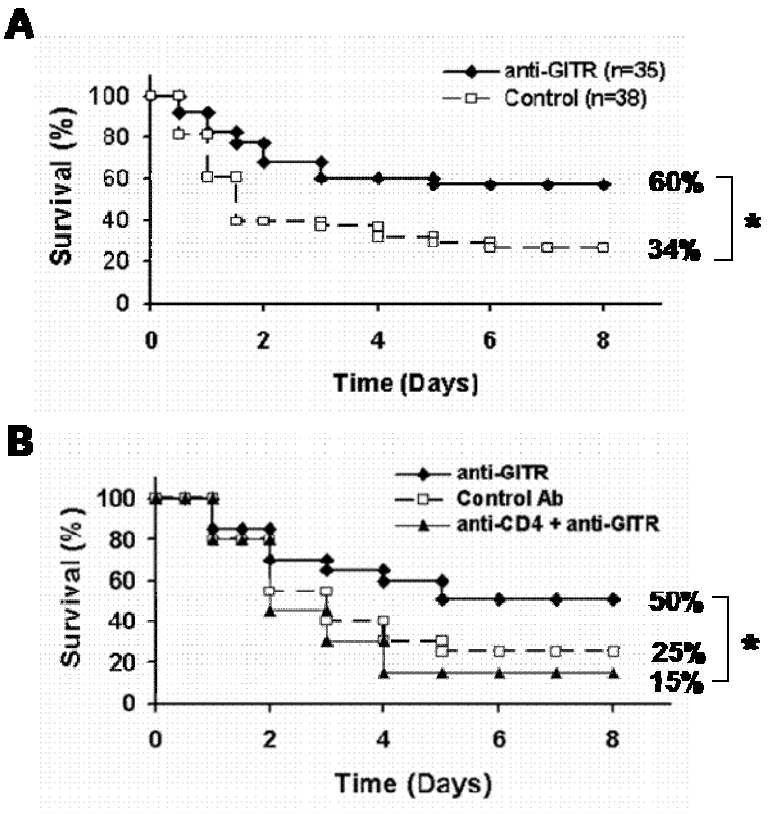

Figure 5-13. Anti-GITR agonistic antibody improvement of sepsis survival depends on $CD4^+$ T cells. A) C57Bl/6 mice were given an intraperitoneal injection of 300 μg anti-GITR or control antibody 30 minutes before CLP surgery and survival was monitored for 10 days. No mortality occurred after Day 5 of sepsis. B) One group of C57Bl/6 mice was given an injection of 500 μg of depleting anti-CD4 (GK1.5) antibody 3 days before CLP surgery and another injection of 250 μg at the time of anti-GITR treatment. The other groups remained the same as in A).

CHAPTER 6
FUTURE WORK, DISCUSSION AND CONCLUSIONS

Future Work

Mechanism of Immune Cell Activation in Sepsis

One question that naturally arises from these studies is: what are the mechanisms of immune activation and immune dysfunction in sepsis? Although we have begun work in this area, no clear answer exists at this time. As we showed in Chapter 1, DC activation occurs in the absence of Type I IFN, MyD88, or TRIF mediated signaling (as well as other, more downstream signaling pathways). Similar to these results, septic IFNAR and wild type SvEv mice demonstrate comparably increased T cell activation levels (both ~2-fold activation), although IFNAR mice start with a lower baseline level of T cell activation (6-1A). However, B lymphocyte activation is dramatically hindered in IFNAR$^{-/-}$ mice (2-fold activation) versus SvEv mice (6-fold activation) (Figure 6-1B). On the contrary, septic MyD88$^{-/-}$ mice demonstrate the same level of B cell activation and the same increase in IgG3 responses to the T cell-independent antigen, NP-Ficoll, as septic B6.129 mice over their sham counterparts (data not shown). However, the significance of the decreased B cell activation in IFNAR$^{-/-}$ mice could not be fully ascertained using the same NP-Ficoll immunization model as septic mice from both wild type SvEv and IFNAR$^{-/-}$ mice demonstrated decreased IgM and IgG3 production instead of the increase seen in all other mouse strains tested (C57/Bl6, B6.129, and BALB/c). Despite this setback, the importance of Type I IFN in outcome to sepsis cannot be understated as IFNAR$^{-/-}$ mice display worsened survival to a low-lethality CLP model (Figure 6-1C). As Type I IFN has an important priming effect on both innate and adaptive immune responses, this is most likely due to a failure to generate an appropriate immune response to the microbial invasion. Although the decreased B lymphocyte activation in IFNAR$^{-/-}$ mice supports this claim, further studies are

needed to determine whether, in fact, IFNAR$^{-/-}$ mice display decreased bacterial clearance in response to sepsis and/or an exaggerated or reduced inflammatory response.

Mechanism of Adaptive Immune Suppression in Sepsis

Determining the cause of sepsis-induced adaptive immune suppression is another important line of investigation that remains to be explored. Since we demonstrated that correcting adaptive immune dysfunction may represent a potential strategy for improving outcome in sepsis, identifying the cause of this dysfunction may lead to more focused therapies. We hypothesized that some component of sepsis-induced inflammation is probably causing T cell anergy, thus leading to adaptive immune suppression. The first pathways we chose to analyze were MyD88 and TRIF dependent pathways, since MyD88 controls most of the inflammatory responses to sepsis, but TRIF also contributes to the induction of several inflammatory genes. However, these experiments were performed at Rhode Island Hospital, thanks to the help of Dr. Alfred Ayala, and we performed one experiment using a limited number of mice. We immunized C57/Bl6, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice at the time of CLP with NP-KLH and examined their ability to produce NP-specific antibodies. However, unlike the experiments performed at the University of Florida, we did not observe any significant decrease in NP-specific antibody production in the control C57BL/6 mice (data not shown), so the data in the transgenic mice were not interpretable. Many reasons could explain the failed experiment, but the most likely cause was the fact that the CLP was performed using a much less severe model in order to ensure survival of the genetically deficient mice for the full duration of the study. The mice therefore may not have developed sepsis, but rather developed a localized peritonitis. The model may not have been severe enough to develop sepsis, thus leading to the failure of the experiment. These experiments need to be repeated with the model of CLP done during the experiments reported in Chapter 5 of this dissertation that generated immune suppression. Since

the transgenic mice are no longer available to us, these experiments are currently on hold until a breeding colony can be established at the University of Florida.

Next we examined whether individual cells or more downstream pathways contribute to the adaptive immune suppression in sepsis. However, we found that sepsis induced suppression of NP-specific antibody production occurs in the absence of several individual factors including iNOS and depletion of NK cells, and deactivation of NK T cells simultaneously with an anti-asialoGM1 antibody and a CD1d blocking antibody. Interestingly, the role of type I IFN in adaptive immune suppression could not be determined as immunization of septic and sham SvEv and $IFNAR^{-/-}$ mice led to results that were not easy to interpret (Figure 6-2). $IFNAR^{-/-}$ sham mice displayed diminished NP-specific IgM and IgG2a responses compared to their SvEv wild type counterparts, but septic $IFNAR^{-/-}$ mice actually had elevated IgG2a to the levels of sham SvEv mice despite no change in the IgM response. What this data probably suggests is that type I IFN signaling is required for normal T cell dependent antibody responses to develop, but up-regulation of one or more inflammatory molecules during the septic response can probably substitute for lack of T cell help in $IFNAR^{-/-}$ mice. Further studies are clearly required to fully assess the role of type I IFN in sepsis-induced adaptive immune suppression.

Although the studies mentioned above are predominantly focused on major signaling pathways involved in early microbial recognition and inflammation, the role of several, more specific downstream pathways in adaptive immune suppression can also be examined. These include immunomodulatory cytokines and molecules such as IL-10, TNF-α, IL-1, NADPH oxidase-dependent free radicals, stress steroids, and catecholamines. Indeed, free radicals may be potentially of interest, as sepsis-induced down-regulation of CD3ζ expression on T cells was partially abrogated in mice treated with a potent antioxidant. Alternatively, cellular mediators or

disruption of cellular interactions (by apoptosis, loss of cell contact, or increased expression of inhibitory receptors) may be just as important in disrupting T cell function following sepsis as soluble mediators. Although antibody-mediated disruption of NK and NK-T cell function did not affect the sepsis-induced decrease of T cell dependent antibody production, it is possible that other cell types such as neutrophils (through arginase expression and depletion of arginine), monocytes (through nitric oxide or IL-10 production), plasmacytoid DCs (through Type I IFN production) or B cells (through inhibitory receptor expression) may be directly inhibiting T cell function.

Discussion and Conclusions

As our understanding of sepsis continues to improve, an expansion in the potential for novel therapeutics should theoretically occur. However, despite advances in supportive care and the availability of antimicrobial agents, mortality from sepsis has not improved (2). Initially, treatments aimed at curtailing the excessive inflammatory response were considered by many to be the most likely therapeutic candidates to provide survival benefits in septic patients. Over the last fifteen years, numerous clinical trials with anti-inflammatory adjunctive therapies, including anti-endotoxin antibodies (137) and inhibitors of the host inflammatory response including treatment with TNF-α blocking antibodies (7, 73), IL-1 receptor antagonist (8), and glucocorticoids (4) have yielded disappointing results. These failed clinical trials have led us to question the paradigms in sepsis that were once considered dogma, and led us to reassess potential therapeutic targets within the pathophysiological response to sepsis. Given the likelihood that multiple processes are simultaneously activated, and septic individuals may exhibit either features of immune hyper-activation or immunosuppression or both

simultaneously, it is likely that targeted therapies or a combination of therapies may be required to improve outcome.

One theory that has developed to account for this apparent lack of improvement of anti-inflammatory therapies is the SIRS/CARS phenomenon (20). According to proponents of this theory, following the dramatic early inflammatory response that is easily overcome by septic patients, their immune system remains in a state of perpetual hyporesponsiveness, known as CARS, where the host has increased susceptibility to secondary infections that eventually lead to their demise. Although this theory has merit, several flaws remain unanswered. For instance, there is no lag between the appearance of pro-inflammatory cytokines and the induction of anti-inflammatory cytokines (21, 22). Instead, both pro- and anti-inflammatory cytokines are elevated simultaneously. Rather, more likely, a "cytokine storm," is induced at the appearance of microbes in the systemic circulation, and is also associated with an oxidative burst and a dramatic release of vasoactive compounds locally and systemically. As a self-limiting mechanism to prevent further elevations in detrimental systemic inflammatory reactions, this cytokine storm renders many immune cells hyporesponsive or anergic to further stimulation until the primary pathogen can be removed. In support of this theory, anergy has been found in T cells of septic patients (63, 66), and those patients that eventually succumb to sepsis display a more severe anergy then those patients who eventually resolve their infection. In another study, septic patients who presented with poor monocyte function and received IFN-γ had improved monocyte function and an improvement in survival over those patients who had identical supportive care but were not administered IFN-γ (65). This study highlights the importance of immune cell function in sepsis and also demonstrates that targeted therapy in a subpopulation of patients may improve outcome.

In further support of this theory, our model of murine polymicrobial sepsis causes a profound, early inflammatory response characterized by the elevation of numerous pro-inflammatory cytokines and chemokines in the systemic circulation that is **accompanied by** a marked increase of anti-inflammatory cytokines (22, 106). Not surprisingly, there is also a profound early activation of dendritic cells, B cells, and T cells following sepsis. The loss of dendritic cells prior to the initiation of sepsis, a key cell mediating the switch between innate and adaptive immunity, predisposes mice to mortality in sepsis. However, the loss of $CD4^+$ helper T cells, the central regulators of the adaptive immune system, does not alter mortality to sepsis.

Septic mice do display profound adaptive immune dysfunction, as mice that are immunized with T cell dependent antigens during this early phase of sepsis develop poor humoral responses despite normal or even enhanced B cell function in response to T cell independent antigens. We then confirmed that adaptive immune dysfunction during sepsis stems from helper T cells as both antigen specific and non-specific proliferation of helper T cells was diminished in the presence of normal antigen presenting cells. Furthermore, helper T cells from septic mice produced dramatically less antigen specific IL-2 and IFN-γ, two crucial cytokines regulating Th1 cell function, and polarizing adaptive immune responses in general.

Since the endogenous $CD4^+$ population during sepsis does not play a major role in outcome, likely due to an already inherent dysfunction in the $CD4^+$ T cell population produced by sepsis. It is known that an increase in apoptotic death occurs in the $CD4^+$ T cell population during sepsis and contributes to the anergy in the T cell compartment. Because the T cell dysfunction that occurs normally during sepsis yields a similar outcome to when the $CD4^+$ population is completely depleted from the animals before the onset of sepsis, these findings

suggest that preserving or stimulating $CD4^+$ effector T cell numbers and function during sepsis might be appropriate therapeutic targets.

Preserving T effector cell numbers by preventing lymphocyte apoptosis has been shown to be a novel strategy to improve survival in murine sepsis (71, 75, 113). We examined whether improving T cell function can similarly improve outcome. To accomplish this, we used an agonistic antibody against glucocorticoid induced TNF receptor (GITR). GITR is found on low levels in T effector cells and on high levels on regulatory T cells. Stimulation through GITR abrogates regulatory T cell mediated suppression and also acts as an activating/costimulatory molecule on T effector cells. Mice pretreated with the anti-GITR agonistic antibody showed a complete reversal of the antigen specific and non-specific T cell anergy and demonstrated improved humoral immunity. Importantly, breaking T cell anergy with anti-GITR treatment improved survival in mice. As GITR expression is not limited to T cells, we needed to assess whether $CD4^+$ effector T cells were necessary for the anti-GITR mediated improvement in survival. Mice previously depleted of $CD4^+$ T cells demonstrated no anti-GITR mediated survival benefit, highlighting the importance of $CD4^+$ T effector cells to this response.

These results demonstrate the complexity of the interactions of cells within the adaptive immune system, especially as they relate to the pathophysiology of the response to sepsis. They also demonstrate the importance of a fully functional adaptive immune system response to combat a microbial challenge. Most importantly, these results suggest that maintaining adaptive immune system function by preventing T cell anergy may represent an appropriate therapeutic strategy to improve outcome in sepsis.

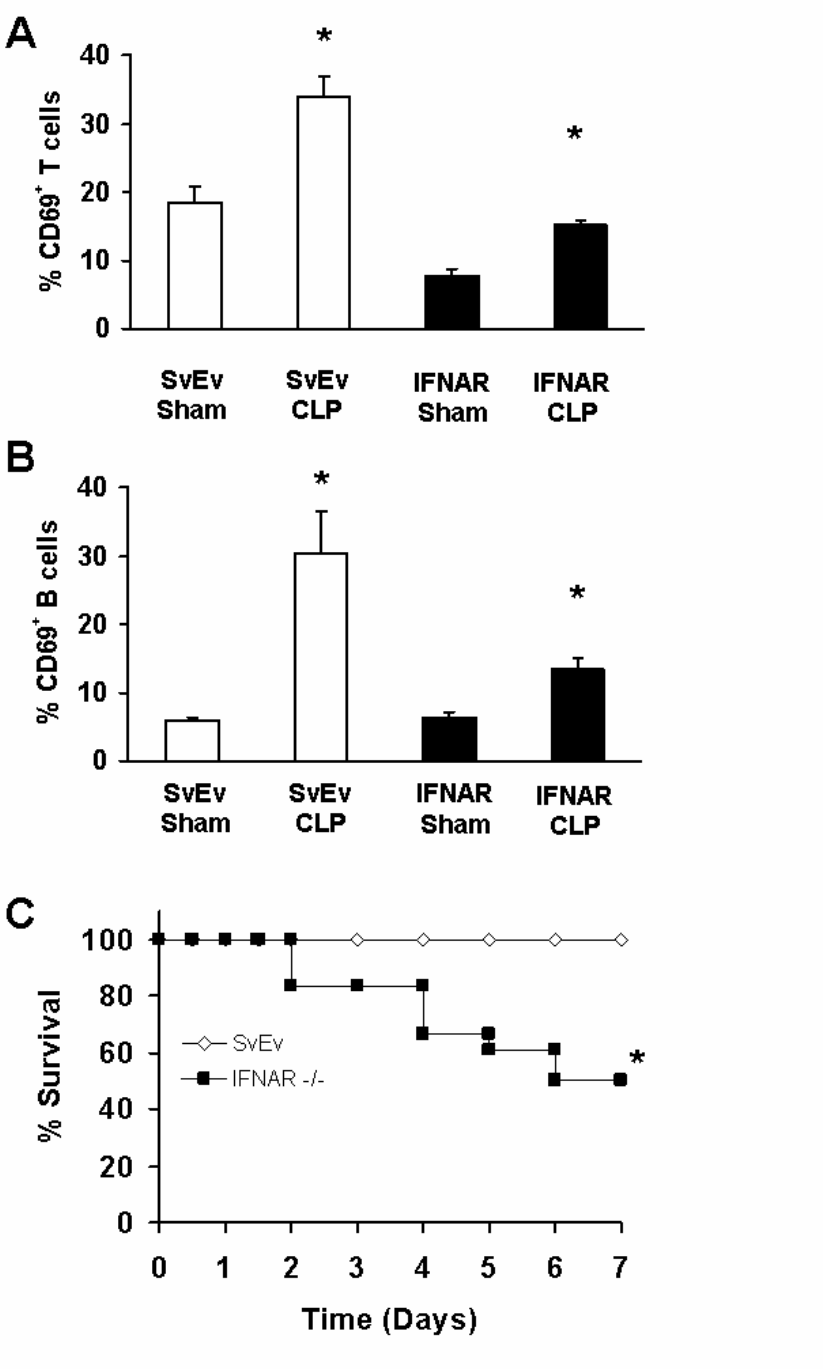

Figure 6-1. Type I IFN is necessary for B cell activation and survival in sepsis. SvEv mice underwent CLP (CLP) or sham surgery and spleens were harvested 24 hours later. Activation of $CD19^{+}B220^{+}$ B cells (A) or $CD3^{+}B220^{-}$ T cells (B) was assessed by the expression of high levels of the marker CD69. Survival was monitored for seven days in $IFNAR^{-/-}$ or SvEv mice (n=18) following CLP. * represents $p<0.05$ versus sham mice (A and B) or versus SvEv mice (C) by student's t test.

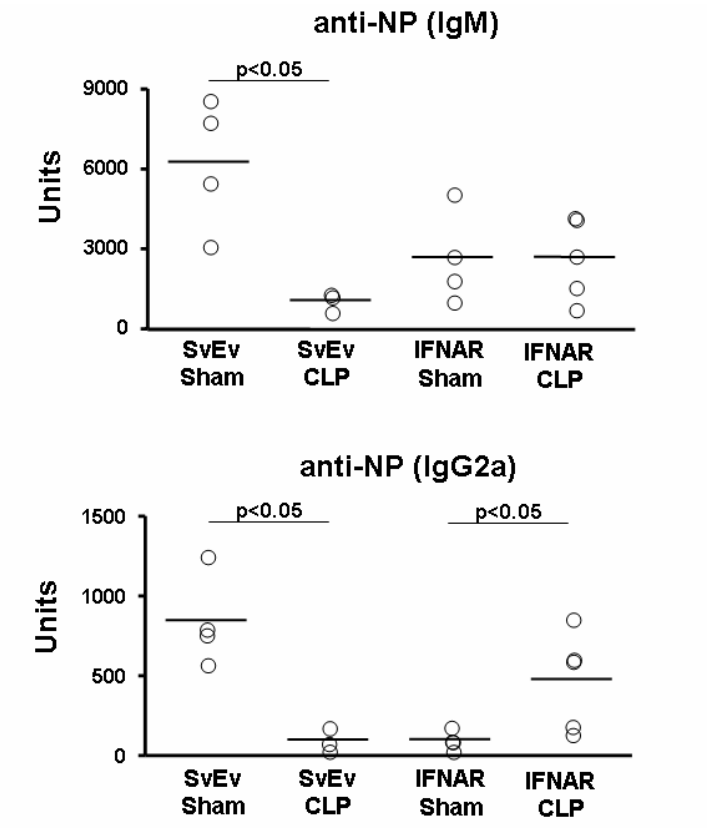

Figure 6-2. Antigen specific antibody responses reveal a potential role for Type I IFN in sepsis-induced adaptive immune dysfunction. SvEv or IFNAR$^{-/-}$ mice were immunized with NP-KLH in alum at the time of CLP (CLP) or sham surgery. Ten days later mice were euthanized and NP-specific IgM and IgG2a responses were measured.

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BIOGRAPHICAL SKETCH

Philip Oliver Scumpia was born on August 12, 1980 in Brooklyn, New York. He lived and attended school during his early youth in Queens, New York, until he moved to Cooper City, Florida, where he attended and graduated from Cooper City High School in 1998. Following high school, Phil attended the University of Florida in Gainesville, where he obtained a Bachelor of Science degree in microbiology and cell sciences. During this period, he worked as a laboratory assistant in the Department of Pediatrics under Dr. Jeffrey Skimming. Phil graduated in May 2002 with highest honors, and was admitted to the Golden Key National Honor Society, Phi Beta Kappa, and the National Society of Collegiate Scholars during his undergraduate tenure at the University of Florida.

Phil was then admitted and entered medical school at the University of Florida as an M.D.-Ph.D. student. During the summer between the first and second year of medical school, Phil participated in the Medical Scientist Training Program and worked for Dr. Bruce Stevens and Dr. Lyle Moldawer. At that time, Phil decided to enter the laboratory of Dr. Moldawer for his Ph.D. training. He then finished his second year of medical school and entered the Interdisciplinary Program in Biomedical Sciences in the College of Medicine at the University of Florida in July 2004. He joined the immunology concentration of the IDP and subsequently joined the laboratory of Dr. Moldawer where he initiated studies investigating the role of the adaptive immune system in sepsis. Following his doctorate work, Phil plans on finishing medical school, then applying for and completing a residency for board certification. His ultimate goals are to become an academic physician and eventually start his own laboratory, and start a family with his wife. Phil has been married to Kindra Kelly-Scumpia since May 2006.