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T CELL DYSFUNCTION AND CHECKPOINT BLOCKADE IN SEPSIS

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Abstract

T CELL DYSFUNCTION AND CHECKPOINT BLOCKADE IN SEPSIS

by Ching-Wen Chen

Sepsis is the leading cause of death in the United States, with mortality rates of septic individuals at ~20%. Immune dysregulation, including T cell exhaustion, has been documented during sepsis. However, the mechanisms underlying sepsis-induced T cell dysfunction remain poorly understood. During my dissertation work, we observed that a co-signaling molecule, 2B4 (CD244, SLAM4) is up-regulated on exhausted $CD4^+$ T cells in septic patients. To determine the role of 2B4 on $CD4^+$ T cell dysfunction during sepsis, cecal ligation and puncture (CLP) was performed on animals as a murine sepsis model. We identified an important inhibitory role of 2B4 on $CD4^+$ T cell-mediated T cell dysfunction and CLP mortality in animals. Of note, therapeutic blockade of 2B4 improved mortality in septic animals.

Cancer represents one of the most common comorbidities in sepsis with the in-hospital mortality at 40%. Approximately 100,000 cancer patients die due to sepsis annually. Checkpoint blockade is a promising therapy for cancer, and PD-1 blockade has recently also shown encouraging results in experimental models of sepsis. Here we aimed to determine the efficacy of PD-1 blockade in a pre-existing cancer sepsis model. We demonstrate that PD-1 blockade failed to improve mortality in cancer septic animals, while 2B4 blockade improved survival in cancer septic animals by modulating the balance of co-signaling receptors on T cells. Moreover, to investigate the mechanisms underlying increased mortality in cancer sepsis, we developed a novel model that allows us to track tumor-specific T cells during cancer sepsis. We showed that tumor-specific T cells in the tumor tissue were less impacted by sepsis-induced dysfunction. In addition, we found that tumor-specific T cells partially contributed to sepsis mortality in cancer septic animals by affecting endogenous $CD8^+$ T cells apoptosis. These findings illustrate a novel role of 2B4 in mediating T cell dysfunction during sepsis and also define the interplay between tumor-specific T cells and sepsis pathophysiology.

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Chapter 1. Introduction

1.1 Introduction of sepsis

1.1.1 The evolution of the definition of sepsis

Sepsis was first described in a medical context in Homer's poems dating back to 2800 years ago. The word sepsis "σηψιζ" in the ancient Greek poems represents "rot, decay," referring to the decomposition of animal or vegetable (1). After hundreds of years, philosopher Hippocrates (400 BC) in his writing described sepsis as the sacred and dangerous biological decay, which could be prevented by alcohol and vinegar. Based on Hippocrates' observation, philosopher and physician Galen (129–199 AD) first developed the very early theory of putrefaction of sepsis. He believed that sepsis resulted from the spontaneous generation of harmful organisms called "miasma". It wasn't until the nineteenth century that Louis Pasteur, Joseph Lister, and Robert Koch illustrated the "germ theory" of infectious disease. Louis Pasteur conducted the groundbreaking experiments that showed that contamination leads to microorganism development and formally proposed the ''Germ Theory'' in 1878. Subsequently, Joseph Lister applied the idea of germ theory to his patients with opened-wound sepsis and demonstrated that adding the carbolic acid in the wound dressings was able to significantly decrease the wound sepsis frequency and death in his hospital. Lister also was the first physician to perform surgery with aseptic technique. Robert Koch at the same time found that tiny organisms were growing in the blood of anthrax-infected sheep, and further he demonstrated that inoculating the microorganisms into healthy animals led to the same disease. Koch's four postulates set up the milestone in infectious diseases, and he is recognized as one of the fathers of modern microbiology (2, 3).

In the modern era, the first clinical definition of sepsis in medicine was announced by The American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) Consensus Conference Committee in Chicago in 1991 (4). Sepsis was defined as the systemic inflammatory response to the presence or suspected of infection. In the clinic, the identifications of the systemic inflammatory response are meeting two or more SIRS (Systemic Inflammatory Response Syndrome) criteria. However, the diagnostic criteria for SIRS published in Sepsis 1 were overly sensitive and nonspecific. The Sepsis 2 was updated in 2001 by the SCCM, the ACCP, the European Society of Intensive Care Medicine (ESICM), the American Thoracic Society and the Surgical Infection Society (5). The definition of sepsis remained the same, but the SIRS signs and symptoms lists were expanded, including general, inflammatory, hemodynamic, and tissue perfusion parameters. However, lacking the defined thresholds for updated SIRS criteria and the confusion between Sepsis 1 and Sepsis 2 became significant deficiencies. Recently, a new sepsis definition (Sepsis 3) was published based on the current knowledge of sepsis pathogenesis and management in 2016 (6). In Sepsis 3, sepsis is defined as the life-threatening organ dysfunction caused by a dysregulated host response to infection. From the clinic side, Sepsis 3 emphasizes the early awareness of sepsis by screening qSOFA (quick Sequential Organ Failure Assessment, Table 1.1). Moreover, the complete SOFA score system (Table 1.2) is recommended to characterize a septic patient clinically. A suspected infected patient with a change in baseline of the total SOFA score of 2 points or more is identified as a septic patient. Further, septic shock is now defined as a subset of sepsis with greater mortality due to profound circulatory, cellular, and metabolic abnormalities. A vasopressor requirement to maintain the arterial pressure of 65 mm Hg and serum lactate level greater than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia are two clinic criteria for meeting the definition of septic shock (6).

1.1.2 **Animal models for sepsis**

Sepsis is a significant healthcare issue in the United States and in global public health. Sepsis affects all age groups, all genders, and all races or ethnic groups, although it has a higher incidence in infants, elderly persons, and patients with chronic diseases (7). In the United States alone, sepsis strikes over one million people and is the leading cause of death in intensive care units (ICU) with over 250,000 patients annually (8, 9). Globally, it is estimated that 7.5 million deaths in children and newborn are associated with sepsis (10). The overall mortality of sepsis in the United States has decreased from 35% to 18% in the past decade because of the early awareness of the disease and the improvement of supportive therapies (8). However, the mortality increases to 55% when the disease progresses to sepsis shock (11). Of note that even patients that survive the initial septic insult still have poor outcomes long-term (12). Data from the Agency for Healthcare Research and Quality showed that U.S. hospitals spent more than $20 billion to treat sepsis patients every year (13). Based on all these facts, sepsis research is an urgent topic with important implications for global public health.

The laboratory mouse is an ideal model animal for preclinical sepsis research. The wide availability of inbred strains and genetically modified mice are essential tools to study the relevance of particular protein in the pathogenesis of sepsis. Mice are also critical to pharmacological intervention; the mortality improvement of the septic animals is the crucial indicator for therapeutic readout. The typical mouse sepsis models are divided into three major types: 1.) Exogenous administration of a toxin, 2.) Exogenous administration of a viable pathogen, and 3.) Alteration of the host barrier. Each model has its similarities with human sepsis and its disadvantages (14, 15).

1.) Exogenous administration of a toxin: Administrations of TLR (Toll-like Receptors) agonists, such as LPS or CpG DNA, can stimulate and induce an overwhelming innate immune response. The endo-toxicosis models are relatively simple to perform and more accessible to control. Human studies have shown that injection of low dose LPS into healthy donor could mimic the first stage of immune-pathogenesis in sepsis patients (16). However, the endo-toxicosis models might not reproduce the complicated host-pathogen interaction, and the immune response to the endo-toxicosis might only represent the short time frame of sepsis immune-pathogenesis. Moreover, most of the mouse strains are relatively resistant to endotoxin challenge (17, 18). A high dose of endotoxin is required to induce septic shock, which is hardly reproduced in human sepsis (15, 17).

2.) Exogenous administration of a viable pathogen: Bacterial infections are the most common type of sepsis in humans. In human sepsis, lung infection is the most common cause, followed by intra-abdominal and urinary tract infections (19, 20). Therefore, administration of bacteria into animals by different infection paths, such as intratracheal infection with *Streptococcus* or intraperitoneal infection of *Escherichia coli*, seem to be a feasible method to mimic human sepsis. However, the specific bacterial strains used in these models are not typically colonized and replicated within the host (21). It needs a high dose of bacteria to induce sepsis pathogenesis in animals. Unfortunately, the high amount of bacteria administration leads to the massive activation of TLRs, which failed to recapitulate the slowly progressive infection in the clinic (22). Moreover, immune response to different types of the bacteria sepsis might have opposite effects on the host. Several studies found contradictory results that IFN-γ contributes to the detrimental pathogenesis of gram-negative sepsis; but provides protective roles in the sepsis with gram-positive pathogens (23). On the positive side, the administration of specific pathogen can provide relevant

mechanisms of the host-pathogen interaction and can be used to develop particular pathogen therapy of sepsis.

3.) Alteration of the host barrier: In these animal models, host barrier is intentionally disrupted to allow host microflora to initiate the infection. The pathogens are various bacteria flora that usually is colonized and replicated inside the host. During these sepsis models, the bacteria caused infection start with a limited number, and continually develop into the systemic infection disease in sepsis. Importantly, this type of model recaptures the immune-pathogenesis in human sepsis patients, including the overwhelming innate immune response and the immunosuppressive stages (24). Cecal ligation and puncture (CLP) is a typical and commonly used surgery for inducing sepsis on the animal (25, 26). The operation requires the ischemia from the ligated cecum and the initial infection from the puncture that allowing fecal material to leak into the peritoneal cavity. The severity of CLP is usually defined by mortality and is adjustable by modulating the cecum ligation length, puncture needle size, and the amount of fecal material leaked out from cecum. Despite the fact that CLP is considered the gold standard for sepsis model, the CLP model has several disadvantages, including the need for mouse surgery, the variability in each mouse, and also the difficulty to control between each different study.

1.1.3 **Sepsis and comorbidity with malignancy**

The top-5 common comorbidities in septic patients is diabetes (35.7%), chronic pulmonary disease (30.9%), renal disease (26.8%), congestive heart failure (25.4%) and cancer (19.7%) (9, 27). Among these comorbidities, cancer patients have a relative risk of 9.77 to develop sepsis, and a relative risk of 2.77 to be hospitalized with sepsis compared to non-cancer patients (28-30). Sepsis also represents 9% of all cancer-related deaths in the United States. In cancer septic patients, lung cancer and pancreatic cancer are the most prevalent type of solid tumor. The most frequent sites of infection in cancer septic patients are lung, intra-abdominal, blood and urinary tract (31). Importantly, the in-hospital mortality is significantly higher for cancer septic patients compared to non-cancer (37.8% versus 24.9%) (30-32).

Neutropenia and defective phagocytosis or chemotaxis of neutrophils are common symptoms after cytotoxic chemotherapy or late-stage malignant myelosuppression (33, 34). The late stage of cancer patient commonly develops impaired immune responses, which are not limited to anti-tumor immunity. Poor vaccination responses and increased susceptibility to opportunistic infection have been shown on cancer patients (35, 36). In addition, global T cell exhaustion has been documented in cancer patients and cancer-bearing animals (37). Different types of cells attribute to the T cell functions impairment in cancer, including regulatory T cells, MDSCs, and erythroid progenitor cells (38-40). Impaired immune system results in increased risk of sepsis in cancer patients. Nonetheless, limited research has focused on how altered immune system in cancer patient contribute to sepsis pathophysiology.

1.2 Immune dysregulation in sepsis

Sepsis occurs when the infection leads to the series of dysregulated host responses that result in life-threatening tissue damages and organ dysfunction (6). Several dysregulated host responses can be assessed and monitored in clinical practice, including hematological, respiratory, cardiovascular, neurological, hepatic, and renal systems; whereas other systems, such as gut and immune systems are less accessible. Dysfunction in different organ systems can be impaired from mild alteration to organ failure during sepsis. Notably, the greater the number of dysregulated organs, the higher the sepsis mortality. Immune pathogenesis during sepsis contains two stages: an early acute phase is later followed by a chronic stage, showed in Figure 1. In the early acute stage, both hyper-inflammatory and hypo-inflammatory responses develop simultaneously (41, 42). At the later chronic stage, the immunosuppression mainly contributes to the immune dysregulation and results in secondary or persistent infections.

1.2.1 The hyper-inflammatory response in sepsis

In the acute stage of sepsis, the hyperinflammatory responses are mainly correlated with the innate immune system. Innate immune cells, including neutrophils and monocytes, are activated by PAMPs (pathogen-associated molecular patterns) from pathogens and DAMPs (damage-associated molecular patterns) from damaged tissue. Recognition of the PAMPs and DAMPs involves several types of PRRs (pattern recognition receptors), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (43). Activation of PRRs on innate immune cells leads to signaling cascades, such as MAPK, MyD88 pathways and further releases multiple inflammatory cytokines and chemokine (44). The high concentration of inflammatory cytokines is known as “cytokine storm” in sepsis and results in persistent inflammatory response

and tissue damages. Clinically, septic patients develop uncontrolled inflammation may develop systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS) (45-48).

Two major inflammatory cytokines, TNF and IL-6, are well studied in early stage sepsis. High levels of TNF and IL-6 were found in the sepsis patients within the first days of diagnosing with sepsis (49, 50). Moreover, serum TNF was observed at peak level when the septic shock occurred (51, 52). Another study showed that serum IL-6 is continually increased in non-survivor sepsis patients (53). In mouse studies, the increased serum level of TNF is found as early as 1 hours post CLP, followed with the subsequent increased IL-6 (54, 55). Besides, serum IL-6 at 6 hours after surgery was able to predict mortality outcomes from experimental mouse sepsis accurately (56). Inflammatory cytokines and the complement system also lead to coagulation activation, thrombin generation and disseminated intravascular coagulation (DIC) (57, 58). The coagulation in sepsis damages the endothelium and the microcirculation in different organs, which recruit more innate immune cells and amplify the inflammation response (59, 60). Activated neutrophils and monocytes recruited into damaged tissue produce reactive oxygen species (ROS) and reactive nitrogen intermediates further contribute to organ failure (61). Although the early release of inflammatory cytokines seems to be harmful to the host, inflammatory cytokines are crucial for appropriate host response during infection. Genetic deficient mouse studies demonstrated that IL-6 deficient leads to early protection of CLP but increases the mortality at the end of survival curve observation (62). Other groups showed that IL-6-deficient mice were unable to efficiently recruit neutrophils to infected site in an *Escherichia coli* sepsis model. TNF-deficient mice are resistant to the lethality doses of LPS challenge but are highly susceptible to *Candida albicans* infections (63). TNF receptors knockout mice exhibited impaired bacterial clearing in *Listeria monocytogenes* infection model (64). Overall, these results indicated that inflammatory

cytokines are functioned as a double-edged sword in sepsis. On one hand, inflammatory cytokines are necessary to activate the immune system and eliminate the pathogens; while on the other hand, excessive production of inflammatory cytokines cause organ damage during sepsis.

Targeting the hyper-inflammatory response during sepsis was the primary strategy to treat sepsis because researchers believed the uncontrolled inflammatory response was the main reason for sepsis mortality. However, hundreds of clinical trials focused on reducing hyper-inflammatory response, including corticosteroids, anti-PPRs agents, and anti-inflammatory cytokine antibodies have failed in the past (65, 66). In some cases, systemic anti-inflammatory treatment even further worsened outcomes of septic patients (67). The primary reasons for the failure of targeting hyper-inflammatory response have been discussed for decades. Firstly, the timing of anti-inflammatory therapy is essential. Blockade of the inflammatory response may be beneficial at the early stage of sepsis, while as it could have detrimental effects at the late stage. There are no ideal biomarkers for identifying the phase of sepsis in the clinic yet, and most septic patients admitted into ICU are already in the late stage of sepsis (68). Therefore, anti-inflammatory drugs are not optimal treatments for current septic patients. Secondly, multiple factors and cytokines are involved in the hyper-inflammatory response; and targeting single cytokine may have an ineffective function in sepsis. Finally, complete blockade of inflammatory signaling during infection have adverse effects on pathogen clearance. Overall, the precise therapy, such as assessing patient's inflammatory status and prioritizing the inflammatory factors in different patients, should be considering if targeting hyper-inflammatory response in sepsis.

1.2.2 **The immunosuppressive response in sepsis**

The immunosuppressive stage in sepsis was believed to start at a later stage of sepsis. However, recent genomic analysis from critical care patients revealed that both pro-inflammatory and anti-inflammatory gene appear simultaneously after systemic inflammatory stress (69). Although the net effect on host immune status still tends toward to hyper-inflammatory response at the initial phase of sepsis, the impact of immunosuppressive responses is revealed after the hyper-inflammation reduction, and it consistently impacts host for weeks or even years. The consequences of persistent immunosuppression are the inability to clear primary infections and the development of secondary infections, which are the major death reasons in late sepsis. A piece of critical evidence supports the idea that immunosuppression dominates the morbidity and mortality in sepsis is the study conducted by Hotchkiss and his colleagues. The study surveyed lymphocyte functionality in patients who died due to sepsis and found septic patients' lymphocytes underwent massive apoptosis, exhibited decreased cellular function and expressed exhaustion markers, PD-1, and BTLA, at a higher frequency (70). Moreover, increased reactivated latent viruses such as EBV, CMV and TTV were detected in septic patients with protracted sepsis compared to critically-ill non-septic patients (71), further supporting the development of an immunosuppressive state at the later stage of sepsis. Immunosuppression in sepsis is often shown on adaptive immune cells, including T cells and B cells. Major mechanisms of sepsis-induced adaptive immune system dysfunction involve: (i) cell apoptosis and (ii) impaired cellular function with exhausted phenotypes.

(i) Cell apoptosis. Extensive cellular loss during sepsis are found in lymph nodes, spleen and circulations, and it is considered as a hallmark of sepsis-induced dysfunction. The extensive depletion of immune cells is due to cell apoptosis, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, and dendritic cells (42). In fact, the severity of lymphopenia in septic patients at day four post

sepsis is also correlated with lower survival and increased development of secondary infections (72). Besides, healthy donor $CD4^+$ T cell apoptosis was observed when healthy donor lymphocytes cultured with septic patient's serum but not healthy serum (73). The rapid decline in T cell number also results in a significant reduction of TCR diversity in septic patients. Venet et al. showed that the diversity of TCR β chains were dramatically reduced once the patients diagnosed with sepsis (74); importantly, the reduction of T-cell receptor diversity was associated with sepsis mortality and also nosocomial infections (74). Mechanistically, the extrinsic pathways (TNF family receptors and caspase-8 dependent) and intrinsic pathways (mitochondrial and caspase-9 dependent) of apoptosis are both involved in sepsis-induced apoptosis (75, 76). Notably, multiple studies have shown that preventing lymphocyte apoptosis improves survival in sepsis. Overexpressing anti-apoptotic protein Bcl-2 in lymphocytes or inhibiting Fas/FasL signaling both significantly improved sepsis survival in the animal model (77-79). Thus, sepsis-induced cell apoptosis plays an essential role in immunosuppression and contributes to sepsis mobility.

(ii) Impaired cellular function with exhausted phenotypes. T and B lymphocytes from septic patients express an increased level of inhibitory molecules and exhibit decreased cellular functions. Increased $CD21^{-/low}$ exhausted B cells and insufficient immunoglobulin M production were correlated with increased secondary infection in septic patients (80). After ex vivo stimulations, decreased effector cytokines (IL-2, IFN-γ, TNF) and cytotoxic functions (Granzyme B, CD107a degranulation) of T cells from septic patients were documented (70, 81). In the post-mortem study, T cells isolated from septic patients had reduced CD28 and IL-7 receptor (CD127) expression and elevated PD-1 expression compared to non-septic controls, which resemble the cellular exhausted phenotypes on T cells (70). T cell exhaustion is a phenomenon that the antigen-specific T cells functions are diminished due to persistent high load antigen, which is first described in a chronic viral infection model (82). Subsequently, T cell exhaustion has been reported in cancer

microenvironment, bacterial infections, HIV, hepatitis C, hepatitis B, polyomavirus, and also sepsis (83, 84). Exhausted T cells express multiple inhibitory molecules, which attenuate the positive signals from T cell receptors (TCR) or co-stimulatory molecules. The mechanisms by which sepsis leads to T cell exhaustion are not yet determined. However, it is now believed that persistent infections and anti-inflammatory cytokines contribute to T cell exhaustion in sepsis. PD-1 (Programmed cell death protein-1) is a well-known exhausted marker (82) and is well-studied in sepsis. Increased PD-1 and PD-1 Ligands (PD-L1, PD-L2) on immune cells are found in septic patients (85-87). Increased PD-1^+ circulating T cells and PD-$L1^+$ on monocytes have been correlated with nosocomial infections and sepsis mortality (88, 89). In support of sepsis-induced T-cell exhaustion, other exhaustion markers, such as BTLA, Lag-3, and Tim-3 have also been shown to have a higher expression on T cells in patients (90-93).

Heterogeneous populations of T cells may respond differently to sepsis-induced apoptosis and dysfunction. Firstly, memory cells are more sensitive to cell death induced by sepsis. In a patient cohort with Staphylococcus aureus Infections, Ardura et al. showed that central memory $CD45RA^-CCR7^+$ $CD4^+$ and $CD8^+$ T cells are significantly decreased but not naive cells (94). Similar results were described in the mouse study showing that $CD44^{hi}CD11a^{hi}$ memory $CD8^+$ T cells are depleted and exhibited higher Annexin-V staining compared to $CD44^-$ naive T cells in a polymicrobial sepsis model (95). Secondly, sepsis affects the T cells in different locations distinctively. Sharma et al. demonstrated that the absolute number of T cells in thymus and spleen are significantly decreased after sepsis; while no significant change was observed on T cells in the liver and lung (96). Furthermore, Badovinac and colleagues showed that LCMV gp33-specific memory $CD8^+$ T cells in the skin are resistant to sepsis-induced dysfunction compared to the memory cells in the circulation (97). Thirdly, antigen-specific T cells display different responses during sepsis. By using segmented filamentous bacterium (SFB)-specific tetramer, Griffith and

colleagues elegantly showed that SFB-specific cells are activated and resistant to cell-death after sepsis induction (98). Next, regulatory $CD4^+$ T cells (Treg) is a specific $CD4^+$ T cell population that can suppress other immune cells by numerous mechanisms. During sepsis, regulatory $CD4^+$ T cells (Treg) are resistant to sepsis-induced apoptosis compared to conventional $CD4^+$ T cells, which results in an increased percentage of Treg cells in the circulation (98). The expanded Treg cells correlate with reduction of effector T cell proliferation and function in sepsis (99-101).

1.2.3 **Targeting immunosuppression as the potential therapy for sepsis**

The standard therapies for sepsis are broad-spectrum antibiotics and supportive care (6). However, there is no approved treatment for sepsis when antibiotics and supportive care fail. Developing new sepsis therapies has become a competitive area for sepsis research. However, hundreds of sepsis clinical trials had failed during the last few decades due to diverse patient populations and complicated sepsis immune-pathogenesis. Over 30 different anti-inflammatory drugs in clinical trials have failed to improve sepsis outcome, indicating that targeting anti-inflammation is not a primary strategy in sepsis therapy. Moreover, current supportive treatments allow septic patients to survive the hyper-inflammatory stage, intensifying the need for effective strategies to combat the systemic immunosuppression in the late stage of sepsis. Targeting the immunosuppressive syndromes with the ideas of replenishment of lymphocytes and restore lymphocytes function have been considered as a compelling strategy. In fact, Phase I clinical trials of therapies targeting immunosuppression have shown some promise (102, 103). Two possible drugs to revive the dysfunction adaptive immune system are recombinant human IL-7 (CYT107) and PD1-/PDL1 blockade antibodies (BMS-936559).

IL-7 is essential for T cell development and effector function (104, 105). In animal studies, IL-7 signaling has shown promising results to improve functional aspects of T cells during sepsis, including upregulation of anti-apoptotic protein Bcl-2 on the T cells, enhancing the resistant to

sepsis-induced apoptosis, sustaining circulating blood T cells number and also increase TCR diversity (106, 107). All of these factors have been correlated with sepsis mortality clinically. In septic animal studies, administration of IL-7 demonstrated survival improvement in a CLP followed with fungal infection model. In addition, ex vivo stimulation of sepsis patients' PBMC with IL-7 exhibited increased T cell IFN-γ production, STAT5 phosphorylation and Bcl-2 expression (108), which indicated that IL-7 might be a potential supplement to reverses sepsis-induced dysfunction in T cell in septic patients.

Blockade of the co-inhibitory molecules PD1 interaction with PD-L1 is another possible strategy to reverse T cell dysfunction. Anti-PDL1 antibodies have been approved for human melanoma therapy due to its ability to restore tumor-specific exhaustion T cell function (109). T cells during sepsis express the similar exhausted phenotypes as do T cells in tumor microenvironment; therefore, anti-PDL1 also holds great potential for sepsis therapy. Encouragingly, delayed administration of anti-PD-1 antibody on CLP treated mice increased T cell viability and improved mouse CLP survival (110, 111). Recently, another mouse study also demonstrated that blockade of PD-1 and LAG-3 signaling together improved the endogenous LCMV-specific T cell functions and enhanced the LCMV control in a polymicrobial sepsis model followed by LCMV infection (112). Moreover, in a two-hit sepsis model, blockade of the PD-1 and CTLA-4 together exhibited increased survival in the secondary fungal infection (113). With many substantial evidence and studies appeared, anti-PDL1 has entered the first stage a clinical trial on sepsis patients and showed favorable results. The antibody blockade treated patients showed no significant changes in cytokine levels, but the HLA-DR (human leukocyte antigen-DR) expression on monocytes was increased, suggesting that PDL1 blockade is safe and partially restore immune status (103). Based on previous literature, restoring T cell functions by targeting exhaustion markers and restoring survival signals have emerged as potential therapies for sepsis.

1.3 Co-stimulatory and co-inhibitory receptors regulate T cell responses

An appropriate immune response to infections demands activation of both the innate immune system and the adaptive immune system. The principal characters in the adaptive immune system are T cells, which are the most diverse and pleiotropic cells in immune systems. The proper activating of T cells is regulated by a precisely and strictly regulated signaling system. T cell activations require three critical signals: Signal 1 is TCR interact with MHC with peptide; Signal 2 is co-stimulatory signals (114); Signal 3 is environment cytokine signals (115). In general, Signal 1 is considered as a switch to turn on or turn off the T cell activation. While Signal 2 functions as a control knob to tightly regulate T cell activation, which is accomplished by the signaling balance of co-stimulatory and co-inhibitory molecules. Co-stimulatory molecules usually cluster closed to TCR complex on cellular membrane and help form an immunological synapse to synergize the TCR signaling (116-118). On the other hand, co-inhibitory molecules deliver the negative signaling to decrease T cell activation, which plays a crucial role in T cell homeostasis and diseases. With increased attention on the immunosuppressive stage of sepsis, strategies to block co-inhibitory pathways during sepsis and reverse sepsis-induced immune dysfunction has become a significant direction of sepsis therapies. In fact, blockade of several molecules like PD-1 and CTLA-4 showed effectively improved survival in sepsis animal studies (91, 110, 113). In this section, we will introduce some particular co-stimulatory and co-inhibitory receptors and their signaling networks.

1.3.1 Co-stimulatory receptor: CD28

CD28 is a glycoprotein with a cytoplasmic region containing tyrosine-based signaling motif and two proline-rich motifs, that are critical for downstream signaling (119). Thompson's group in 1987 first demonstrated that CD28 is associated with TCR signaling in interleukin-2 gene

expression (120). Moreover, follow up studies from Lee Nadler identified CD80 (B7.1), and CD86 (B7.2) are the ligands for CD28 (121). CD28 is highly expressed on naive T cells (122), TCR ligation (Signal 1) and CD28 stimulation (Signal 2) are critical for T cell IL-2 secretion and survival. Early studies suggest that ligation TCR without stimulation of CD28 induces T cell anergy and apoptosis, representing an important tolerance mechanism in controlling auto-reactive T cells (123, 124). While during infection conditions, activated APC express both B7 family and MHC with pathogen peptides to stimulate naive T cells fully (122). Recent studies elucidated that CD28 signaling promotes T cell proliferation by increasing cell cycle proteins and anti-apoptotic proteins (125). Further molecular studies showed that tyrosine-based signaling motif (YMNM) and proline-rich motif in CD28 cytoplasmic domain recruit several critical proteins and results in three crucial signal cascades (126, 127), including 1.) PI3K and Lck recruitments lead to Akt activation; 2.) Grb2: GADS complex and phosphorylation of LAT, SLP-76, and Vav lead to PKCϴ activation; 3.) Grb2:Sos:Vav1 complex lead to JNK activation. These signal cascades culminate in nuclear activation of NFAT, AP-1, and NFκB and enhance IL-2 transcription (126, 128-131). Moreover, CD28 activation in cytoplasmic tail promotes TCR micro-clustering and stabilizes the lipid rafts, which are essential for APC-T cell synaptic localization (126).

CD28 signaling has been involved in regulating both sepsis-induced hyper- and hypo-inflammatory responses. First, $CD28^{-/-}$, $CD80^{-/-}$, and CD86 animals showed improved survival and reduced inflammatory cytokines in the CLP model (132). Moreover, blockade of CD28 signaling with CTLA-4Ig in a *Staphylococcus aureus* sepsis model showed reduced serum IL-6 and attenuate the migrations of neutrophils and monocytes into draining lymph nodes (133). In addition, AB103, a novel CD28 antagonist peptide that dampen CD28 signaling attenuated TNF and IL-6 secreted from human PBMC stimulated with LPS (134). In the same study, single dose

administration of AB103 improved survival in animals that challenged with LPS, *Escherichia coli 018:K1* peritonitis, and CLP model (134). These results indicated that CD28 signaling is involved in the hyper-inflammatory response in sepsis and interrupting CD28 signaling is protective during sepsis. On the other hand, Hotchkiss and his group demonstrated that CD28 expression is significantly reduced on T cells in ICU patients who died due to sepsis (70). Decreased CD28 expression on septic patients also correlated with impaired proliferation after in vitro stimulation. Moreover, decreased CD28 expression on $CD4^+$ T cells was found in patients with Candida sepsis (87). In animal studies, CD28 is dramatically reduced during sepsis at early time-point and is considered as a hallmark as a T cell dysfunction.

1.3.2 **Co-inhibitory receptor: CTLA-4**

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, CD152), discovered by Ledbetter group back in 1991, is a coinhibitory molecule with a fundamental role in immunotolerance and downregulation of the T cell-mediated immune response (135, 136). CTLA-4 is a homologous CD28 family protein that also binds to CD80 and CD86, but with higher binding affinity (137, 138). CTLA-4 controls T cell activation in a variety of mechanisms. First, CTLA-4 competes with CD28 for CD80 and CD86 on APCs with higher affinity and deprives the CD28 co-stimulatory signaling on activated T cells (139). Second, the cytoplasmic tail of CTLA-4 binds to SHP2 via a YVKM motif, which dephosphorylates TCR zeta chain, ZAP70, and PKCθ in TCR signaling (140). Third, PP2A can be recruited to lysine-rich motif on CTLA-4 cytoplasmic tail. PP2A has been shown inhibiting Akt-mTOR pathway that is associated with T cell proliferation (141). Finally, CTLA-4 is known to impede lipid raft stability of the TCR complex (142). The inhibitory function of CTLA-4 is important nearly T cells tolerance, supported by the evidence that CTLA4-deficiency mice only survive 2-3 weeks and die from massive lymphoproliferative and inflammation (143).

Recent studies showed that CTLA-4 serves as an essential molecule for regulatory T cells (Treg) function in mediating tolerance. Conditionally knockout CTLA-4 on Treg dramatically deprives Treg suppressive functions and lead to massive inflammation in animals (144).

Several studies have shown that CTLA-4 expression is elevated during sepsis. Gao et al. showed that $CD4^+CTLA\text{-}4^+$ was positively correlated with APACHE II score, a representative scoring system for sepsis severity (145). Interestingly, the functional single-nucleotide polymorphism (SNP) rs231775 in CTLA-4 gene has been shown to correlate to lower 90-day mortality risk in Caucasian patients with sepsis (146, 147). Mouse studies showed that septic animals displayed increased CTLA-4 expression on $CD4^+$ T cells and $Foxp3^+$ Treg cells at the early and later time points of sepsis (bimodal distribution, peak at day 1 and day 7 post-CLP) (148). Of note, blockade of CTLA-4 in septic animal modulates survival with a dose-dependent effect. A higher dose of CTLA-4 blockade antibody (200 μg/mouse, two doses) worsens the CLP survival; in contrast, low dose blockade antibody (50 μg/mouse, two doses) results in improved survival (148).

1.3.3 **Co-inhibitory receptor: PD-1**

PD-1 (Programmed death 1) is an Ig superfamily member with a critical function on negatively regulating T cell responses. PD-1 binds to PD-L1 (B7-H1; CD274), and PD-L2 (B7-DC; CD273) on activated APCs or some non-hematopoietic cells, where the expressions contribute to T cell peripheral tolerance (149-151). PD-1 receptor gene was first discovered in a T cell hybridoma with TCR activation-induced cell death in 1992 (150). The cytoplasmic tail of PD-1 contains ITIM and ITSM domains, which recruit inhibitory phosphatases SHP-1 and SHP-2 (152, 153). SHP-1 and SHP-2 target ZAP-70 in TCR signaling and PI3K in CD28 signaling and attenuates the T cell activation (154). PD-1 signaling is also responsible for reducing anti-apoptotic

protein Bcl-xL expression and inhibiting IL-2 secretion in T cells (152). Interestingly, the inhibitory effect of PD-1 can also be overcome by CD28 co-stimulation or IL-2 cytokine stimulation (155). Different from the phenotype of CTLA4$^{-/-}$ mice, PD-1$^{-/-}$ knockout mice do not die at an early age, and their autoimmune symptoms are relatively minor (156). In C57BL/6 and BALB/c mouse backgrounds, PD-1 deficient mice developed lupus-like glomerulonephritis and dilated cardiomyopathy symptoms (157), revealing the importance of the PD-1 in immune tolerance.

Extensive research has focused on the biological function of PD-1 in chronic virus infection model and cancer field, where PD-1 is highly expressed on exhausted T cells. Blockade of PD-1 and PD-L1 have been successful in treating several types of cancer, including melanoma, non-small cell lung cancer, bladder cancer, head and neck cancers, and Hodgkin lymphoma (158). With the achievements in cancer research, PD-1 has received increased attention in sepsis and infection diseases research as we described in the previous section. For sepsis animal studies, administration of anti-PD-1 and anti-PD-L1 both result in significantly improved CLP survival. Mechanistically, PD-1/PD-L1 blockade leads to decreased T cell apoptosis during sepsis, increased Bcl-xL expression on T cells, and enhanced bacterial clearance (110). Interestingly, the pivotal functions of PD-1 signaling are not limited on T lymphocytes during sepsis. Dr. Alfred Ayala and colleagues showed that high levels of PD-1 expression on peritoneal macrophages during sepsis are correlated with decreased phagocytosis ability and reduced mortality (159).

1.3.4 **Dual function receptor: 2B4**

2B4 (CD244) is a CD2 related surface protein and one of the members of SLAM (signaling lymphocyte activation molecule) family (160, 161). 2B4 interacts with CD48, a glycosylated GPI-anchored protein widely expressed on hematopoietic cells and endothelial cells. 2B4 is recognized

as a dual function receptor that has both co-stimulatory and co-inhibitory properties (162, 163). 2B4 contains four ITSM (immuno-tyrosine switch motif) domains in the cytoplasmic tails. The ITSM domains in 2B4 have been shown to recruit different properties of signaling proteins: 1.) SLAM-associated protein (SAP) and EAT-2 adaptor proteins, which recruits Fyn, LAT, PLCγ and Erk kinase to activate immune cells (164-166). 2.) SHP-1 and SHP-2 that competes for the binding of SAP and attenuate TCR signaling by dephosphorylation of ZAP-70 (167). Moreover, the net effect of 2B4 signaling on immune cells is context dependent. The amount of 2B4 expression at the cell surface, the extent of the CD48 ligand, and the availability of SAP or EAT-2 inside the cells all contribute to the outcome of 2B4 signaling (168).

2B4 is highly expressed on NK cells and the dual functions of 2B4 on mediating of NK cells response have been described. First, antibody-mediated crosslink of 2B4 on mouse NK cells increases cell proliferation and enhances tumor lysis in vitro (169, 170). Furthermore, murine NK cells express the short isoform of 2B4 (2B4-S) that lack three of the ITSM domains in its cytoplasmic domains, which showed co-stimulatory properties after ligation (161). On the other hand, 2B4-deficient on NK cells or blockade of 2B4-CD48 interaction showed enhanced NK cells cytotoxicity and IFN-γ production against $CD48^+$ tumor cells in vitro and in vivo (171). Further support came from one study found that 2B4 profoundly accumulated at the interface between NK and nonlytic target cells, but not with lytic target cells (161). Finally, one recent study showed that lack of 2B4 on NK cells dampens the anti-viral CD8 responses by enhancing NK cell-mediated killing of activated $CD8^+$ T cells. This research reinforces the inhibitory roles on NK cells but also highlight the interaction of the immune cells can be controlled by 2B4 (172). While both stimulatory and inhibitory function of 2B4 was found on NK cells, 2B4 appears to exhibit dominantly inhibitory functions on $CD8^+$ T cells. Studies found that 2B4-deficient animals exhibited expanded Th1 populations, developed the spontaneous lupus-like disease and increased

rejection of $CD48^+$ melanoma cells in vivo (173, 174). Moreover, 2B4 expressed on exhausted $CD8^+$ T cells has been shown to limit secondary memory T cells proliferation under chronic infection (175). A recent report on allograft-specific CD8 T cell responses also indicated that 2B4 attenuates antigen-specific $CD8^+$ T cell responses under selective CD28 blockade (176). These findings suggest that 2B4 expressed on $CD8^+$ T cells results in augmentation of co-inhibitory signaling. The role of 2B4 in regulating T cells during sepsis will be discussed further in Chapters 2 and 3.

In summary, these studies highlight that the balance of co-stimulatory and co-inhibitory signaling modulates T cell responses. Importantly, different co-stimulatory and co-inhibitory on T cells may have distinct roles in controlling T cell responses under a particular disease or infection model. Here in this study, we aimed to understand the role of co-inhibitory receptors in the setting of sepsis and cancer sepsis models.

1.4 Figure and Tables

Table 1.1 qSOFA Criteria for early awareness of sepsis

Table 1. qSOFA (Quick Sequential Organ Failure Assessment) Criteria for early awareness of sepsis
1. Respiratory rate ≥ 22/min
2. Altered mentation
3. Systolic blood pressure ≤ 100 mm Hg

This table is adapted from a published paper "The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)" (6)

Table 1.2 Sequential Organ Failure Assessment (SOFA) Score

Table 2. Sequential Organ Failure Assessment (SOFA) Score					
Score	**0**	**1**	**2**	**3**	**4**
Coagulation, Platelets, $10^3/\mu L$	≥ 150	< 150	< 100	< 50	< 20
Liver, Bilirubin, mg/dL	< 1.2	1.2-1.9	2.0-5.9	6.0-11.9	> 12.0
CNS, Glasgow Coma Scale score	15	13-14	10-12	6-9	< 6
Renal, Creatinine, mg/dL	< 1.2	1.2-1.9	2.0-3.4	3.5-4.9 Urine output < 500 ml/day	> 5.0 Urine output < 200 ml/day
Respiration, PaO_2/F_{IO2}, mm Hg	≥ 400	< 400	< 300	< 200 with support	< 100 with support
Cardiovascular	MAP ≥ 70 mm Hg	MAP < 70 mm Hg	Dopamine < 5 or dobutamine (any dose)	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1

This table is adapted from a published paper "The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)" (6)

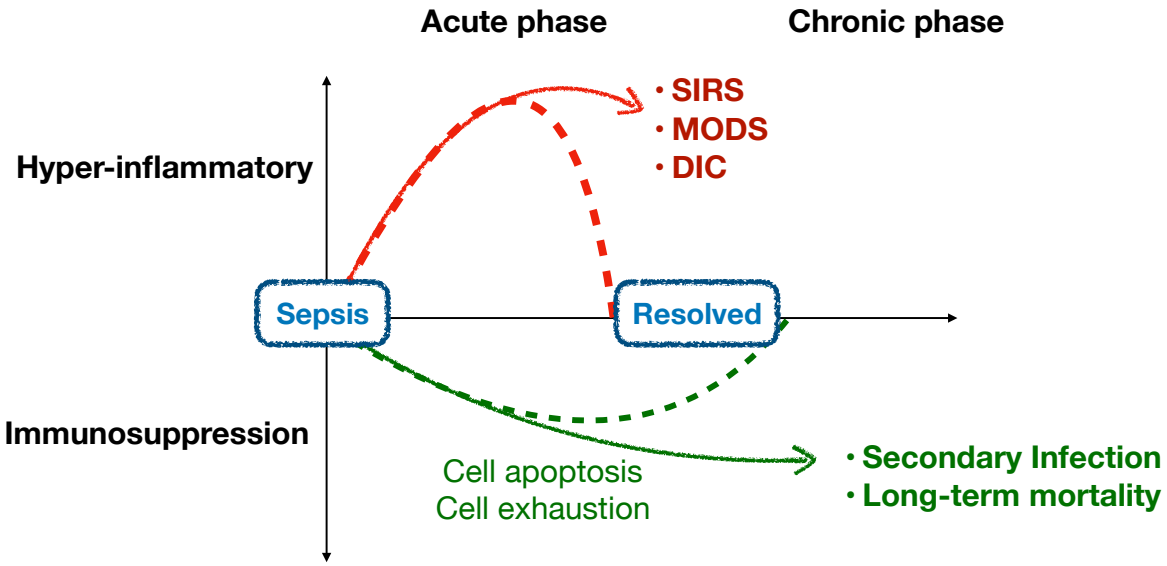

Figure 1.1 The model of immune pathogenesis in sepsis

Hyper-inflammatory responses in the early stage of sepsis lead to SIRS (systemic inflammatory response syndrome), MODS (multiple organ dysfunction syndromes) and DIC (disseminated intravascular coagulation). These inflammatory symptoms all contribute to organ failure during sepsis. Simultaneously, the adaptive immune systems encounter cell apoptosis and have cellular exhaustion. At the later stage of sepsis, the defective adaptive immunes develop into immunosuppression status and results in increased risk of secondary infection and the long-term death in sepsis.

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Chapter 2. 2B4-mediated co-inhibition of $CD4^+$ T cells underlies mortality in experimental sepsis

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2.1 Abstract

Sepsis is a leading cause of death in the United States, but the mechanisms underlying sepsis-induced immune dysregulation remain poorly understood. 2B4 (CD244, SLAM4) is a co-signaling molecule expressed predominantly on NK cells and memory $CD8^+$ T cells that has been shown to regulate T cell function in models of viral infection and autoimmunity. In this article, we show that 2B4 signaling mediates sepsis lymphocyte dysfunction and mortality. 2B4 expression is increased on $CD4^+$ T cells in septic animals and human patients at early time points. Importantly, genetic loss or pharmacologic inhibition of 2B4 significantly increased survival in a murine cecal ligation and puncture model. Further, CD4-specific conditional knockouts showed that 2B4 functions on $CD4^+$ T cell populations in a cell-intrinsic manner and modulates adaptive and innate immune responses during sepsis. Our results illuminate a novel role for 2B4 coinhibitory signaling on $CD4^+$ T cells in mediating immune dysregulation.

2.2 Introduction

Sepsis is the leading cause of death among critically ill patients in the United States (1). Importantly, no approved therapeutics are available for sepsis once antibiotics and supportive therapy fail. Recently, studies assessing the immune phenotypes and functionality of septic patients have shed light on the immune dysregulation that occurs during sepsis, as well as its ability to result in profound and protracted immune suppression (2). Previous studies have identified a role for cell surface inhibitory receptors PD-1 and BTLA in con- trolling macrophage activation and APC function following sepsis (3, 4); however, the mechanisms underlying sepsis- induced immune suppression remain poorly understood.

It is well known that $CD4^+$ T cells are heavily impacted during sepsis; human septic patients and experimental mouse models of sepsis exhibit profound $CD4^+$ T cell apoptosis and loss of functionality (2, 5). Importantly, preventing $CD4^+$ T cell apoptosis has been shown to reverse sepsis-induced mortality in mouse models. In this study, we find that the coinhibitory molecule 2B4 is significantly upregulated on $CD4^+$ T cells in human septic patients and in an experimental model of mouse cecal ligation and puncture (CLP). 2B4 (CD244, SLAMf4) is a member of the CD2 subset of Ig superfamily molecules. Previously best known for its role on NK cells (6), more recent work has shown that, in certain settings, 2B4 can be inducibly expressed on $CD4^+$ and $CD8^+$ T cells and possesses coinhibitory function on these cell populations (7, 8). Therefore, we sought to interrogate the role of 2B4 in mediating immune dysregulation and mortality during sepsis.

2.3 Results

2B4 is upregulated on T cells but not NK cells following sepsis

To interrogate the mechanisms underlying immune dysregulation during sepsis, WT mice were subjected to polymicrobial sepsis via CLP, and expression of 2B4 was assessed by flow cytometry. As negative controls, 2B42/2 splenocytes and isotype controls were used. 2B4 expression was confirmed by staining with three distinct anti-2B4 clones [eBio244F4, m2B4 (B6)458.1, and 2B4], and all yielded similar results (data not shown). Results indicated that NK cells isolated from the sham surgery and CLP groups expressed high levels of 2B4 and maintained 2B4 expression over 7 d post- surgery (Figure 2.1 A). Intriguingly, 2B4 expression on $CD8^+$ and $CD4^+$ T cells was increased at 24 h post-CLP in septic animals. The elevated expression of 2B4 on T cells was maintained for 3 days post-CLP and declined by day 4 (Figure 2.1 B, C). PD-1 and BTLA were significantly higher on $2B4^+$ T cells, potentially indicative of an exhausted phenotype (Figure 2.1 D). In the murine CLP model, PD-1 and CTLA-4 begin to be upregulated on $CD4^+$ and $CD8^+$ T cells 48–96 hours post-CLP and steadily increase until day 7 (10, 11). In contrast, our study found that 2B4 is expressed with distinct kinetics from PD-1 or CTLA-4, suggesting that 2B4 might have a unique and nonredundant coinhibitory function relative to PD-1 or CTLA-4, acting as an earlier inhibitory molecule during T cell activation.

2B4 upregulation was primarily observed on $CD44^{hi}$ memory T cells during sepsis (Figure 2.1 D), which is consistent with previous findings and also suggests that 2B4 functions as a co-signaling receptor on memory cell populations. Analysis of expression of other SLAM family members revealed that, although the mean fluorescence intensity of SLAMf2 (CD48, the ligand of 2B4) decreased significantly on $CD4^+$ and $CD8^+$ T cells following sepsis, it remained highly expressed on all T cells (Figure 2.1 E), thus confirming ligand availability for 2B4 signaling on T cells during sepsis. Furthermore, we found no difference on SLAMf1 and SLAMf6 between CLP

group and sham group. Although SLAMf3 expression on $CD4^+$ T cells was decreased during CLP, the expression on $CD8^+$ T cells was similar (Figure 2.2). For SLAMf5 (CD84), we could not detect the expression on $CD3^+$ populations (data non-shown). Combined together, 2B4 (SLAMf4) is the only SLAM receptor was upregulated on T cell surfaces, which might emphasize the importance of 2B4 co-signaling in the regulation of T cell responses during sepsis. Congruent with previous findings that memory cell populations are more susceptible to sepsis-induced dysfunction (12), our data indicated that 2B4 expression on memory T cell populations might produce inhibitory signaling and lead to sepsis-induced dysfunction.

$2B4^{-/-}$ animals display increased survival and effector T cells after sepsis

Given the above results, we sought to determine the effect of disrupting 2B4 signaling during sepsis. Strikingly, $2B4^{-/-}$ mice were significantly protected from death during sepsis following CLP compared with WT controls (82% survival compared with 13%, Figure 2.3 A). Although no difference was found in the bacterial load in peritoneal fluid or blood at 24 h post-CLP, $2B4^{-/-}$ mice possessed increased numbers of $CD4^+$ T cells at 24 h relative to WT controls (Figure 2.3 B, Figure 2.6 a). Analysis of caspase 3/7 activity in $CD4^+$ T cells revealed that $2B4^{-/-}$ $CD4^+$ populations contained a lower frequency of apoptotic cells (Figure 2.3 C) and increased numbers of live cells relative to WT $CD4^+$ cells (Figure 2.6 b, c). To further determine the functionality of T cells in $2B4^{-/-}$ and WT mice, surface markers and intracellular cytokines were assessed. $2B4^{-/-}$ and WT animals exhibited no difference with regard to CD25 and CD69 expression in the $CD4^+$ or $CD8^+$ T cell compartments prior to CLP. In contrast, CD25 was significantly upregulated in the $CD4^+$ and $CD8^+$ T cell compartments in $2B4^{-/-}$ CLP mice relative to WT CLP animals. Furthermore, CD69 was upregulated in $2B4^{-/-}$ CLP mice relative to WT CLP animals in the $CD4^+$, but not the $CD8^+$, T cell compartment (Figure 2.3 D). After CLP, increased

secretion of IFN-γ was also observed in 2B4$^{-/-}$ CD4^{+} and CD8^{+} T cell populations relative to WT controls (Figure 2.3 E). Taken together, our data indicate that 2B4$^{-/-}$ CD4^{+} T cells exhibit a more activated phenotype and have higher functionality during sepsis, a finding that could underlie the increased survival observed in 2B4-deficient mice.

CD4^{+} T cell-specific deletion of 2B4 enhances sepsis survival

To elucidate which immune compartment contributes to the survival benefit obtained in 2B4$^{-/-}$ mice, we first targeted NK cells because they express high levels of 2B4. NK cells were depleted in 2B4$^{-/-}$ mice prior to CLP. No survival difference was observed between intact and NK-depleted 2B4$^{-/-}$ mice (Figure 2.6 d). This result indicated that NK cells likely did not contribute to the survival benefit in 2B4$^{-/-}$ animals. Next, to further dissect the function of 2B4 on different T cell compartments, we generated BM chimeric conditional knockout mice in which 2B4 is knocked out exclusively in the CD4^{+} or CD8^{+} T cell compartment. Briefly, the CD4-specific 2B4 conditional knockouts were generated by a BM transplant of 80% CD4$^{-/-}$Thy1.2^{+} (2B4-intact) BM mixed with 20% 2B4$^{-/-}$Thy1.1^{+} BM (or 20% WT BM as control). After 10 weeks of reconstitution, all hematopoietic lineages, with the exception of CD4^{+} T cells, were reconstituted primarily from the CD4$^{-/-}$ WT BM. In contrast, CD4^{+} T cells were reconstituted from the 2B4$^{-/-}$ BM. The same procedure was done with CD8$^{-/-}$ BM, thus generating CD4- and CD8-specific 2B4 conditional knockouts (CD4$^{2B4-/-}$ and CD8$^{2B4-/-}$, Figure 2.4 A, B). There was no difference in the number of B or T cells between control chimera and CD4$^{2B4-/-}$ chimera animals at baseline (Figure 2.6 e). We confirmed our chimera phenotype by subjecting CD4$^{2B4-/-}$ chimeras to CLP and found that CD8^{+}, but not CD4^{+}, T cells upregulated 2B4 expression post-CLP (Figure 2.4 C). Although CD8$^{2B4-/-}$ animals did not exhibit significantly altered survival relative to controls (Figure 2.4 D), CD4$^{2B4-/-}$ chimeric mice exhibited significantly improved survival following CLP compared with CD4$^{2B4+/+}$

control chimeras (Figure 2.4 E). Functional assays showed that $CD4^{2B4-/-}$ mice exhibited increased numbers of IFN-γ-secreting $CD4^+$ T cells in splenocytes relative to controls (Figure 2.4 F), although they exhibited comparable bacterial loads (Figure 2.6 f). To further investigate the functional effect of 2B4 specifically on $CD4^+$ T cells on sepsis-induced immune dysregulation, we analyzed the innate immune compartments in $CD4^{2B4-/-}$ mice and found that macrophages expressed elevated levels of CD86, suggesting increased activation of macrophages in the spleens of $CD4^{2B4-/-}$ mice (Figure 2.4 G). Indeed, macrophage expression of CD86 has been correlated with reduced immune dysregulation and increased number of intensive care unit free days in human septic patients (13). In addition, reduced levels of CD86 mRNA were identified in lethal pediatric septic shock (14). Of note, increased activated IFN-γ-secreting macrophages did not correlate with higher serum TNF and IL-1ß (Figure 2.6 g). Taken together, our data strongly suggest that 2B4 signaling on $CD4^+$ T cells results in distinct functionality relative to 2B4 signaling on $CD8^+$ T cells and contributes significantly to septic mortality.

Blockade of 2B4 results in improved CLP survival

Next, to determine whether 2B4 could be pharmacologically targeted to improve mortality following sepsis, we blocked 2B4 signaling in septic animals with a mAb to 2B4 (clone 2B4) given on days 0, 2, 4, and 6 post-CLP. We first confirmed that this clone results in 2B4 blockade and not depletion of 2B4-expressing cells in vivo using GFP^+ 2B4-expressing retrogenic T cells transferred into WT animals (Figure 2.6 h). Results showed significantly increased survival of animals treated with anti-2B4 relative to PBS-treated controls (Figure 2.4 H), suggesting that anti-2B4 might be a potential treatment during sepsis.

Septic patients exhibit increased 2B4 expression on $CD4^+$ T cells

Finally, to interrogate the expression of 2B4 on distinct immune cell types in human septic patients, PBMCs were collected from patients within the first 24 hours of a diagnosis of clinically defined sepsis (15). Our results showed no difference in 2B4 expression between septic patients and healthy donors within NK cells and the $CD8^+$ T cell compartment (Figure 2.5 A-C). However, although $2B4^+$ $CD4^+$ T cells are rare in healthy human controls, we observed that 2B4 expression was significantly upregulated within the $CD4^+$ T cell compartment of septic patients compared with non-septic controls (Figure 2.5 D). Further immunophenotyping on human $2B4^+$ $CD4^+$ T cells revealed that these cells possess an exhausted phenotype that is characterized by increased PD-1 expression and decreased expression of the costimulatory molecules ICOS and CD28 (Figure 2.5 E). Overall, these results, combined with our mouse data, highlight the critical role of 2B4 on $CD4^+$ T cell populations during sepsis.

2.4 Discussion

The balance of costimulatory and coinhibitory molecules is critical in determining T cell function during infection. In this article, we show that increased 2B4 expression at early time points during sepsis can dampen T cell functionality. Importantly, blockade of 2B4 signaling improves sepsis mortality, thus identifying this pathway as a potential therapeutic target for sepsis immunotherapy. With increased attention on the immunosuppressive stage of sepsis, reversing sepsis-induced immune dysfunction has become a major direction of sepsis therapies. For example, anti-PD-1 has been shown to improve sepsis survival in mice and has entered clinical trials for septic patients. However, it is increasingly apparent that different coinhibitory molecules play distinct and nonredundant roles in inducing T cell dysfunction (16). Our data show that 2B4 expression kinetics is different from those of PD-1 and CTLA-4, indicating potentially distinct roles for these coinhibitory molecules during sepsis. Recent work has also shown that the ability of anti-PD-1 to rescue antitumor T cells is dependent upon CD28 expression (17). In this study, we found that $2B4^+$ $CD4^+$ T cells express little surface CD28, suggesting that anti-PD-1 therapy may not effectively rescue the $2B4^+$ T cell compartment within septic patients, further highlighting the concept that targeting multiple coinhibitory molecules during sepsis might be required to adequately reverse immune dysfunction.

Another important and novel finding of the current study is that 2B4 functions in a cell-intrinsic manner on $CD4^+$, but not $CD8^+$, T cells during sepsis and contributes to decreased macrophage activation. Previous work has shown that 2B4 functions as a co-stimulatory or co-inhibitory molecule on NK cells and primarily as a co-inhibitory molecule on memory $CD8^+$ T cells, depending upon external stimulation and the downstream adaptor protein SAP and phosphatase SHP-1. Noticeably, distinct 2B4 expression patterns were observed in specific pathogen–free–housed murine versus healthy human $CD8^+$ T cells, likely due to differential

frequencies of memory and naive T cell populations. Indeed, our murine data showed that 2B4 is expressed primarily on $CD44^{hi}$ memory cell populations following CLP (Figure 2.1 D). Because we have previously reported that memory T cells are more susceptible to sepsis-induced dysfunction compared with naive T cells (12), understanding the impact of 2B4 signaling specifically on memory T cell subsets remains an important goal. However, despite the differences in memory T cell composition between murine and human immune populations, our results show that 2B4 expression was significantly increased after sepsis on human and mouse $CD4^+$ T cells. Unlike 2B4 expression on human $CD8^+$ T cells, which is high at baseline, our data indicate that sepsis selectively induces 2B4 expression on $CD4^+$ T cells. However, further investigation is needed to identify the molecular signaling downstream of 2B4 in $CD4^+$ T cells during sepsis. In summary, our results suggest a novel therapeutic target for the treatment of septic individuals, as well as highlight the unique roles for 2B4 within $CD4^+$ and $CD8^+$ T cell compartments.

2.5 Acknowledgments

We thank the patients and healthy donors who contributed to this study, with special thanks to Leona Wells and Mona Brown for collecting patient samples.

2.6 Materials and Methods

Mice

C57BL/6, $CD4^{-/-}$, $CD8^{-/-}$ mice were originally purchased from The Jackson Laboratory; they were maintained at Emory University and used at 8-12-week-old. To generate $CD4^{2B4-/-}$ chimeric mice, $CD45.1^{+}$ mice received 1200 cGy irradiation and 16×10^{6} $CD4^{-/-}$ $Thy1.2^{+}$ bone marrow (BM) cells mixed with 4×10^{6} $2B4^{-/-}Thy1.1^{+}$ BM cells. Control chimeric mice received wild-type (WT) BM cells instead of $2B4^{-/-}$ cells. $CD8^{2B4-/-}$ chimeric mice were generated by the same method.

CLP model

Sepsis was induced by CLP (two punctures with a 25-gauge needle) following the method of Baker et al. (9). Septic animals received subcutaneously antibiotics (50 mg/kg ceftriaxone and 35 mg/kg metronidazole) at 0, 12, 24, and 36 hours after surgery. For anti-2B4 treatment, animals received anti-2B4 mAb (clone 2B4, 250 mg per dose, i.p.; Bio X Cell) on days 0, 2, 4, and 6 after CLP. For NK cell depletion, anti-asialo GM1 (100 mg i.p.) was given 1 day before CLP.

Human patients

Healthy donor and septic patient PBMCs were isolated under Emory University Institutional Review Board protocol no. 00002503. Blood was collected from septic patients within the first 24 h of meeting the consensus clinical definition of sepsis. Patients' demographics are provided in Table 2.1.

Flow cytometry and intracellular cytokine staining

Murine 2B4 expression was assessed using clone eBio244F4 (eBioscience). CountBright Beads (Thermo Fisher) were used to determine absolute cell numbers. For intracellular cytokine staining

(ICCS), splenocytes were incubated with 30 ng/ml PMA and 400 ng/ml ionomycin in the presence of GolgiStop for 4 hours at 37℃. Apoptotic cells were determined by caspase 3/7 staining (Thermo Fisher).

Statistics

The Student t test, Mann–Whitney U test, and log-rank test were used. Data are presented as mean ±SEM.

2.7 Figures and Figure Legends

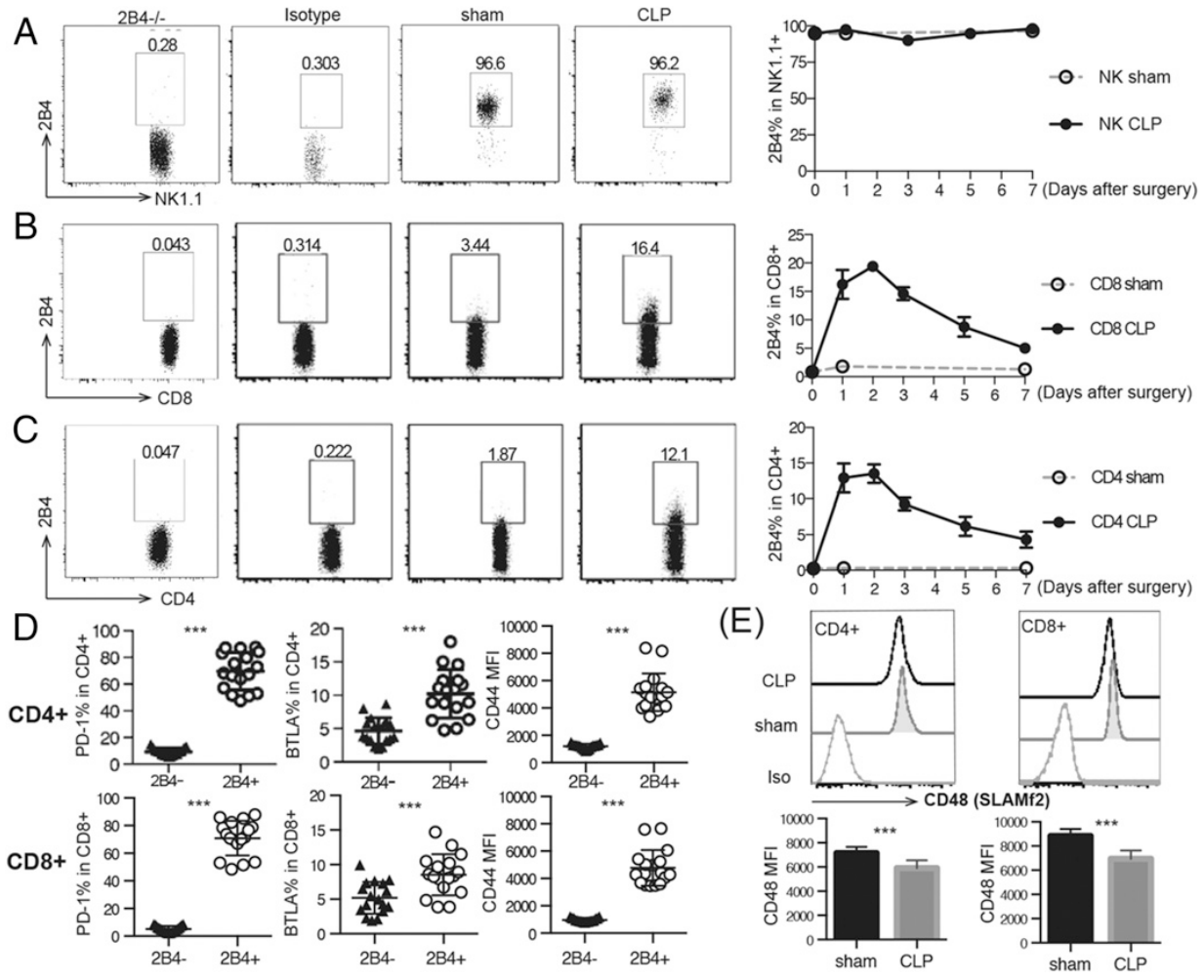

Figure 2.1. 2B4 expression is upregulated on T cells following CLP.

WT mice underwent CLP or sham surgery and were sacrificed at the indicated time points. Splenocytes were harvested and stained for NK cells (A), $CD8^+$ T cells (B), and $CD4^+$ T cells (C). Isotype control and $2B4^{-/-}$ animals were used to gate on $2B4^+$ cells. (D) PD-1, BTLA, and CD44 expression was assessed at 24 hours. (E) SLAMf2 expression was assessed at 24 hours. ***$p < 0.001$.

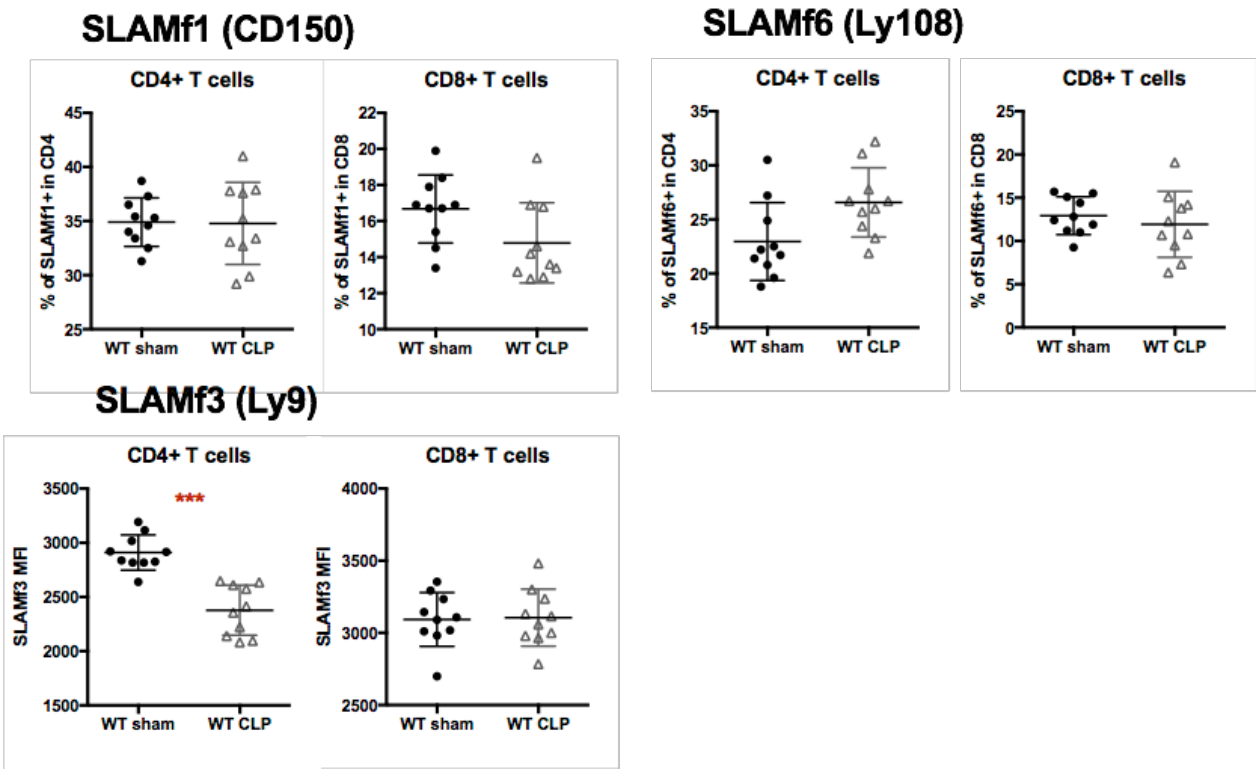

Figure 2.2 Other SLAM family expressions are examined after CLP.

WT mice underwent CLP or sham surgery and were sacrificed at 24 hours post-CLP. Splenocytes were harvested and stained for SLAMf1, SLAMf3, SLAMf6 at 24 hours. ***$p < 0.001$.

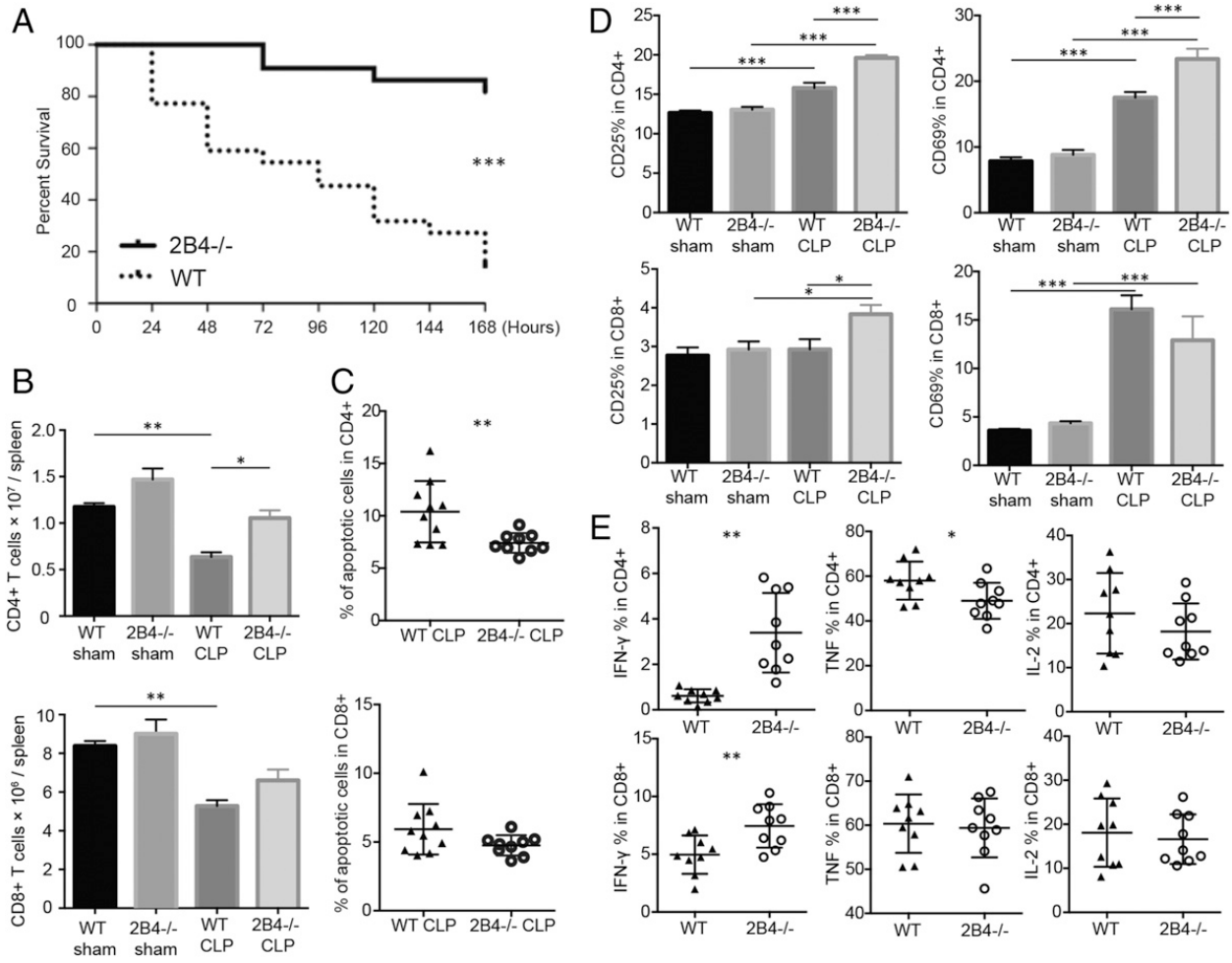

Figure 2.3 2B4-deficient mice are protected from CLP and exhibit increased effector T cell functions during sepsis.

(A) CLP was performed on WT and 2B4- deficient mice, and animals were monitored for survival for 7 days. (B) Splenocytes were harvested at 24 hours post-surgery, and lymphocyte counts were assessed by flow cytometry. (C) Cell apoptosis was measured by caspase $3/7^+$ $SYTOX^-$ in both groups. (D) Frequencies of $CD25^+$ and $CD69^+$ $CD4^+$ and $CD8^+$ T cells in WT mice and 2B4-deficient mice are shown. (E) For ICCS, splenocytes were stimulated in PMA and ionomycin for 4 hours, and cells were stained with IFN-γ, TNF, and IL-2. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

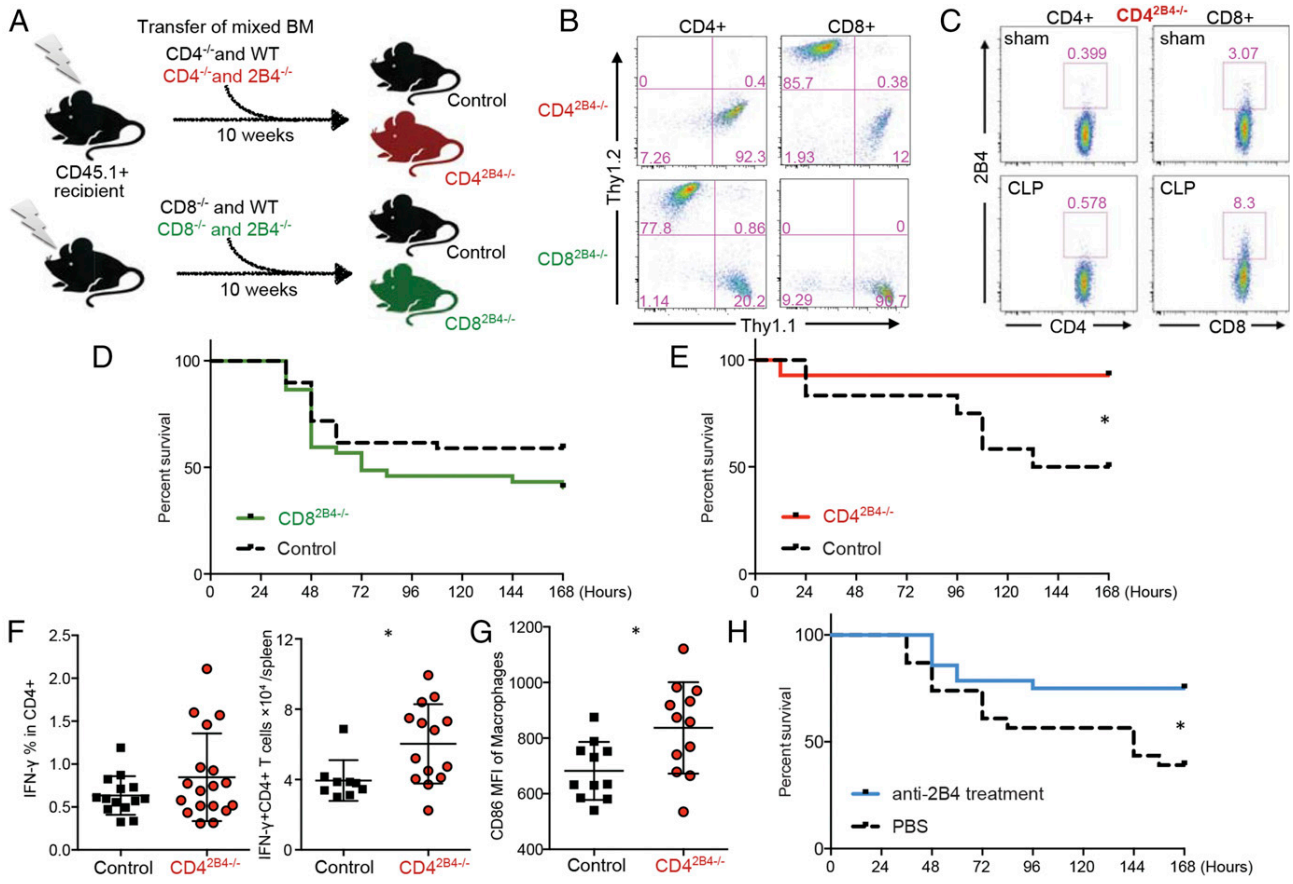

Figure 2.4 Loss of 2B4 specifically on CD4$^+$ T cells provides a survival benefit during sepsis, and blockade of 2B4 results in decreased mortality following CLP.

(A) Generation of CD4$^{2B4-/-}$ and CD8$^{2B4-/-}$ chimeric mice. (B) After 10 weeks of reconstitution, frequencies of CD4$^+$ and CD8$^+$ T cells were assessed (gated on CD45.2$^+$CD3$^+$). (C) Representative 2B4 expression on T cells from CD4$^{2B4-/-}$ chimeric mice after sham or CLP surgery. (D) Control chimeras and CD8$^{2B4-/-}$ chimeras were subjected to CLP and monitored for survival. (E) Control chimeras and CD4$^{2B4-/-}$ chimeric animals were subjected to CLP and monitored for survival. (F) Chimeric splenocytes were harvested at 24 hours post-CLP and stimulated for ICCS. Frequencies and numbers of IFN-γ-secreting CD4$^+$ T cells are shown. (G) Splenic macrophages (CD11b^{hi}CD11c^{lo}F4/80$^+$) were assessed for CD86 expression at 24 hours post-CLP. (H) WT mice underwent CLP and were treated with PBS or anti-2B4 mAb. *$p < 0.05$.

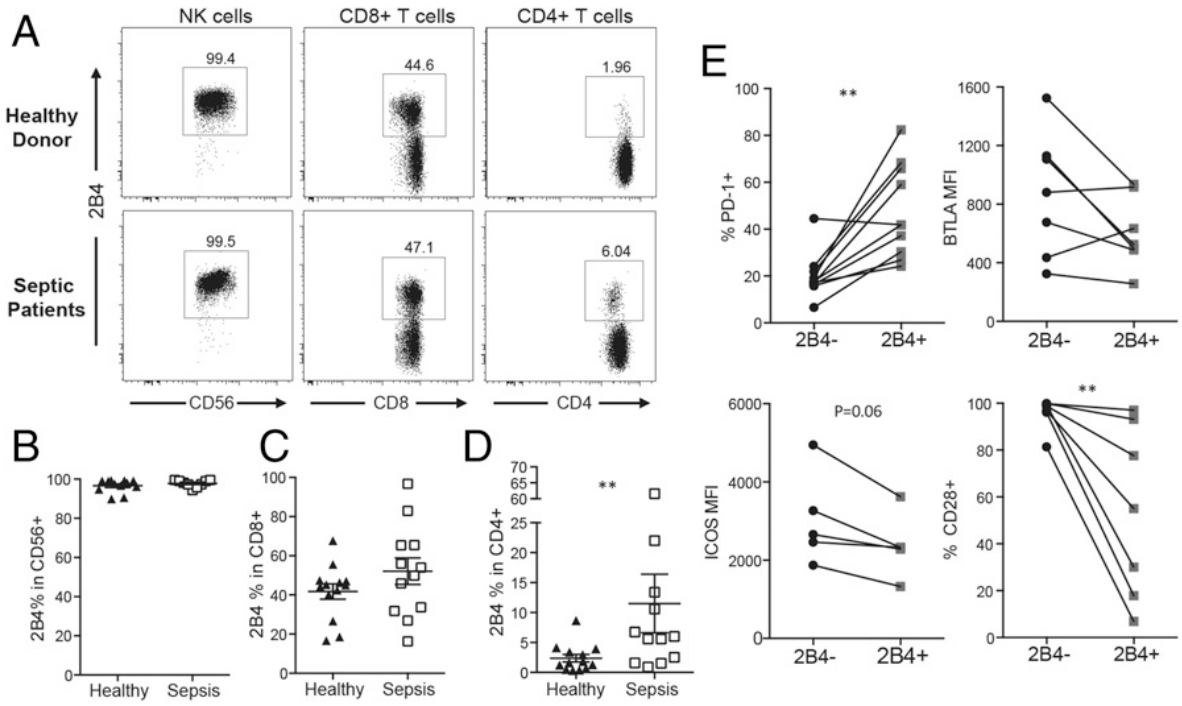

Figure 2.5 PBMCs isolated from septic patients exhibit upregulation of 2B4 on CD4$^+$ T cells.

PBMCs isolated from septic patients and healthy donors were analyzed for 2B4 expression on CD4$^+$ T cells, CD8$^+$ T cells, and NK cells. (A) Representative flow plots. (B-D) Summary data from 13 healthy and 12 septic patients per group. (E) Expression of PD-1, BTLA, ICOS, and CD28 on 2B4$^+$ and 2B4- CD4$^+$ T cells from septic patients. ** $p < 0.01$.

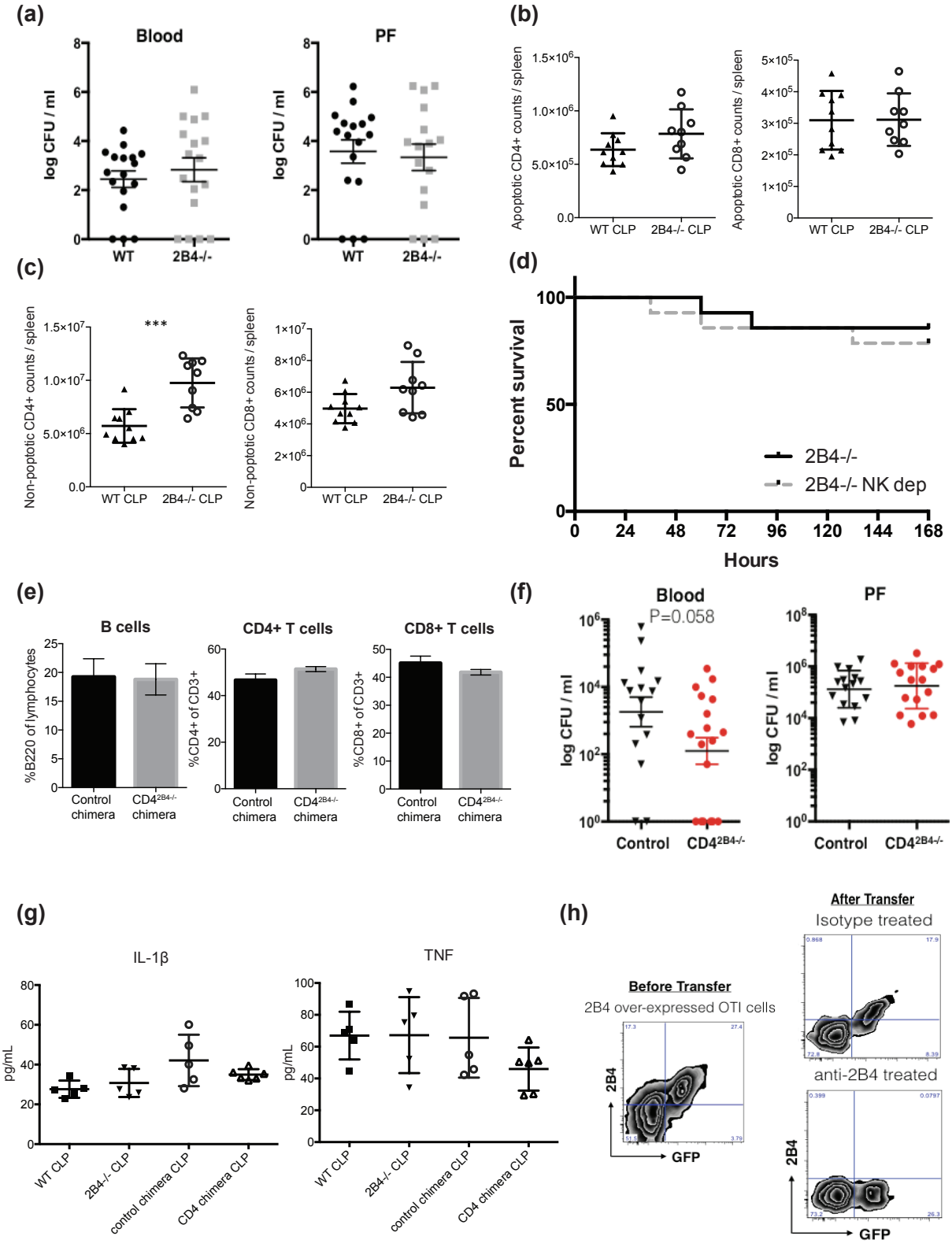

Figure 2.6 Supplemental figures and information

(a.) Bacterial cultures showed no difference between WT and $2B4^{-/-}$ animals. WT and $2B4^{-/-}$ animals were subjected to CLP. After 24 hours post-surgery, blood was collected by cardiac puncture and peritoneal fluid was obtained by injection of 2mL 0.9% sterile saline into the mouse peritoneum at 24 hours post-sepsis. Samples were serially diluted and a 100 μL aliquot of each sample was plated on sheep's blood agar plates and incubated at 37°C in 5% CO_2 for 24 hours. Bacterial colony counts were obtained from plates containing fewer than 300 colonies and the colony-forming units (CFUs) per ml of original specimen were determined. Data in this figure were generated by C.W. Chen and E. M. Burd.

(b.) $2B4^{-/-}$ and WT animals exhibited similar numbers of apoptotic cell after CLP. Age and gender matched WT and $2B4^{-/-}$ animals were subjected to CLP and sacrificed at 24 hours. Apoptotic cells were identified as Caspase3/7^{+}SYTOX^{-} and the absolute cell counts are determined.

(c.) $2B4^{-/-}$ animals retained higher number of non-apoptotic cell numbers after CLP. Age and gender matched WT and $2B4^{-/-}$ animals were subjected to CLP and sacrifice at 24 hours. Non-apoptotic and non-necrotic cells were identified as Caspase3/7^{-}SYTOX^{-} and the absolute cell counts are determined.

(d.) NK depletion in $2B4^{-/-}$ animals displayed no survival disadvantages. $2B4^{-/-}$ animals were treated with anti-asialoGM1 one day before surgery to deplete NK cell population. NK cells depletion has confirmed by flow cytometer (data not shown). The CLP survival was performed on $2B4^{-/-}$ animals and $2B4^{-/-}$ animals with NK cells depletion.

(e.) The lymphocyte compositions of chimera animals are the same. Blood samples collected from control chimera and $CD4^{2B4-/-}$ chimera mice prior to CLP and composition of individual immune cell compartments was analyzed.

(f.) Bacterial cultures showed no difference between $CD4^{2B4-/-}$ chimeric animals and control chimera. The control chimera and $CD4^{2B4-/-}$ chimeric animals were subjected to CLP. The blood

and peritoneal fluid were collected, and bacterial load was determined at 24 hours post-surgery. Data in this figure were generated by C.W. Chen and E. M. Burd.

(g.) Serum cytokine levels are similar between chimeric animals. Serum were collected and the level of TNF and IL-1β at 24 hours post-CLP in WT, $2B4^{-/-}$, control WT chimeric and $CD4^{2B4-/-}$ chimeric animals were measured by Bio-Plex ProTM (BIO-RAD).

(h.) Monoclonal anti-2B4 antibody (clone: 2B4) displayed no depletion effect on 2B4-expressing retrogenic OT-I cells. 2B4 over-expressed retrogenic OTI cells were generated by a previously described method [19]. Two million 2B4 over-expressed OTI cells were adoptively transferred into WT animals which were treated with intraperitoneal anti-2B4 mAb (250μg) to determine the depletion effect. Animals were sacrificed 24 hours after mAb treatment and transferred OTI T cells were analyzed by flow cytometry.

Table 2.1 Septic Patients Demographics

Table 1 – Septic Patients Demographics

Patient	Age	Gender	Weight (kg)	Race	Hospital LOS* (Days)	ICU LOS* (Days)	Intubated	Discharge status	Sepsis source	Sepsis Organism	WBC**	ALC***	2B4% of CD4+ T cells	2B4% of CD8+ T cells
#1	74	M	78	Black	4	3	Y	Dead	Respiratory	Bacilus species- not anthracis and coagulase negative Staphylococcus	25.5	2.5	22.0	31.8
#2	32	M	61.7	Black	18	2	N	Home	Urine	Escherichia coli and Stenotrophomonas maltophilia	3.3	0.1	1.5	65.4
#3	52	M	65.9	Black	17	7	Y	Skilled Nursing	Respiratory	Methicillin resistant staphylococcus aureus and Escherichia coli	14.9	0.4	5.6	65.5
#4	79	M	77	Black	10	4	Y	Skilled Nursing	Respiratory	Escherichia coli, Enterococcus faecalis, and Klebsiella pneumoniae ssp pneumoniae and Haemophilus influenzae	4.8	0.2	10.6	82.9
#5	87	F	82	Black	28	2	N	Skilled Nursing	Respiratory	Not identified	7.5	1	13.4	54.0
#6	19	F	64	Other	7	4	Y	Home	Respiratory	MSSA	7.5	0.8	61.6	96.8
#7	47	M	70	Caucasian	31	8	Y	Home	Urine	Staphylococcus aureus	12.5	0.4	2.5	16.3
#8	51	F	65	Black	11	10	Y	Hospice	Respiratory	Escherichia coli, yeast, Staphylococcus aureus, and Coagulase negative Staphylococcus	18.6	1.1	5.6	49.9
#9	49	M	90	Black	49	25	Y	Home	Respiratory	Klebsiella pneumonae	13.9	1	6.0	33.7
#10	33	M	136	Black	11	4	Y	Rehab	Blood	Not identified	7.1	1.4	0.9	45.9
#11	59	M	106	Black	21	2	Y	Home	Respiratory	Staphylococcus aureus, Alpha (Viridans) Streptococcus, Enterobacter aerogenes, Staphylococcus aureus	9.1	0.5	6.8	56.0
#12	46	M	121	Caucasian	30	16	Y	Rehab	Respiratory	Acinetobacter calcoaceticus-baumannii complex	11.2	2.1	1.6	26.9

* Length of stay (LOT) **White Blood cell Counts (WBC), ***Absolutely Lymphocyte Counts (ALC)

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Chapter 3. 2B4 but not PD-1 blockade improves mortality in septic animals with pre-existing malignancy

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3.1 Abstract

In addition to its well-known beneficial effects for the treatment of several types of cancer, PD-1 blockade has shown encouraging results in pre-clinical models of sepsis and in a recent clinical trial in sepsis. Because cancer is the most common co-morbidity in septic patients, here we aimed to determine the efficacy of PD-1 checkpoint blockade in the setting of sepsis complicated with pre-existing malignancy. While previous reports demonstrate that PD-1 blockade shows efficacy in models of both cancer and sepsis separately, in a model of established lung cancer followed by cecal ligation and puncture (CLP)-induced sepsis, PD-1 blockade exhibited no therapeutic effect on sepsis survival. Mechanistically, this diminished efficacy of PD-1 blockade in cancer septic animals was characterized by a reduction in both the quality and quantity of PD-1^+ responder cells. Specifically, $CD8^+$ T cells isolated from cancer septic animals exhibited decreased CD28 expression and a reduction in the $CXCR5^+PD\text{-}1^+$ subset. Further, flow cytometric analysis of T cells isolated from cancer septic animals revealed that 2B4 is another possible checkpoint blockade target under these conditions. Administration of anti-2B4 to cancer septic animals significantly improved sepsis survival and was associated with increased in T cell co-stimulatory receptor expression and decreased co-inhibitory receptor expression. These results illustrate unique functions of distinct inhibitory receptors in the setting of sepsis complicated with cancer.

3.2 Introduction

Every year, more than 1.7 million patients in the United States develop sepsis (1-3). The most common co-morbidity in septic patients is cancer, present in 19% of septic patients, resulting in about 200,000 cancer septic patients per year (1, 4). Cancer patients also have a relative risk of 9.77 to develop sepsis, and a relative risk of 2.77 to be hospitalized with sepsis compared to non-cancer patients (5, 6). Among those cancer septic patients, lung cancer (18%) is the most prevalent type of cancer (5, 7). Importantly, the in-hospital mortality was significantly higher for cancer septic patients compared to non-cancer (37.8% versus 24.9%) (5, 8, 9).

The immune responses to cancer and sepsis share several similarities, including the induction of T cell exhaustion. In the tumor microenvironment, continual exposure to tumor antigens leads to T cell exhaustion, including up-regulation of co-inhibitory receptors and decreased cytokine production and cytotoxicity (10-12). Furthermore, this deficiency in the adaptive immune system functions systemically, resulting in increased expression of co-inhibitory molecules on T cells in the circulation, inadequate response to vaccination and an increase in opportunistic infections (13-15). While the mechanisms leading to T cell exhaustion during sepsis remain unknown, the phenotypes of T cell exhaustion are well characterized (16). Postmortem analysis of septic patients and a plethora of animal studies on sepsis reveal that reduced number of T cells, increased expression of co-inhibitory receptors, and decreased cytokine production are observed in sepsis (17). Given this evidence of immunosuppression during sepsis, several checkpoint blockade therapies have been investigated in different animal sepsis models. PD-1/PD-L1 blockade has also entered sepsis clinical trials with encouraging results (18, 19). For animal studies, administration of anti-PD-1 and anti-PD-L1 both result in significantly improved CLP survival (20, 21). Blockade of PD-1 also increases the survival in the second hit sepsis model (CLP followed by Candida infection (22)). Mechanistically, PD-1/PD-L1 blockade leads to decreased cell apoptosis during

sepsis, increased Bcl-xL expression on T cells, and enhanced bacterial clearance (20, 21). Other co-inhibitory molecules such as CTLA-4, BTLA, and 2B4 also exhibit some therapeutic effect during sepsis. CTLA-4 blockade also resulted in increased survival in the CLP model and decreased cell apoptosis in splenocytes (22, 23). Anti-BTLA antibody treatment in a model of dual insults with hemorrhagic shock and sepsis resulted in enhanced recruitment and functionality of innate immune cells; however, anti-BTLA reduced overall survival in this specific sepsis model (24, 25). Administration of anti-2B4 antibody increased CLP survival, and studies on 2B4-deficient animals demonstrated the protection is $CD4^+$ T cell dependent. (26). Taken together, these studies highlight the potential therapeutic implications of checkpoint blockade during sepsis.

Efficacy of PD-1/PD-L1 blockade has been shown to be dependent on several mechanisms in both models of cancer and chronic virus infection. First, CD28 expression on T cells is vital for PD-1 therapy. Blockade of B7 signaling or conditional-knockout of CD28 on CD8 T cells results in ineffective tumor control and inadequate T cell response after PD-1 therapy. Moreover, the proliferating $CD8^+$ cells from cancer patients receiving PD-1 therapy are predominately $CD28^+$ cells (27, 28). Second, $CXCR5^+PD\text{-}1^+$ population provides the proliferative burst after PD-1 therapy. During chronic virus infection, $CXCR5^+PD\text{-}1^+$ cells are found in lymphoid tissues and express higher co-stimulatory receptors and memory-related transcription factors. After PD-1 blockade, $CXCR5^+$ populations are capable of expanding and differentiating into $CXCR5^-$ cells to mediate tumor control (29). Importantly, $CXCR5^+$ $CD8^+$ T cells with high functionality have been found in both circulation and tumor microenvironment on human pancreatic cancer and lung cancer (30, 31). Third, host PD-L1 expression is crucial for PD-1 therapy. By using $PD\text{-}L1^{-/-}$ mice, two groups have shown that PD-L1 expression on host APCs but not tumor cells determine the efficacy of PD-1 blockade therapy (32, 33). Additionally, in metastatic melanoma and ovarian

carcinoma patients treated with checkpoint blockade therapies, patients with clinical response exhibited significantly higher PD-L1 expressed on host APC compared to the non-responders (32).

Here, we thought to determine the efficacy of PD-1 checkpoint blockade in a cancer sepsis model. In this study, we found that anti-PD-1 blockade failed to impact sepsis mortality in animals with pre-existing cancer and was associated with a reduced number of $CXCR5^{+}PD\text{-}1^{+}$ cells and lower CD28 expression on $PD\text{-}1^{+}$ cells. On the other hand, anti-2B4 blockade conferred improved sepsis survival in cancer animals. This study highlights the differential impact of PD-1 checkpoint blockade in cancer septic animals and reveals the non-redundant roles of two individual checkpoint inhibitors in the setting of cancer and sepsis.

3.3 Results

Anti-PD-1 administration failed to exert a therapeutic effect on cancer-bearing animals in sepsis

In order to determine anti-PD-1 efficacy during sepsis in cancer-bearing animals, we first tested the delayed anti-PD-1 administration protocol described by Brahmamdam et al. (20) In brief, LLC1 tumor cells were subcutaneously implanted in the right thigh of 8-12-week-old C57BL/6J mice. After three weeks of tumor growth, animals were subjected to CLP for sepsis induction. Anti-PD-1 antagonistic mAb (clone RMP1-14) was then given at 48hr and 72hr post-CLP. In contrast to its known effect on previously healthy (PH) animals, PD-1 blockade treatment failed to improve CLP survival in cancer animals: 57% of isotype-treated animals survived (11 of 19 animals) while only 37% of anti-PD-1 treated animals survived (7 of 19 animals) (Figure 3.1 A). It is known that PD-1 blockade improves sepsis survival by decreasing lymphocyte apoptosis and increasing anti-apoptotic protein expression. To further evaluate anti-PD-1 efficacy on a cellular level, we assessed caspase3/7 activity and anti-apoptotic protein Bcl-xL expression on $CD4^+$ and $CD8^+$ T cells isolated from both PH and cancer animals. In PH animals, anti-PD-1 was able to reduce cell apoptosis and increase Bcl-xL expression on both $CD4^+$ and $CD8^+$ T cells as previously described (20). However, in cancer animals, the anti-PD-1 effect was diminished, as both caspase3/7 and Bcl-xL expression were unchanged compared to isotype treatment (Figure 3.1 B, C). Thus, PD-1 blockade is ineffective at inhibiting lymphocyte apoptosis in cancer septic animals.

We previously demonstrated that T cells isolated from LLC1 cancer animals displayed higher co-inhibitory receptor expression at the baseline compared to PH animals. It is possible that PD-1 signaling on T cells is already impelled before the CLP insult. Therefore, we adjusted our administration time points from delayed time points (D2, D3 post-CLP) to early anti-PD1 blockade (D0, 2, 4 and 6 post-CLP, Clone 29F.1A12). However, PD-1 blockade still failed to improve survival in cancer septic animals (Figure 3.1 D).

Both PD-1 and PD-L1 are highly expressed during cancer sepsis

We next sought to determine the mechanisms underlying the ineffectiveness of PD-1 blockade in the setting of cancer and sepsis. We first confirmed adequate expression of both the receptor and ligand in the setting of cancer sepsis. In previously healthy animals, PD-1 expression on T cells was significantly increased after the CLP insult (20, 21). However, in cancer septic animals, PD-1 expression on both $CD4^+$ and $CD8^+$ T cells was maintained at the same level as sham surgery controls from Day 1 to Day 3 post-CLP (Figure 3.2 A). We also tested the PD-L1 expression on host antigen-presenting cells (APCs) to confirm whether cancer septic APCs could promote negative signaling through PD-1/PD-L1 pathway. Results showed that dendritic cells, macrophages and MDSC-liked cells in spleen all strongly up-regulated PD-L1 expression after sepsis (Fig 3.10).

Cancer sepsis is associated with reduced CD28 expression and lower frequencies of $CXCR5^+$ $PD\text{-}1^+$ stem cell-like $CD8^+$ T cells

Several recent publications have defined the mechanisms by which PD-1 blockade works in models of chronic viral infection. First, Kamphorst et al. showed that CD28 expression is absolutely required for PD-1 blockade to rescue $CD8^+$ T cell responses (27). Thus, we queried whether CD28 expression was reduced during sepsis. $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ from sham cancer animals and 24-hour post CLP cancer animals were stained with a series of markers (Figure 3.2 B, C). For activation markers on $CD4^+PD\text{-}1^+$ cells, although CD44 expression was similar between sham and CLP groups, the percentage of $CD69^+$ cells were significantly increased in CLP group, indicating that cells in the $PD\text{-}1^+$ population became activated during CLP. Interestingly, we found that CD28 expression was significantly reduced on septic $CD4^+PD\text{-}1^+$ T cells (Figure

3.2 B). For $CD8^+PD\text{-}1^+$ cells, an increased percentage of $CD69^+$ was also observed. Of note, $CD8^+PD\text{-}1^+$ cells dramatically lost CD28 expression during sepsis, similar to $CD4^+PD\text{-}1^+$ cells (Figure 3.2 C). Thus, compromised CD28 expression on both $CD4^+$ and $CD8^+$ T cells may underlie the reduced efficacy of PD-1 blockade during cancer sepsis.

Im et al. recently showed that $CXCR5^+$ $PD\text{-}1^+$ $CD8^+$ T cells exhibit self-renewal potential and the ability to reconstitute the response following PD-1 blockade in the setting of chronic viral infection. $CXCR5^+$ $PD\text{-}1^+$ responder cells are predominantly $TIM\text{-}3^-$ and $2B4^-$ (29). We thus tested the hypothesis that PD-1 blockade may be ineffective during cancer sepsis due to a dearth in this critical population of $CXCR5^+$ $PD\text{-}1^+$ $CD8^+$ T cells. Interestingly, we observed that the percentage of $CXCR5^+$ of $PD\text{-}1^+CD8^+$ cells in splenocytes is significantly declined in CLP animals at 24 hours after surgery compared to sham group (Figure 3.3 A, B). Furthermore, the absolute cell counts of $CXCR5^+PD\text{-}1^+$ $CD8^+$ in splenocytes also significantly decreased in CLP animals at 24 hours post CLP (Figure 3.3 C). Analysis of their co-inhibitory molecules on $PD\text{-}1^+$ $CD8^+$ T cells showed no difference in TIM-3 expression but significantly increased expression of 2B4 (Figure 3.3 D), suggesting that cancer animals lost the PD-1 blockade responder cell populations during sepsis. Therefore, not only the qualities of the $PD\text{-}1^+$ cells but also the quantity of the responder population is reduced during sepsis.

SPADE and CITRUS analysis revealed unique $CD8^+$ T cell populations in cancer septic animals

Given these results suggesting that the factors critical to the effectiveness of PD-1 blockade are lacking during cancer sepsis, we sought to identify additional coinhibitory signals that might be effectively targeted in order to control dysregulated T cell responses during sepsis. Cancer and PH animals both received CLP and sacrificed at 24-hours post-surgery. Splenocytes were harvested and stained with CD44, 2B4, PD-1, BTLA, and LAG-3. $CD3^+CD8^+$ cells were gated in

FlowJo and exported to Cytobank to perform algorithm analysis. These algorithms provide an unbiased and comprehensive analysis rather than traditional two dimensional and hierarchical analysis using FlowJo. First, Spanning-tree Progression Analysis of Density-normalized Events (SPADE) was performed, and use of 50 nodes conferred better resolution in our model. After adjustments, multiple t-test comparisons identified two distinct clusters as being significantly elevated in cancer septic animals compared to PH septic animals (Figure 3.4 A, B). The first cluster, located on the right side of the SPADE tree-like figure, contained cells expressing a $CD44^{hi}PD\text{-}1^{hi}2B4^{hi}LAG\text{-}3^{int}$ phenotype compared to total $CD8^{+}$ (Figure 3.4 A, C). The second cluster up-regulated in cancer septic animals contained cells possessing a $CD44^{hi}PD\text{-}1^{lo}2B4^{hi}LAG\text{-}3^{int}$ phenotype (Figure 3.4 A, C).

Next, the CITRUS (cluster identification, characterization, and regression) algorithm was performed on the same data sets to confirm the SPADE findings. CITRUS is a specific algorithm for fully automated discovery of statistically significant differences between groups. By choosing Nearest Shrunken Centroid (PAMR) association model and minimum false discovery date (FDR) in CITRUS, the algorithm identified three populations that are changed between cancer septic animals and PH septic animals (Figure 3.5 A). The summary plots and CITRUS plot showed two groups of cells are increased in cancer septic animals: 1.) Node-209985; 2.) Node-209991 and 209995 (clustered together) (Figure 3.5 A, B). The phenotype of cells in the first node 209985 is $CD44^{hi}PD\text{-}1^{lo}2B4^{hi}LAG\text{-}3^{int}$; and the phenotype of cells in the second group 209991 and 209995 are $CD44^{hi}PD\text{-}1^{hi}2B4^{hi}LAG\text{-}3^{int}$ (Figure 3.5 C, Figure 3.11). Notably, these results are consistent with our SPADE data. We also performed CITRUS on $CD4^{+}$ T cells and observed that similar populations ($CD44^{hi}PD\text{-}1^{lo}2B4^{hi}$ and $CD44^{hi}PD\text{-}1^{hi}2B4^{hi}$) that are increased in cancer septic animals (Figure 3.12).

Finally, to further confirm this result, traditional flow analysis was performed by manually gated on 2B4 and PD-1 (Figure 3.6 A). Both $2B4^{+}PD1^{-}$ and $PD1^{+}2B4^{-}$ in $CD8^{+}$ T cells are increased in cancer septic animals compared to PH septic animals (Figure 3.6 B). Thus, these data suggest that 2B4 is expressed and potentially functioning on both overlapping and unique populations relative to PD-1 in cancer septic animals. Because anti-PD1 was ineffective at improving sepsis survival in the setting of cancer sepsis, we shifted out interest toward blockade of 2B4 signaling in cancer septic animals.

Blockade of 2B4 improves cancer septic animal survival

In order to determine the timing of 2B4 blockade, we first assessed 2B4 expression kinetics on T cells in cancer septic animals. 2B4 expression in $CD4^{+}$ and $CD8^{+}$ T cells was significantly increased at day 1 and day 2 post-CLP and then decreased at day 3 post CLP (Figure 3.7 A). The timing of peak expression of 2B4 is similar to PH septic animals; therefore, we decided to treat the cancer septic animals following the previously described protocol (26).

To determine the efficacy of 2B4 blockade in the setting of cancer sepsis, animals were treated with anti-2B4 or isotype after CLP at day 0, 2, 4, 6. Results indicated that anti-2B4 significantly improves sepsis survival in cancer animals (39% survival in anti-2B4 treated verses 13% survival in isotype treated, Figure 3.7 B). To understand the impact of 2B4 blockade on T cell dysregulation during cancer sepsis, cancer septic animals after receiving one dose of anti-2B4 treatment were sacrificed at 24 hours post CLP. One mechanism that leads to T cell dysfuntion during sepsis is the loss of co-stimulatory molecules and increased co-inhibitory molecules on T cells. Therefore, we assessed the co-stimulatory receptor expression on $CD4^{+}$ T cells and found that anti-2B4 treatment significantly increased CD48 expression (Figure 3.8 A), which may provide adjacent T cells with stronger CD2 co-stimulation signals during sepsis. No change in

CD28 expression was observed, but significantly lower ICOS expression on $CD4^+$ T cells in anti-2B4 treated animals was noted (Figure 3.8 A). The expression of CD48 was significantly elevated, and CD28 exhibited a trend toward increased expression after 2B4 blockade on $CD8^+$ T cells during sepsis (Figure 3.8 B).

Next, co-inhibitory receptor expression on T cells after treatments was determined. Interestingly, anti-2B4 treatment significantly decreased PD-1 expression on $CD4^+$ T cells but not $CD8^+$ T cells and significantly reduced TIGIT expression on $CD8^+$ T cells but not on $CD4^+$ T cells (Figure 3.8 C, D). Strikingly, we found that anti-2B4 treatment significantly down-regulated CTLA-4 on both $CD4^+$ and $CD8^+$ T cells (Figure 3.8 C, D).

Further, anti-2B4 treatment significantly reduced both the frequency of $Foxp3^+$ among $CD4^+$ cells and the MFI of Foxp3 within that subset (Figure 3.9 A-C). The decrease in Foxp3 expression in Treg cells after 2B4 blockade (Figure 3.9 D) may indicate a loss of suppressive function and/or higher Treg plasticity following 2B4 blockade. Thus, we next examined GITR and CTLA-4 expression in Treg cells, which are both important for Treg cells to regulate immune response. We found no different in GITR expression, but we also observed that CTLA-4 expression in $Foxp3^+$ cells is significantly decreased after 2B4 blockade, suggesting Treg in 2B4 blockade group may exhibit lower suppressive activity (Figure 3.9 E). We also confirmed that CTLA-4 expression is significantly reduced in T conventional population (Figure 3.9 F).

3.4 Discussion

In this study, we showed that anti-PD1 blockade did not improve sepsis survival in cancer animals. This impaired efficacy of PD-1 blockade was associated with a decrease in CD28 expression on PD-1^+ cells and a loss of PD-1^+CXCR5^+ $CD8^+$ stem cell-like responder cells during sepsis. Next, multi-parameter flow analysis identified the expression of another co-inhibitory receptor, 2B4, as a potential therapeutic target in cancer septic animals. Importantly, we found that blocking 2B4-mediated co-inhibitory signals is sufficient to improve sepsis survival in cancer animals. These results highlight the importance of personalized therapy in sepsis and highlight the potential utility of animal models in modeling the physiology of comorbid conditions that may impact sepsis pathophysiology.

Altered sepsis pathophysiology has been previously described in hosts with pre-existing malignancy. For example, the inhibition of lymphocytes apoptosis is well-known to improve sepsis survival in previously healthy animals (37-39). However, in pre-existing cancer animals, prevention of lymphocyte apoptosis by overexpression of Bcl-2 or using $Bim^{-/-}$ mice lead to increase mortality in pneumonia sepsis model (40). Moreover, we have previously shown that during sepsis, the dysregulated host response to infection may be exaggerated in cancer animals compared to previously healthy animals (35, 41). Significantly increased activation markers (CD25, CD69) and co-inhibitory receptors (PD-1, 2B4) expression were found on both $CD4^+$ and $CD8^+$ T cells. In addition, increased TNF secretion was observed following ex vivo T cell stimulation. These results demonstrate that the host response to sepsis in cancer animals is characterized by increased activation and inflammation relative to that observed in healthy animals (35). Therefore, blockade of PD-1 may be insufficient to overcome the immune dysregulation in cancer septic animals, and potentially may even further amplify the overwhelming inflammation during cancer sepsis.

Other immunologic features known to be required for optimal response to PD-1 blockade were dysregulated in cancer septic hosts. First, Kamphorst et al. (27) found that CD28 signals are absolutely required for the efficacy of PD-1 blockade. CD28 deletion on T cells results in impaired expansion during PD-1 blockade on virus infection model. Further, Kamphorst et al. (28) demonstrated that in non-small lung cancer patients treated with PD-1 targeted therapy, the responding PD-1$^+$Ki-67$^+$ cells highly expressed CD28 costimulatory molecules. These results both emphasized that PD-1$^+$ T cells require costimulation signals in order to respond to PD-1 blockade therapy. In our cancer sepsis model, PD-1$^+$ cells lost CD28 and increased other coinhibitory molecules during sepsis, indicating those PD-1$^+$ T cells exhibit lower responding capacity to PD-1 blockade therapy. In addition, Im et al (29) found that CXCR5$^+$ CD8$^+$ T cells correlated with response to PD-1 blockade. Interestingly, the PD-1 blockade responder cells (PD-1$^+$CXCR5$^+$) are predominately 2B4- and have transcriptional profile characteristic of memory precursor T cells with the expression of Bcl-6, TCF-1. In contrast, the PD-1$^+$2B4$^+$ cells were mostly CXCR5- with more terminal differentiation profiles and are incapable of responding to PD-1 blockade. In our study, we found decreased frequencies of CXCR5$^+$ cells and increased frequencies of 2B4$^+$ cells among PD-1$^+$ T cells in cancer septic animals (Figure 3.3), suggesting the composition of the PD-1$^+$ population during sepsis shifted toward becoming more terminally differentiated. Moreover, these data are also consistent with the SPADE and CITRUS data (Figure 3.4, Figure 3.5) that PD-1hi2B4hi was identified in cancer septic animals when compared to PH septic animals. Taking together, we have shown that both the loss of the quality and quantity of PD-1$^+$ cells may lead to the lack of efficacy of PD-1 blockade in cancer septic animals.

SPADE and CITRUS analysis revealed that 2B4 and PD-1 expressing CD8$^+$ T cells constitute different populations during sepsis, suggesting these co-inhibitory receptors may have different roles during sepsis. These data are similar with our previously published data on CD4$^+$ T cells,

which we found one population is $2B4^{hi}PD1^{lo}CD4^{+}$ T cells and $2B4^{lo}PD1^{hi}CD4^{+}$ T cells are elevated in cancer septic animals (35). Similar populations are identified in both $CD4^{+}$ and $CD8^{+}$ T cells (Figure 3.12) strength the idea that 2B4 and PD-1 play distinct functions during cancer sepsis. We previously showed that 2B4-deficient animals are protected from CLP and blockade of 2B4 on previously healthy WT animals improves sepsis survival. We found that the survival benefits of 2B4-deficient animals are derived from increase IFN-γ secretion on $CD4^{+}$ T cells (26); however, the mechanisms underlying the 2B4 blockade remain unclear. Here we demonstrated anti-2B4 treatment increases CD48 expression and decreases PD-1 and CTLA-4 expression on T cells. CD48 is the ligand for CD2, which provides another T cell co-stimulatory signal. We speculate that during sepsis, T cells might rely more on co-stimulatory signals downstream of CD2 due to a significant decline in CD28 expression (Fig 3.8 A, B). Blockade of 2B4 not only reduces the inhibitory signaling to T cells but also decreases competition for CD48, potentially allowing increased CD2 co-stimulatory signaling. These possibilities warrant further investigation. Interestingly, PD-1 blockade does not affect 2B4 expression on T cells (data not shown). These results strengthen the idea that 2B4 and PD-1 play distinct and unique roles in the pathophysiology of sepsis in cancer animals. However, we are not able to rule out a role for other 2B4-expressing immune cells, such as NK cells and gamma-delta T cells in our model.

One of the limitations of this study is that the result might be tumor-type specific or sepsis model-specific. LLC1 is Lewis lung carcinoma cell line, which is highly tumorigenic and widely used as a model of metastasis and to evaluate the efficacy of therapeutic agents (42). We chose LLC1 as our tumor model for several reasons: first, lung cancer represents the most common cancer type in cancer septic patients. Second, PD-1 blockade therapy has previously been shown to not reduce LLC1 tumor size in the mouse model (32); this was also true in our system (data not shown), allowing us to isolate the impact of PD-1 blockade on cancer sepsis survival.

This study emphasizes the complexity and contradiction in pre-existing host comorbidity with sepsis. Despite the negative results using PD-1 blockade in our model, targeting immune dysfunction via 2B4 blockade remains a promising direction for sepsis with cancer comorbidity. Our results should be repeated using different types of tumors and other models of sepsis to determine the generalizability of the impact of PD-1 or 2B4 blockade in cancer septic hosts. In the future, combination therapy targeting multiple checkpoints could represent promising strategies for successful immune therapy in the setting of cancer sepsis. For example, we observed that $CD4^{+}PD\text{-}1^{+}$ T cells in cancer animals up-regulated CD127 (IL-7 receptor) during sepsis (not shown); therefore, combined PD -1 blockade IL-7 administration could be investigated (43). Furthermore, clinical factors or potential biomarkers should be considered carefully in the criteria for future clinical sepsis trials aimed at checkpoint therapy. The septic patients with cancer comorbidity should be excluded from general septic patient populations and precision medicine strategies should be developed to more optimally improve sepsis-induced immune dysregulation in the setting of pre-existing malignancy.

3.5 Materials and Methods

Animals and cancer model

C57BL/6 mice with age from 8-12 weeks old, both male and female were purchased from Jackson Laboratory. All animals were housed and maintained by following Emory IACUC guidelines. Animal studies were approved by Emory IACUC (Protocol: DAR-2003199-ELEMNT-N). For cancer animal model, 500,000 murine lung cancer cell line LLC1 (ATCC, CRL-1642) cells were implanted in the right thigh via subcutaneous injection. After three weeks, the cancer-bearing animals were randomly divided into different groups for experiments. LLC1 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% FBS, Penicillin-Streptomycin (100 U/ml, Thermofisher) and HEPES (0.01M, Sigma). The LLC1 cancer cell culture and cryopreservation methods were followed by ATCC guidelines.

Cecal ligation and puncture

Polymicrobial animal sepsis model, cecal ligation and puncture (CLP) was performed to induce sepsis on cancer animals. Following by the method of Baker et al. (36), the cecum was ligated and punctured twice with a 25-gauge needle. After surgery, septic animals received 1ml of subcutaneous saline at 0h, and four does of antibiotics (50 mg/kg ceftriaxone and 35 mg/kg metronidazole, Sigma) at 0, 12, 24, and 36 h. Sham surgery was performed as a control group. Animals were provided with pain medication (0.1 mg/kg buprenex, McKesson Medical) to minimize pain before the surgery.

Antibody blockade

For anti-PD-1 treatment, animals were treated with two different dosing strategies. The delayed administration of anti-PD-1 was followed by the method of Brahmamdam et al. (20) 250μg of PD-

1 antagonistic mAb (clone: RMP1-14, isotype Rat IgG2a, Bio X Cell) or isotype control antibody were administered by intraperitoneal injection on days 1, 2 after CLP. An alternative method of anti-PD1 blockade was performed by administration of 250μg anti-PD-1 mAb (clone: 29F.1A12, isotype Rat IgG2a, Bio X Cell) on days 0, 2, 4, and 6 after CLP. For anti-2B4 treatment, animals received anti-2B4 mAb (clone 2B4, 250 mg per dose, i.p.; Bio X Cell) on days 0, 2, 4, and 6 after CLP.

Flow cytometry antibodies and Reagents

Antibodies (clone) for flow cytometry were purchased from the following companies: BioLegend: CD2(RM2-5), CD8 (53-6.7), CD28 (E18), CD44(IM7), CD127(A7R34), CD62L(MEL-14), CD48(HM48-1), Tim-3(RMT3-23), PD-1 (29F1A12), ICOS (C398.4A), CTLA-4 (UC10-4B9), LAG-3 (C987W), TIGIT (IG9), CXCR5(L138D7) and PD-L1(10F.9G2). eBioscience Thermo Fisher Scientific: 2B4 (eBio244F4), BTLA (6F7) and Foxp3 (FJK-16s). BD Pharmingen: CD3 (500A2) and CD4(RM4-5). Cell signaling: Bcl-xL (54H6). For Foxp3, Bcl-XL and total CTLA4 staining, splenocytes were stained with surface markers then fixed and permeabilized by eBioscienceTM Foxp3/Transcription Factor Staining Buffer Set (Catalog: 00-5523-00). CellEventTM Caspase-3/7 Green Flow Cytometry Assay Kit (Catalog: C10427) was used for activated Caspase 3/7 staining. Absolute cell counts were obtained by utilized CountBrightTM Absolute Counting Beads (Catalog: C36950). Flow analysis was performed on Flowjo and Cytobank.

SPADE and CITRUS analysis

Cancer and previously healthy animals were subjected to CLP, and 24 hours post CLP, splenocytes were collected and stained for CD3, CD4, CD8, CD44, BTLA, PD-1, 2B4, LAG-3. Files contained

only $CD3^+CD8^+$ was exported from traditional flow data (FlowJo, LLC) and uploaded to Cytobank (Cytobank.org) for automated analysis. Two different algorithms were performed: SPADE (Spanning-tree Progression Analysis of Density-normalized Events) and CITRUS (cluster identification, characterization, and regression). For SPADE analysis, previously healthy septic animals were set as baseline and cancer septic animals were defined as the fold changed group. The node size was defined as 50 nodes to have at least 100 cells in one node. The SPADE trees were generated by the parameter "percent total ratio log" to visualize the difference between groups. For SPADE statistic, the percent of total $CD8^+$ cells in each node was compared by multiple t-tests with adjust p values (FDR=1%), and significantly different nodes were grouped into clusters based on the phenotypically similarly. For CITRUS, the PAMR association model and minimal FDR were selected. CITRUS program generated the tree-like figures, phenotype figures, defined clusters, and statistics automatically without manual input.

Statistics

The Student t-test, multiple t-test, one-way ANOVA, and log-rank test were used. The outlier is removed by performing Grubbs' test. Data are presented as mean ± SEM. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

Author Contributions

C.W.C, C.M.C and M.L.F. designed and directed the project; C.W.C performed and analyze all the experiments; W.Z. and J.X. helped the experiments on PH animals; C.W.C. wrote the manuscript with support from C.M.C and M.L.F. All authors discussed the results and contributed to the final manuscript.

3.6 Figures and Figure Legends

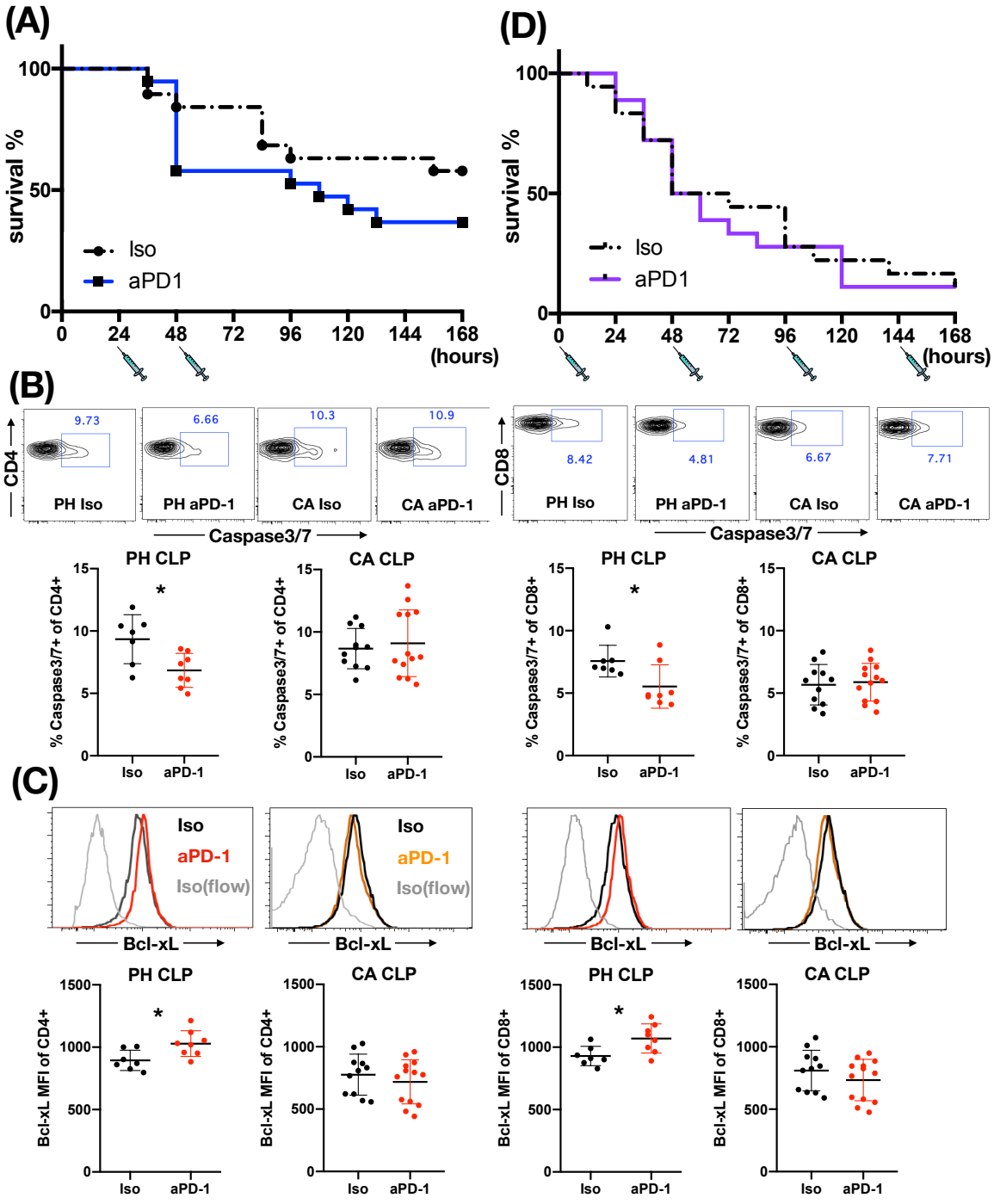


Figure 3.1 PD-1 blockade has an ineffective impact on sepsis survival and no effect on reversing T cells apoptosis in cancer sepsis model.

LLC tumor was subcutaneously injected on mouse thigh and allowed to grow for three weeks. At day 21, cancer animals were subjected to CLP surgery to induce sepsis. (A) PD-1 antagonistic monoclonal antibody (clone RMP1-14) and isotype control antibody were delayed administrated to cancer septic animals by intraperitoneal injection at day 1 and day 2 post CLP. The survival curve was followed for seven days. N=19 in each group. (B) Previously healthy septic animals were treated with PD1 blockade or isotype at Day1 post CLP and sacrificed at Day2, splenocytes were collected and activated caspase 3 were stained on T cells (N=7-8). Cancer animals with the same procedure of PD1 blockade or isotype treatments were sacrificed and stained for activated caspase 3 (N=11-12). (C) Anti-apoptotic protein Bcl-xL were stained on previously healthy septic animals (N=7-8) or cancer septic animals (N=11-12). (D) Anti-PD-1 monoclonal antibody (clone: 29F.1A12) and isotype control were given at Day 0, 2, 4 and 6 post-CLP as an early administration strategy. The survival curve was followed for 7 days. N=18 in each group.

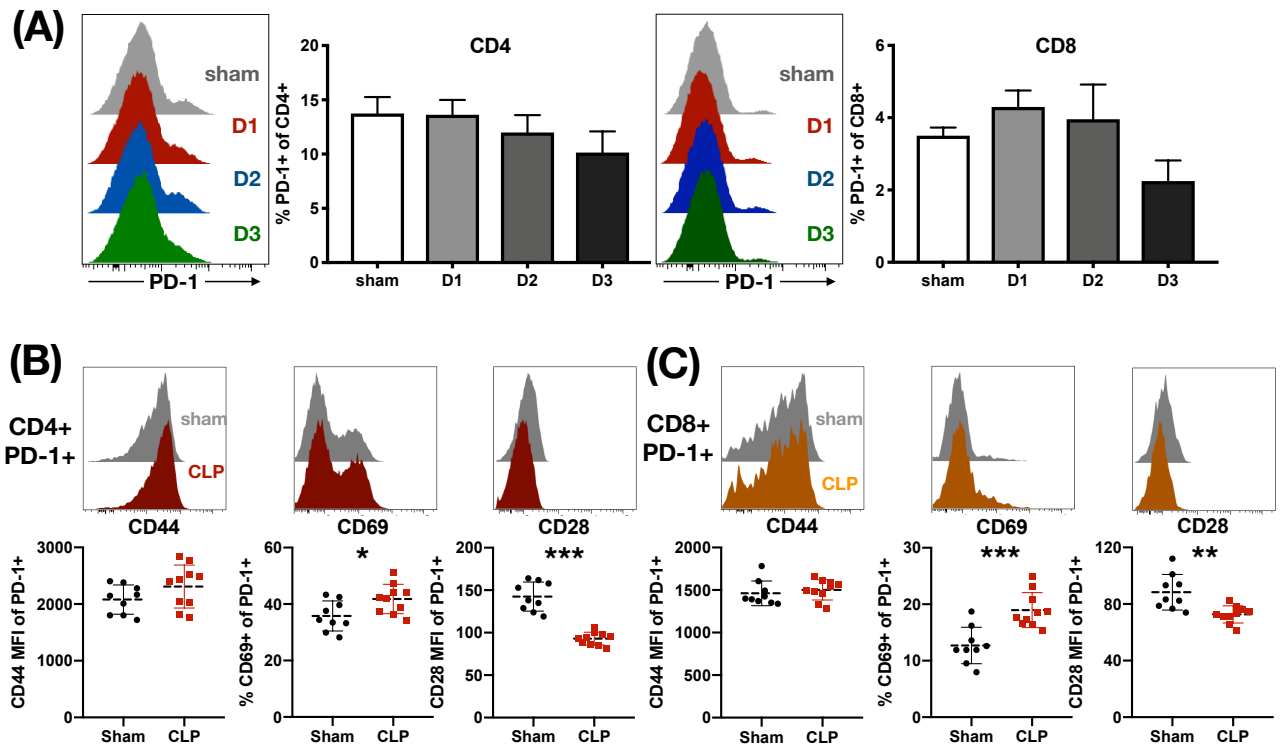


Figure 3.2 PD-1 expression is steady after sepsis in cancer animals, but PD-1$^+$ cells showed dysregulated phenotypes during sepsis.

(A) Cancer septic or sham animals were sacrificed at different time points. Spleen was harvested, and PD-1 expression was determined (N=6-8). Serial markers on PD-1$^+$ populations were assessed on Day 1 post CLP; cancer sham animals were defined as a control group. Different marker expressions on CD4$^+$PD-1$^+$ cells (B) and CD8$^+$PD-1$^+$ cells (C). N=9-14.

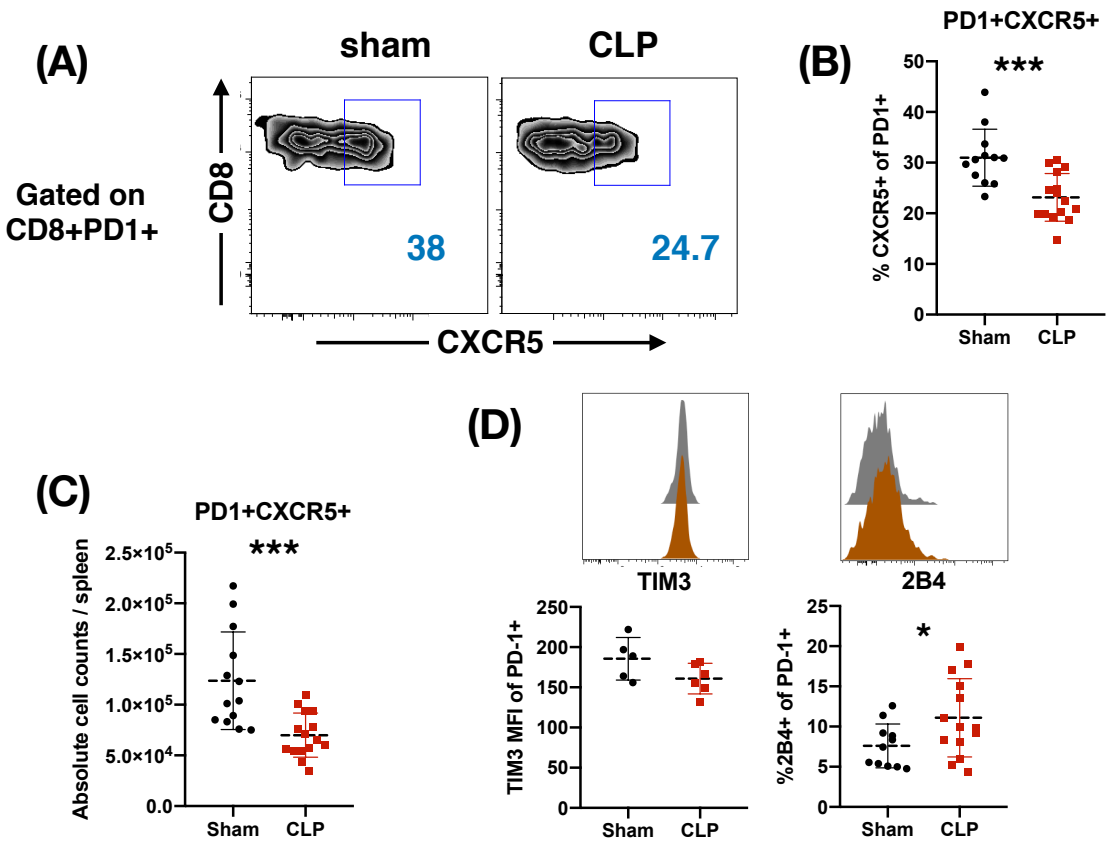


Figure 3.3 CXCR5$^+$PD-1$^+$CD8$^+$ cells significantly decreased during sepsis.

Cancer animals were subjected to CLP and sacrificed at 24 hours post CLP. The CXCR5$^+$PD-1$^+$CD8$^+$ population was analyzed. (A) CXCR5 representative flow plots from cancer sham and cancer CLP. Cells are gated on CD8$^+$PD-1$^+$population. (B) The summary plot of CXCR5$^+$ percentage in CD8$^+$PD-1$^+$ cells. (C) The summary plot of the absolute number of CXCR5$^+$PD-1$^+$CD8$^+$ cells in the spleen. (D) The summary plot of Tim-3 and 2B4 expression on PD-1$^+$CD8$^+$ cells.

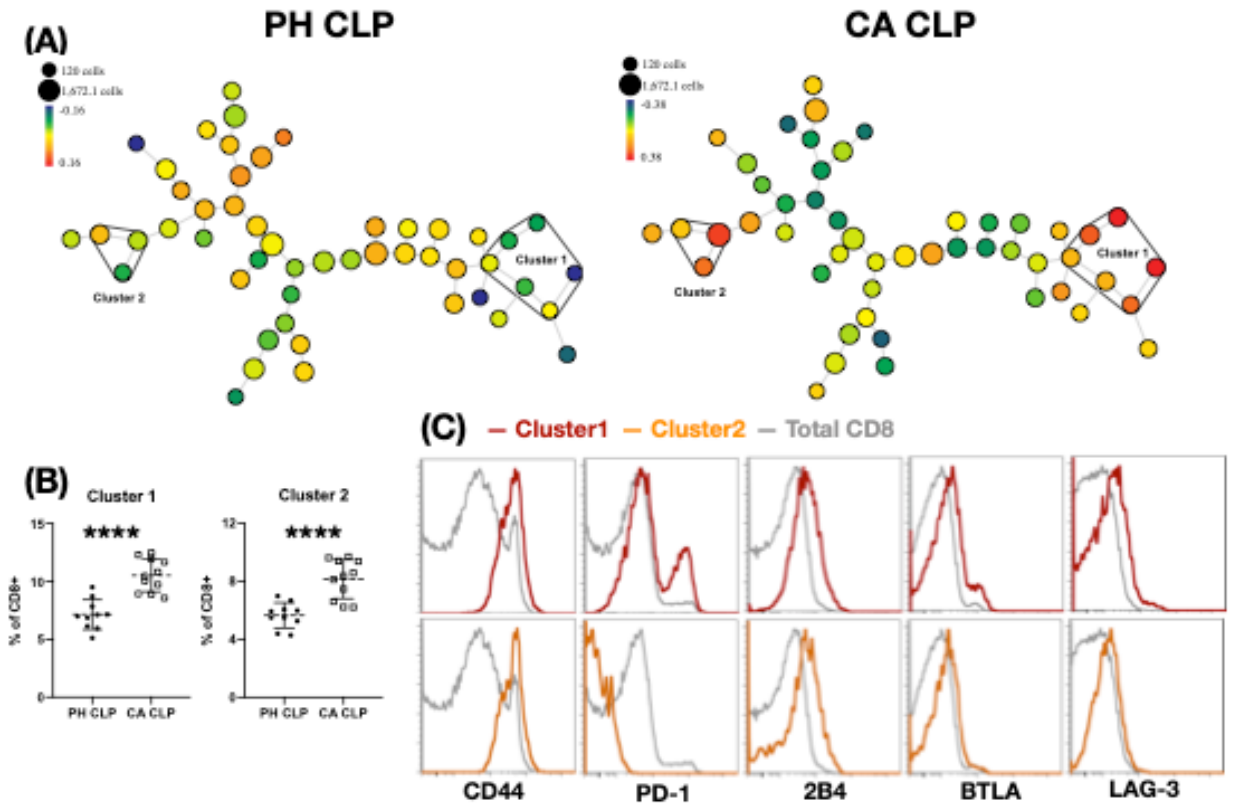


Figure 3.4 SPADE algorithm identified two CD8$^+$ T cell clusters are up-regulated in cancer septic animals.

Previously healthy animals and cancer animals were subjected to CLP and euthanized at 24 hours post CLP. Splenocytes were stained with different markers. CD3$^+$CD8$^+$ T cells were pre-gated in Flowjo and exported to Cytobank. SPADE analyzed were performed under the setting of 50 nodes. Previously healthy animals are defined as a baseline to benefit group comparisons. (A) Representative SPADE tree figure generated by the algorithm. Left tree figure represents previously healthy septic animals, and right tree figure represents cancer septic animals. The clusters were discovered by multiple t-tests with adjustment. Only the clusters up-regulated in cancer animals are shown here. (B) The summary plot of percentage of two clusters in total CD8$^+$ T cells. (C) Phenotype histograms of two clusters. The gray line represents total CD8$^+$ T cells from PH CLP mice, serving as a baseline. N=10-11.

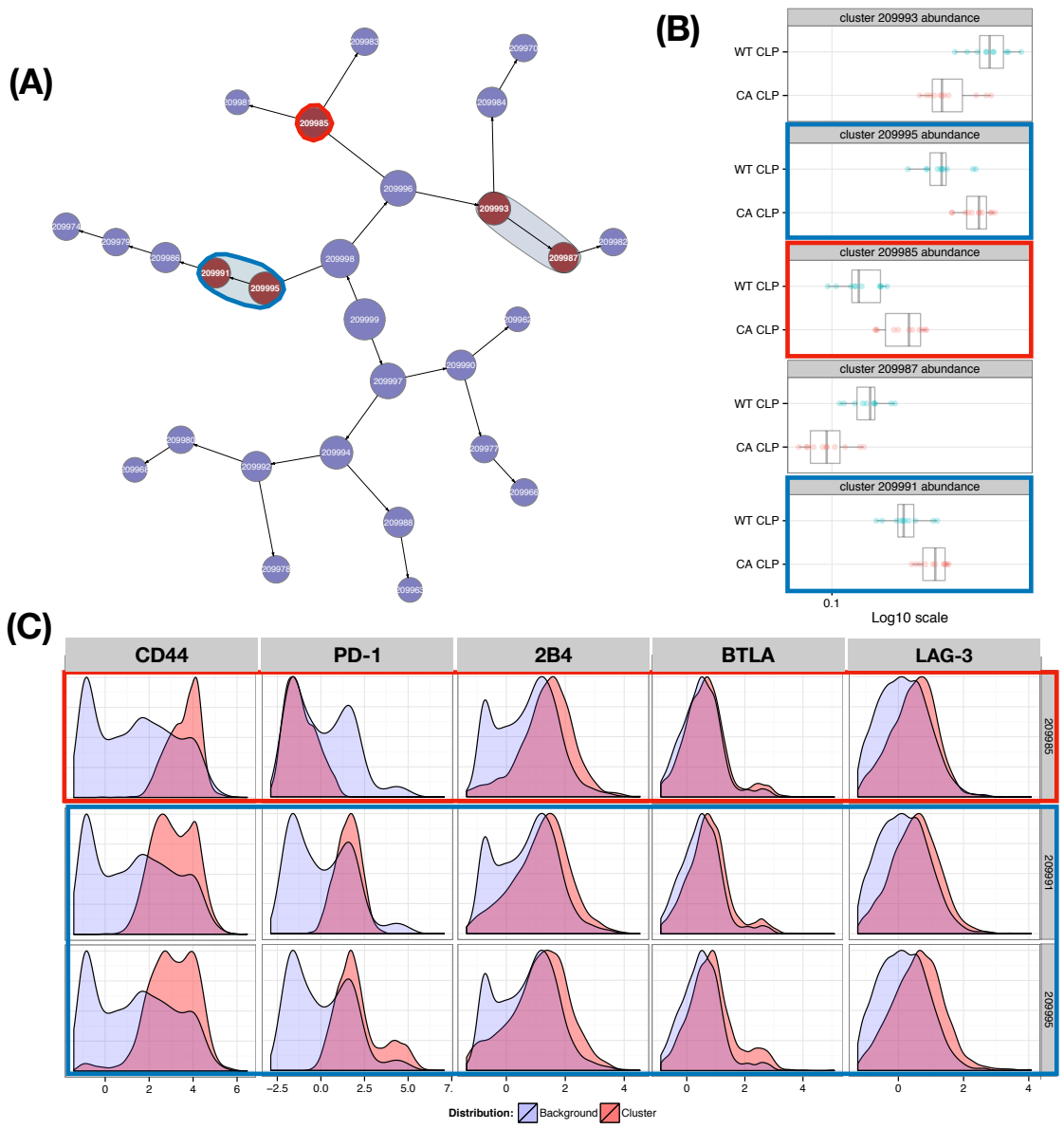


Figure 3.5 CITRUS identified similar CD8$^+$ populations that are elevated in cancer septic animals.

Same CD8$^+$ data sets were exported into CITRUS algorithm and PAMR association model with minimum FDR was selected. (A) CITRUS plot of abundance and significantly changed cluster were automatically generated by CITRUS. (B) The abundance summary plot of the changed nodes between PH CLP and CA CLP groups. (C) Phenotype histograms of the nodes that are increased in cancer septic animals. N=10-11.

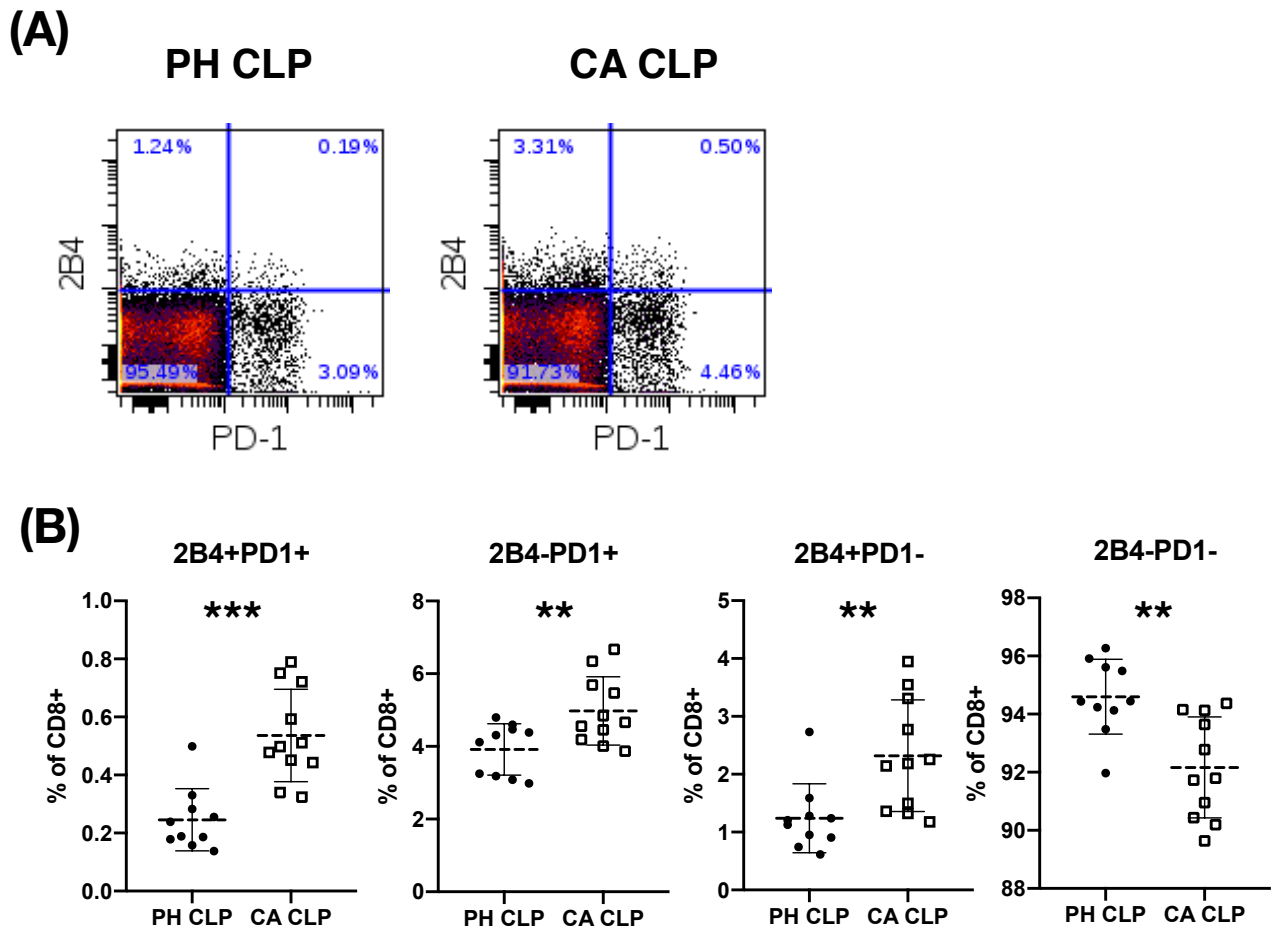


Figure 3.6 Traditional flow gating confirmed the findings from SPADE and CITRUS.

Utilizing traditional flow gating strategy, PD-1 and 2B4 co-expressing or single expressing cells were analyzed in $CD8^+$ T cells. (A) Representative flow plots for PD-1 and 2B4 expression in PH CLP and CA CLP animals. (B) The summary plots of different populations in total $CD8^+$ cells.

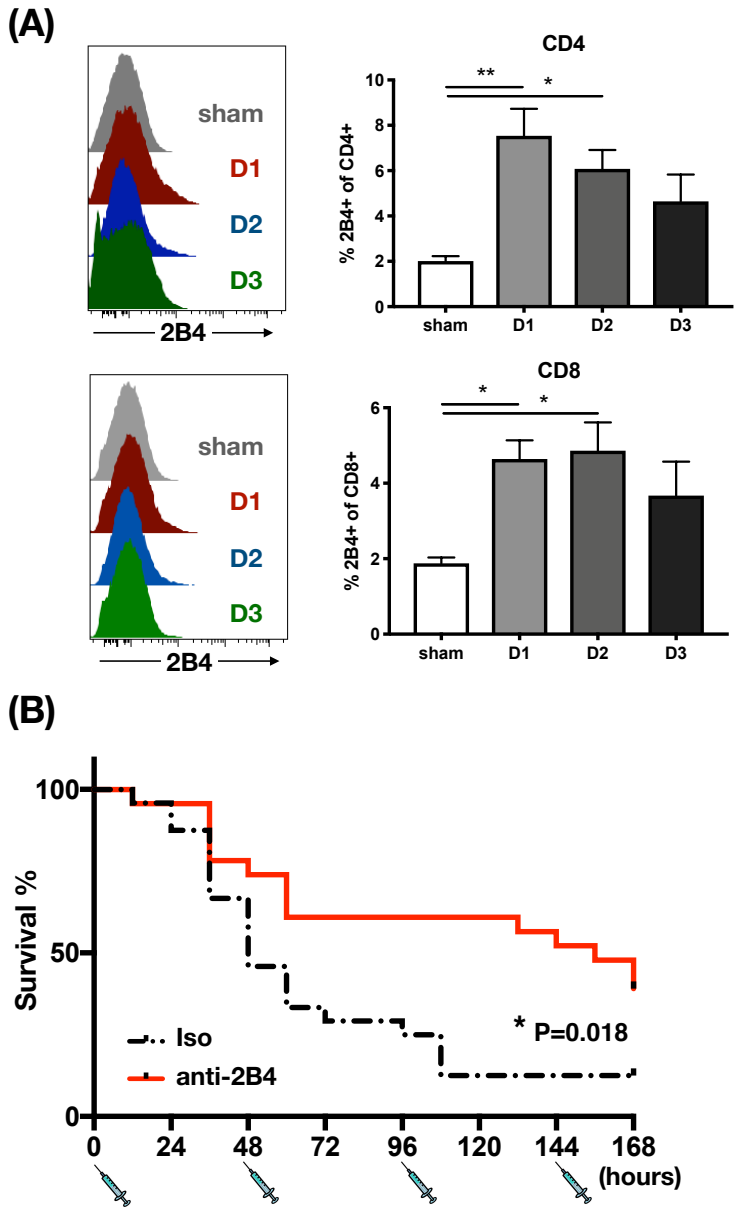
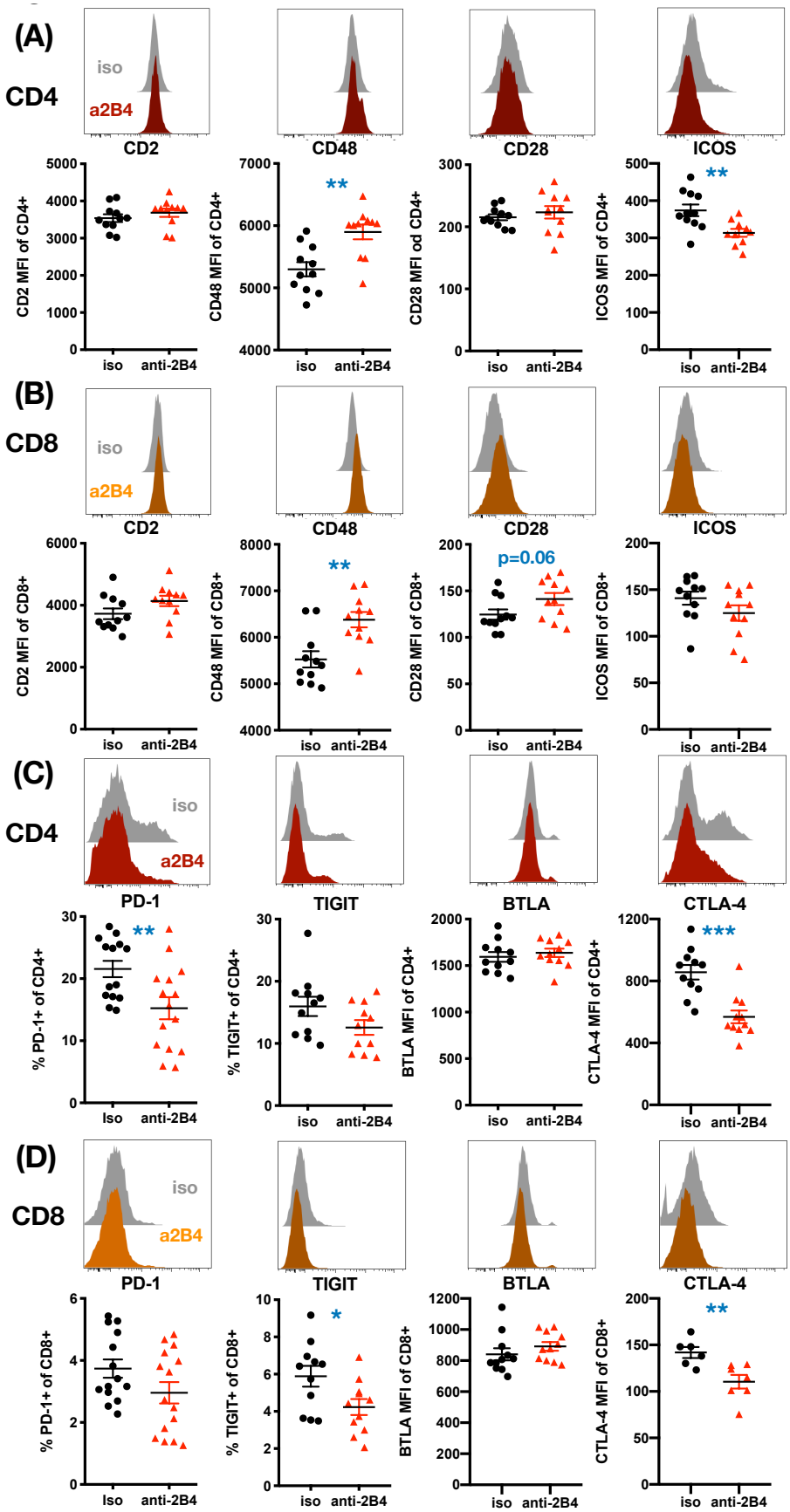


Figure 3.7 2B4 is increased on T cells and the blockade improves sepsis survival on cancer animals

(A) Cancer septic or sham animals were sacrificed at different time points to determine 2B4 expression on spleen T cells. N=6-8. (B) 2B4 blockade antibody (clone 2B4) and isotype control antibody were administrated to cancer septic animals by IP injection at Day 0, 2, 4 and 6 post CLP. The survival curve was followed for seven days. N=23, 24 in two groups.


(A)
CD4
iso
a2B4
CD2
CD48
CD28
ICOS
CD2 MFI of CD4+
CD48 MFI of CD4+
CD28 MFI od CD4+
ICOS MFI of CD4+
**
**
iso
anti-2B4
(B)
CD8
iso
a2B4
CD2
CD48
CD28
ICOS
CD2 MFI of CD8+
CD48 MFI of CD8+
CD28 MFI of CD8+
ICOS MFI of CD8+
**
p=0.06
iso
anti-2B4
(C)
CD4
iso
a2B4
PD-1
TIGIT
BTLA
CTLA-4
% PD-1+ of CD4+
% TIGIT+ of CD4+
BTLA MFI of CD4+
CTLA-4 MFI of CD4+
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anti-2B4
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CD8
iso
a2B4
PD-1
TIGIT
BTLA
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% PD-1+ of CD8+
% TIGIT+ of CD8+
BTLA MFI of CD8+
CTLA-4 MFI of CD8+
*
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Iso
anti-2B4

Figure 3.8 Further analysis showed co-signaling receptors on T cells are revived by anti-2B4 treatment during sepsis.

The expressions of different co-stimulatory and co-inhibitory receptors are checked after 2B4 blockade. Septic animals received anti-2B4 or isotype antibody after surgery were sacrificed at 24 hours post CLP. (A) the summary plots of co-stimulatory receptors on total $CD4^+$ T cells. (B) the summary plots of co-stimulatory receptors on total $CD8^+$ T cells. (C) the summary plots of co-inhibitory receptors on total $CD4^+$ T cells. (D) the summary plots of co-inhibitory receptors on total $CD8^+$ T cells. N=6-14.

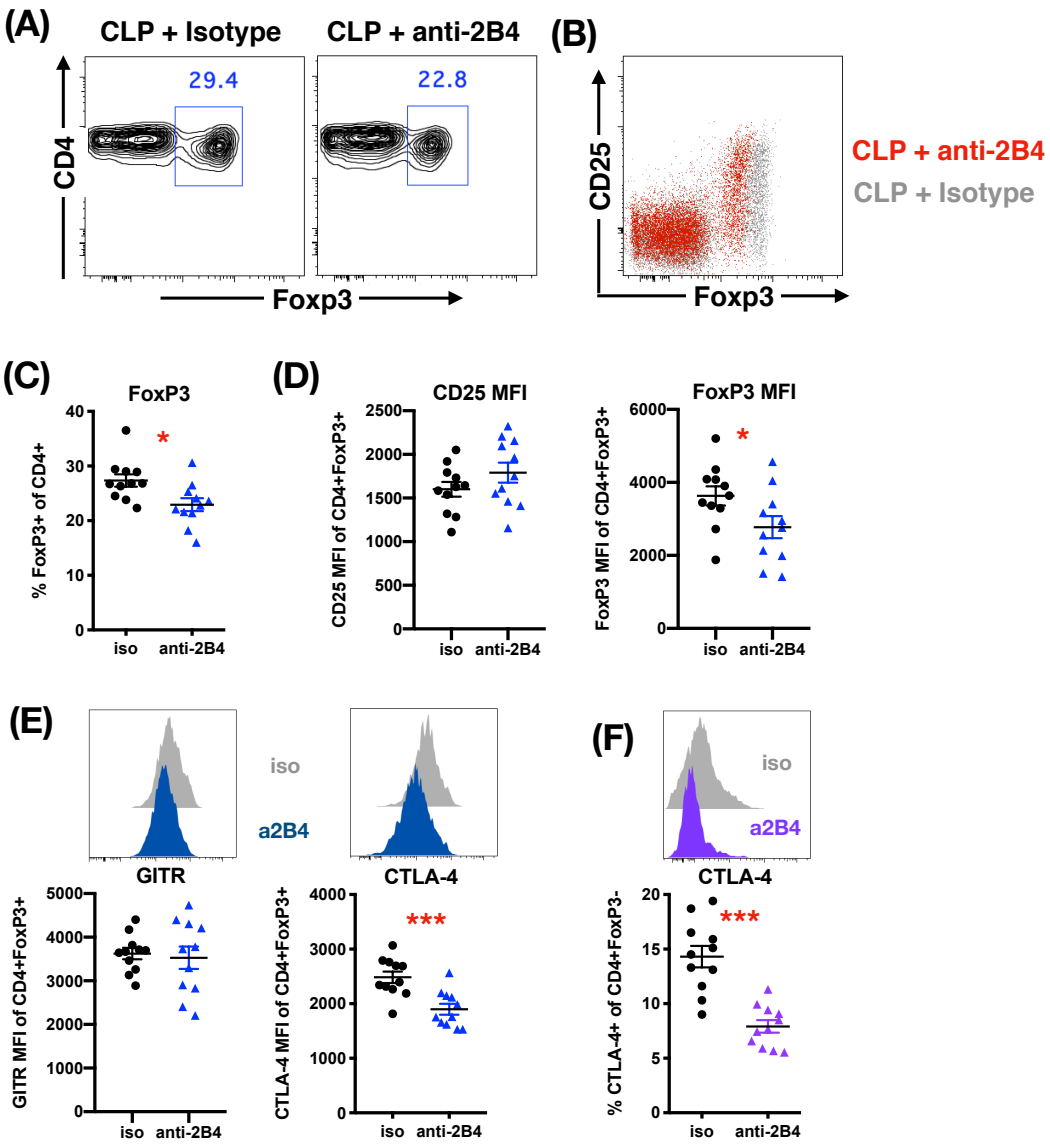


Figure 3.9 2B4 blockade reduce FoxP3$^+$ cells and it's CTLA-4 expression during sepsis.

Septic animals received one does of anti-2B4 or isotype antibody after surgery were sacrificed at 24 hours post CLP. (A) Representative flow plot for FoxP3$^+$ staining in different groups of cancer septic animals. The cells were gated on CD3$^+$CD4$^+$ T cells. (B) Overlap total CD3$^+$CD4$^+$ T cells flow plot between anti-2B4 treated or isotype treated. (C) The summary figure for percentage of FoxP3$^+$ cells in total CD4$^+$ T cells. (D) The summary figure for CD25 MFI and FoxP3 MFI of CD4$^+$ FoxP3$^+$ T cells. (E) Functional markers of Treg cells summary on CD4$^+$ FoxP3$^+$ T cells. (F) The summary figure of CTLA-4 of CD4$^+$ FoxP3$^-$ T cells. N=11.

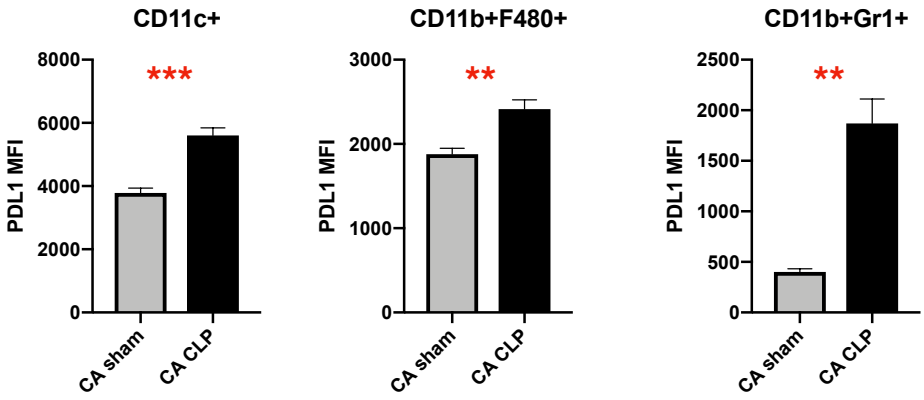


Figure 3.10 PD-L1 expression on different APCs during sepsis

Cancer sham or septic animals were sacrificed at 24 hours post-surgery, the splenocytes were gated on different cell subsets to determine PD-L1 expression. N=4-5.

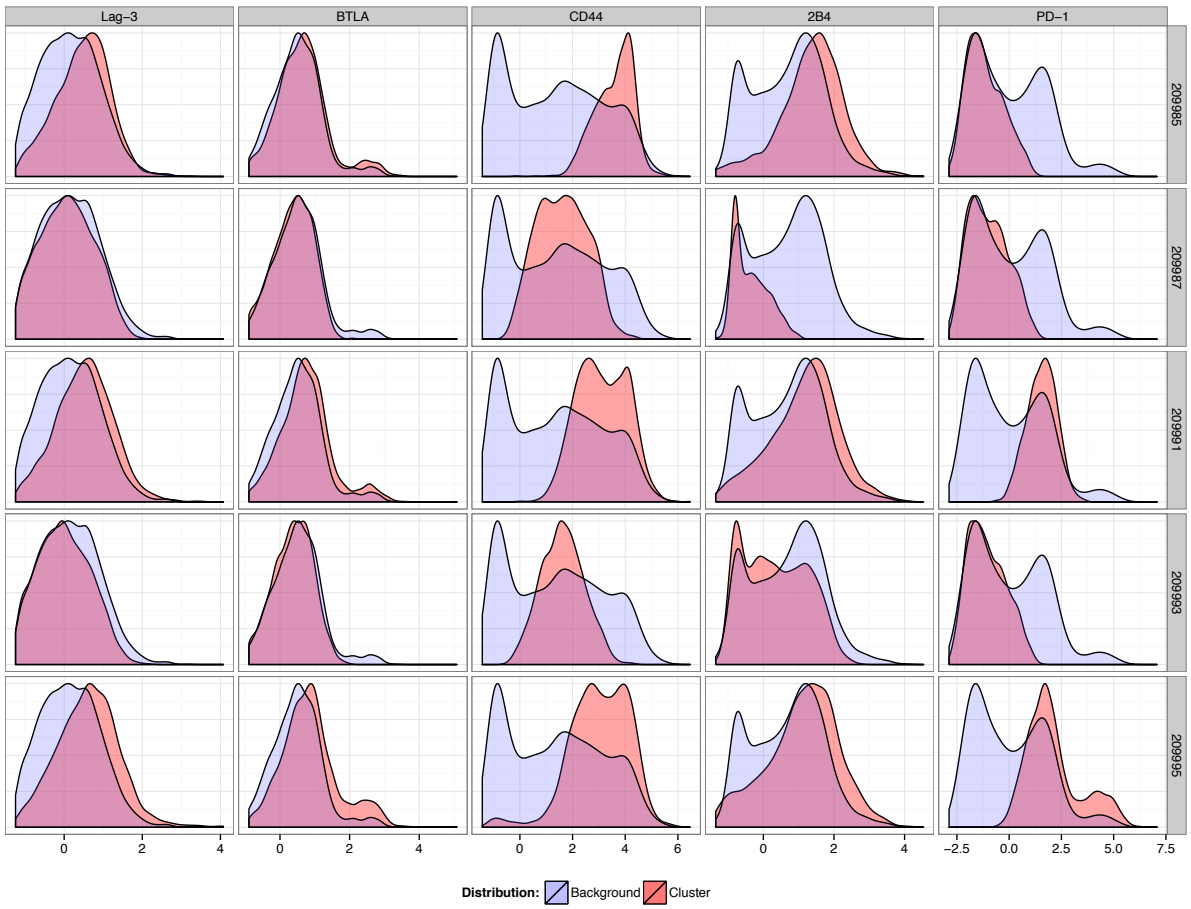
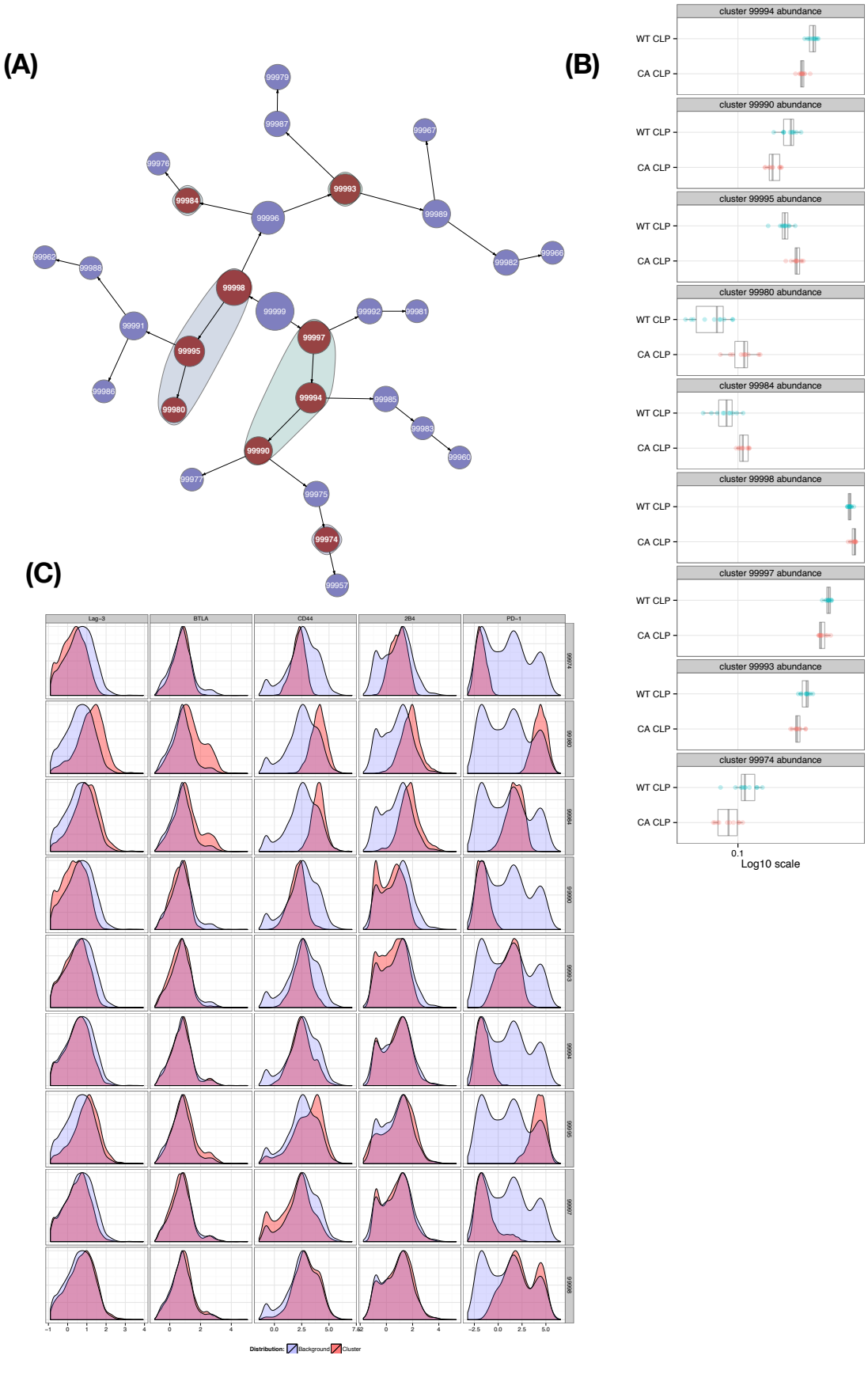


Figure 3.11 Complete CITRUS phenotype table of changed CD8$^+$ T cells

PH sepsis and CA sepsis CD8$^+$ T cell data set were analyzed by CITRUS (Figure 5). The phenotypes of five different nodes that are identified by CITRUS program is shown. The purple histogram represents total CD8$^+$ T cells (background), and the red histogram represents the specific cluster.


(A)
99979
99987
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99976
99984
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99962
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99982
99966
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99983
99990
99960
99977
99975
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99957
(B)
cluster 99994 abundance
cluster 99990 abundance
cluster 99995 abundance
cluster 99980 abundance
cluster 99984 abundance
cluster 99998 abundance
cluster 99997 abundance
cluster 99993 abundance
cluster 99974 abundance
WT CLP
CA CLP
0.1
Log10 scale
(C)
Lag-3
BTLA
CD44
2B4
PD-1
Distribution:
Background
Cluster

Figure 3.12 CITRUS identified changed $CD4^+$ populations that are elevated in cancer septic animals.

Previously healthy animals and cancer animals were subjected to CLP and euthanized at 24 hours post CLP. Splenocytes were stained with different markers. $CD3^+CD4^+$ T cells were pre-gated in Flowjo and exported to Cytobank. $CD4^+$ data sets were exported into CITRUS algorithm and PAMR association model with minimum FDR was selected. (A) CITRUS plot of abundance and significantly changed cluster were automatically generated by CITRUS. (B) The abundance summary plot of the changed nodes between PH CLP and CA CLP groups. (C) Phenotype histograms of the nodes that are increased in cancer septic animals. N=10-11.

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Chapter 4. Tumor specific T cells exacerbate mortality and immune dysregulation in cancer sepsis

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4.1 Abstract

Sepsis induces significant immune dysregulation characterized by lymphocyte apoptosis and alterations in the cytokine milieu. While the impact of this dysregulation on the host's response to infection has been well studied, it is unknown how this dysfunction impacts the immune response to co-occurring but non-infectious insults. As cancer patients have a ten-fold greater risk of developing sepsis compared to the general population, we were particularly interested in understanding how sepsis-induced immune dysregulation alters anti-tumor response. To investigate the impact of sepsis on tumor-specific $CD8^+$ T cell responses, an OVA-specific tumor model was used. Briefly, OVA-specific $CD8^+$ T cells (OTI) were adoptively transferred into mice one day prior to the implantation of OVA-expressing lung cancer cells (LLC-OVA). Three weeks later, animals were subjected to cecal ligation and puncture (CLP) or sham surgery to induce sepsis. We found that CLP reduced the frequency of OTI and endogenous $CD8^+$ T cells in the spleen and draining lymph nodes compared to sham mice. In contrast, the frequency of OTI $CD8^+$ T cells infiltrating the tumor was not reduced. In addition, IFN-γ secretion by the tumor-specific $CD8^+$ T cells was impaired in all locations at early stage sepsis but recovered at Day 4 post CLP. Importantly, we found that the presence of tumor-specific $CD8^+$ T cells is associated with increased apoptosis of endogenous $CD8^+$ T cells, which may contribute to the increased mortality associated with cancer sepsis. Our research provides a novel insight into the way that sepsis shapes the immune response to solid tumors and how tumor-specific $CD8^+$ T cells impact sepsis pathogenesis.

4.2 Introduction

Cancer is one of the most common co-morbidities of sepsis, affecting at least 200,000 sepsis patients each year in the United States alone (1, 2). Cancer patients have a 10-times higher risk of developing sepsis compared to non-cancer patients and have increased risk of mortality (3, 4). Moreover, cancer is the comorbidity associated with the highest underlying cause of death in sepsis patients (5). Lung cancer is the most prevalent type of solid tumor in cancer septic patient (3). Importantly, cancer septic patients show elevated in-hospital sepsis mortality compared to non-cancer septic patients (6, 7). While around 50% of cancer septic patients recover from sepsis, many experiences persistent organ dysfunction, particularly affecting their immune system (6).

It is well appreciated that sepsis induces T cell dysfunction. Recent post-mortem human studies and studies using murine models of sepsis have demonstrated that sepsis induces T cell apoptosis and exhaustion, leading to the immunosuppression (8, 9). Interestingly, recent studies suggest that heterogeneous populations of T cells have different impacts from sepsis-induced immune dysfunction. In a patient cohort with Staphylococcus aureus infection, Ardura et. al showed that central memory CD45RA-CCR7$^+$ CD4$^+$ and CD8$^+$ T cells are significantly decreased, but not naive cells (10). Similar results were described in a mouse study showing that CD44^{hi}CD11a^{hi} memory CD8$^+$ T cells are depleted and exhibit higher Annexin-V staining compared to CD44$^-$ naive T cells in a polymicrobial sepsis model (11). Sharma et al. have demonstrated that the absolute number of T cells in the thymus and spleen are significantly decreased during sepsis, but no significant change occurs in the liver or lung (12). In addition, Danahy et. al showed that antigen-specific memory CD8$^+$ T cells resident in the skin are resistant to sepsis induced dysfunction compared to the memory cells in the circulation (13).

It is well-known that tumor-specific CD8$^+$ T cells are crucial to control cancer. However, only a few studies have addressed how sepsis or bacterial infection might impact the tumor-specific T

cells, and their results are inconsistent. In 1920, Dr. William Coley published a study claiming that infection with Streptococcus led to tumor remission in over 25% of his sarcoma patients (14, 15). While bacterial infection could theoretically lead to improved anti-tumor lymphocyte responses due to TLR activation, no other studies have replicated these results. In the context of sepsis, one animal study has reported that sepsis leads to the expansion of regulatory T cells that suppress anti-tumor immunity (16). After tumors are inoculated into animals that have recovered from sepsis, sepsis-recovered animals have enhanced tumor growth compared to animals who underwent sham surgery. Although this research emphasizes the immunosuppression after sepsis, the tumor-recognizing T cells present in this model were naive cells prior to the sepsis insult, in which is not an ideal model to investigate the activated anti-tumor immunity during sepsis in patients with existing cancer. Here we report a clinically relevant model that allow us to assess the tumor-specific T cells response during sepsis. In this study, we demonstrate that there are location-dependent alterations in the function of tumor-specific $CD8^+$ T cells during sepsis. Moreover, we demonstrate that tumor antigen-specific $CD8^+$ T cells may increase mortality during sepsis mortality by modulating the apoptosis of other populations of $CD8^+$ T cells. This study provides an insight into how the immunosuppressive phase of sepsis affects anti-tumor immunity in cancer sepsis patients and may help lead to novel therapeutic strategies specific to cancer patients with sepsis.

4.3 Results

LLC-OVA activated OTI cells in vitro and in vivo

Compared to previously healthy animals, septic mice with pre-existing tumors induced by Lewis lung cancer (LLC) cells had significantly increased mortality (17). In order to understand how tumor-specific T cell responses during sepsis and whether this population contributes to exaggerated sepsis morality in cancer animals, we developed a cancer animal model that lung carcinoma cells are over-expressed OVA protein (LLC-OVA), and OVA specific and $CD8^+$ T cells (OTI) with the congenic marker Thy1.1 are served as tumor-specific T cell. First, to confirm OTI cells recognize OVA antigen on LLC-OVA, LLC-OVA cells were co-cultured with OTI in vitro. After four days of co-culture, OTI cells had increased expression of CD25 and CD69 expression compared to OTI cells co-cultured with LLC cells without OVA expressing, indicating that the LLC-OVA cells can specifically induce OTI activation (Figure 4.1 A). Next, OTI cells were adoptively transferred into naïve animals and LLC-OVA were implanted on the thigh (Figure 4.1 B). Three weeks after LLC-OVA implantation, many of the splenic OTI cells in cancer mice displayed a memory phenotype ($CD44^{hi}$), suggesting that tumor specific OTI cells are activated by LLC-OVA in the host. The frequency of OTI cells in the tumor infiltrating lymphocytes (TILs) and in the tumor draining lymph node (dLN) were assessed using flow cytometry (OTI gating strategy is shown in Figure 4.7). We found that OTI cells constituted a larger proportion of $CD8^+$ T cells in the dLN and TILs compared to spleen (Figure 4.1 C). Moreover, OTI cells found in TILs displayed higher frequency of $CD44^+$ and the exhaustion markers PD-1, 2B4, and Tim-3 as compared to OTI cells in the spleen (Figure 4.1 D). These phenotypes are consistent with previous literature and indicated that OTI cells develop exhausted phenotypes when continually exposed to tumor antigens. Overall, these results demonstrated that OTI cells are specifically activated by LLC-OVA in our model.

Tumor specific T cells in the tumor microenvironment are resistant to sepsis-induced cell death

To investigate how sepsis shapes the tumor-specific T cell response, LLC-OVA cancer animals were subjected to CLP or sham surgery three week after tumor inoculation (Figure 4.2 A). The absolute cell counts of tumor-specific T cells (OTI) and endogenous (non-OTI) $CD8^+$ T cells were determined for the spleen, dLN, and TILs. Since sepsis induces T cell apoptosis and reduces the number of circulating T cells, but has less impact on tissue resident T cell populations, we hypothesized that sepsis would result in a greater reduction of tumor specific T cells in the circulation compared to the dLN or tumor site. Consistent with previous reports, both the number of OTI and non-OTI $CD8^+$ T cells were significantly reduced in spleen 24 hours post-CLP (Figure 4.2 B, C). In the dLN, the absolute number of $CD8^+$ T cells was significantly reduced after CLP (Figure 4.2 B, C). Of note, the fold change in the dLN was much higher than spleen in both OTI and non-OTI $CD8^+$ T cells (4.4-fold vs. 1.5-fold). In agreement with our hypothesis, we found that the number of OTI cells in the tumor was not different between mice receiving CLP and sham surgery. In contrast, the number of non-OTI $CD8^+$ T cells decreased by two-fold after CLP (Figure 4.2 B, C). These results suggest that sepsis induced the apoptosis of tumor-specific T cells in the circulation but not in tumor microenvironment.

Tumor specific T cells in the tumor microenvironment are resistant to sepsis-induced exhaustion compared to cells in the circulation

Systemic T cell exhaustion is considered a hallmark of sepsis-induced immune dysfunction and is characterized by the expression of multiple inhibitory receptors and decreased functionality. In our model, OTI cells in the tumor microenvironment already express high levels of co-inhibitory molecules prior to the induction of sepsis (Figure 4.1 D). To determine whether tumor-specific T cells become more exhausted during sepsis, the phenotype of OTI T cells was assessed 24 hours

post-CLP. We found that after sepsis, OTI cells in the spleen exhibited decreased CD28 expression, and elevated 2B4 and TIGIT expression (Figure 4.3 A). In the dLN, OTI T cells did not change their expression of CD28 or 2B4 but had increased expression of PD-1 and TIGIT (Figure 4.3 B). These results are consistent with the literature showing that sepsis drives T cell exhaustion in the circulation. Interestingly, OTI T cells infiltrating the tumors did not up-regulate their expression of co-inhibitory receptors, although CD28 expression declined after sepsis (Figure 4.3 C). These results are coherent with our findings that sepsis-induced T cell dysfunction preferentially affects circulating lymphocytes (Figure 4.2 C).

Tumor specific T cells produce less IFN-γ during sepsis

Decreased cellular function is a critical marker of T cell exhaustion during sepsis. As IFN-γ and TNF secretion are also important for anti-tumor immunity, we measured the effect of sepsis on T cell cytokine secretion. At 24 hours post CLP, we found that the frequency of IFN-γ secreting OTI cells was reduced in spleen, dLN and TILs after sepsis (Figure 4.4 A). In contrast, TNF secretion results varies in different locations. We found that the frequency of TNF-secreting OTI cells in the dLN increased following CLP; however, the percentage of TNF secreting OTI cells declined in the tumor (Figure 4.4 B). Overall, this data suggests that tumor specific T cells have decreased cytokine secretion, specifically on IFN-γ, during sepsis.

The function of tumor specific T cells is restored at later stages of sepsis

Around 50% of cancer septic patients recover from sepsis (6); however, whether their anti-tumor response recovers from immunoparalysis after sepsis has not been studied. Clinically, the secretion of cytokines by tumor-specific $CD8^+$ T cells, especially IFN-γ, is crucial to control tumor growth. In the early stage of sepsis, we observed that IFN-γ secretion by tumor specific T cells

was reduced (Figure 4.4 A). To determine whether its secretion was restored at later stages of sepsis, OTI T cells were assessed at days 4 and 7 post CLP. At day 4 post CLP we found that IFN-γ secretion by OTI cells was slightly restored in the spleen, dLN and tumor (Figure 4.5 A). By day 7 post CLP, IFN-γ secretion by OTI cells was returned to baseline levels in the spleen and dLN (Figure 4.5 B). Surprisingly, we noticed that OTI secretion of IFN-γ in TILs was significantly higher in mice following CLP compared to mice that underwent sham surgery (Figure 4.5 B). To understand whether this increase in function could lead to enhanced control of the tumor, we measured tumor volume at various time points before and after CLP. We found that cancer septic mice had a reduced rate of tumor growth compared to sham animals (Figure 4.5 C). In sum, the function of tumor-specific T cell is restored at a late stage during sepsis and leads to reductions in tumor growth.

Depletion of tumor specific T cells prior to sepsis results in improved survival

Finally, to test whether tumor-specific T cells contribute to sepsis pathogenesis, we depleted OTI and OTII cells in LLC-OVA cancer mice using an anti-Thy1.1 antibody and measured their survival. Flow cytometer data confirmed that depletion antibody treated animals have no OTI cells in the spleen (Figure 4.6 A). Strikingly, depletion of tumor-specific T cells improved survival compared to sham treatment with an isotype antibody (Figure 4.6 B). This indicates that tumor-specific T cells contribute partially to the increased mortality from sepsis in mice with cancer compared to previously healthy animals. To further determine the mechanism by which tumor-specific T cells contribute to sepsis mortality during cancer, we surveyed endogenous $CD8^+$ T cells phenotypes ($Thy1.1^-$, "endo. CD8" in Figure 4.6 A) at day 2 after CLP after CLP. No significant difference was seen in serum cytokines at 24 hours post-CLP (Figure 4.8). The frequency of $CD44^+$ endogenous $CD8^+$ T cells in the anti-Thy1.1 treated group was significantly reduced, while there

was an overall trend toward higher absolute cell counts compared to isotype treated group (Figure 4.6 C). Next, we assessed T cell apoptosis by staining for Caspases 3/7 activity. We found that reduced percentage of endogenous $CD8^+$ T cells were $Caspase3/7^+$ in the anti-Thy1.1 treated group compared to isotype (Figure 4.6 D), supporting the survival curve result we observed (Figure 4.6 B). In sum, our results imply that tumor-specific T cells contribute to sepsis pathogenesis by inducing the apoptosis of endogenous $CD8^+$ T cells.

4.4 Discussion

In this study, we used an antigen specific cancer system to investigate how sepsis shapes anti-tumor immunity, representing a novel and clinically relevant field. We first showed that sepsis-induced dysfunction is less pronounced in tumor-specific $CD8^+$ T cells found in the tumor itself and is indicated by reduced cellular apoptosis and less expression of exhaustion markers. Cytokine secretion by tumor specific $CD8^+$ T cells were impaired early after CLP but recovered at later time points. We observed that at day 7 post-CLP, tumor-specific $CD8^+$ T cells in the tumor exhibited increased cytokine secretion, correlating with reduced tumor growth in septic animals. Using a Thy1.1 depletion antibody to remove tumor-recognizing T cells prior to sepsis, we demonstrated that tumor-specific T cells contribute to sepsis mortality by inducing the apoptosis of endogenous $CD8^+$ cells.

In our model system, using ovalbumin expressing LLC cells and OVA-specific T cells provided us several benefits but also had limitations. Since ovalbumin is a foreign antigen in mice, less than 0.01% of endogenous $CD8^+$ T cells, about 100-200 $CD8^+$ T cells in a spleen, are expected to be able to recognize OVA (18, 19). As these endogenous $CD8^+$ cells lack the congenial marker Thy1.1, we do not know what role the endogenous OVA-specific T cells played during sepsis. In addition, although OVA was over-expressed by the LLC cancer cells, it only represents one antigen from a pool of hundreds antigen present on cancer cells. While OTI served as tumor antigen-specific cells in our model, it is also possible that some endogenous $CD8^+$ T cells may have recognized an antigen on the tumors. Another limitation of our model was that few OTII could be detected three weeks after tumor inoculation (data not shown). Therefore, in this study, we could not elucidate the role of tumor-specific $CD4^+$ T cells in cancer sepsis and instead focused on the $CD8^+$ T cell response.

Tissue resident T cells in lung and skin have been shown to be less impaired during sepsis (12, 13), possibly because there are fewer signals to induce apoptosis and lower levels of inflammatory cytokines in these microenvironments. Our finding that OTI T cells within the tumor were retained and did not express increased levels of exhaustion markers are consistent with previous studies. Of note, although the OTI cell count was preserved during sepsis in the tumor, non-OTI cells at this site were decreased. This implies that TCR-activation of OTI cells at the site of the tumor may provide survival signals to these cells. Therefore, we administrated antibody that specifically block MHCI with SIINFEKL peptide (Clone: 25-D1.16) to block TCR-activation on OTI cells. No difference was observed in this experiment (data not shown), indicating that survival benefits on OTI in TILs are not solely TCR-dependent. Other mechanisms behind the survival benefits of OTI T cells in the tumor microenvironment need to be investigated.

We found that the cytokine secretion by OTI cells in the TIL improved at later stages of sepsis and were associated with reduced tumor growth. While these results are surprising, they need to be carefully interpreted. Recently, TLR agonist have been shown to improve checkpoint blockade efficacy in tumor microenvironment (20-23). In general, cancer cells harness the adaptive immune response and dispel the T cells that enter tumor microenvironment, rendering them immunologically “cold”. TLR9 and STING agonist administration activates monocytes and dendritic cells and turns the immunologically “cold tumor” into a “hot tumor”. TLR agonists have been combined with checkpoint blockade in several cancer clinical trials (24, 25). TLR signals are increased during sepsis, which may be responsible for the increase in function of the TILs. On the other hand, the cancer septic mice that survived until day 7 post-CLP may have a survival bias These sepsis-surviving animals may have distinct immune responses as compared to the mice that do not survive, such as a reduced propensity to cellular apoptosis or enhanced responses by innate cells. Therefore, we included an earlier time point (day 4 post-CLP). Overall, our results

demonstrate that the anti-tumor T cell responses recover even after undergoing sepsis-induced dysfunction.

In the depletion experiment, we found that tumor-specific T cells are contributed to the apoptosis of endogenous $CD8^+$ T cells (Figure 4.6 D). The extrinsic pathways (TNF family receptors and caspase-8 dependent) and intrinsic pathways (mitochondrial and caspase-9 dependent) of apoptosis are both involved in sepsis-induced apoptosis (26, 27). The serum cytokines at 24 hours imply that the apoptosis differences were not due to changes in inflammatory cytokines (extrinsic pathways). Therefore, we hypothesize that the $CD8^+$ T cells survival signals (intrinsic pathways) in isotype treatment animals are diminished by tumor-specific $CD8^+$ T cells. The limited survival signals for $CD8^+$ T cells during sepsis include the cytokines (IL-2, IL-7, and IL-15) and binding of the co-stimulatory molecule (CD28 to B7 family). In cancer septic animals, pre-activated tumor-specific T cells might be competing for survival signals with the endogenous $CD8^+$ T cells. In fact, we noticed that the OTI exhibited increased CD25 (IL-2 receptor alpha) expression compared to endogenous $CD8^+$ T cells during sepsis (data not shown). Interestingly, this competition is only specific to $CD8^+$ T cells as the frequency of Caspase3/7^+ $CD4^+$ T cells was similar between the groups (data not shown). Therefore, we hypothesis that selective depletion of tumor-specific T cells may have resulted in an increase of the survival signals for the endogenous $CD8^+$ T cells, leading to lower cellular apoptosis and improved sepsis survival in the host. Collectively, our model helps illustrate how anti-tumor immunity interplays with sepsis immune pathogenesis.

4.5 Materials and Methods

Cancer cell lines and in vitro co-culture experiments

LLC1 cells were acquired from ATCC (CRL-1642) and LLC-OVA was kindly provided by Dr. Gabrilovich at the Wistar Institute (28). LLC1 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% FBS, 1% P/S and 1% HEPES. LLCOVA was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% FBS, 1% P/S, 1% HEPES and 0.5 mg/ml of G418. The ATCC guidelines for LLC1 culturing and cryopreservation were followed. For in vitro co-culture experiments, 100,000 LLC or LLCOVA cancer cells were seeded into each well of the 24 well plate at day -1. The next day (day 0), 500,000 OTI mouse splenocytes were added to each well. On day 4, the cells were harvested and analyzed with a flow cytometer.

Cancer animal model and antibody treatment

Eight to twelve-week-old C57BL/6 male mice were acquired from Jackson Laboratory. All animals were housed and maintained in accordance with Emory University IACUC guidelines and studies were approved by Emory IACUC (Protocol: 201700361.M001). Two million of OTI and OTII cells were adoptively transferred into animals via intravascular injection one day before cancer inoculation. For cancer inoculation, 100,000 LLC-OVA or 50,000 LLC cells were implanted in the right thigh via subcutaneous injection as a solid tumor model. The tumor volume was calculated by length, width and height, and measured using a caliper. After three weeks, the cancer-bearing animals were randomly divided to different groups for experiments. For anti-Thy1.1 depletion experiment, 250μg of InVivoMAb anti-mouse Thy1.1 and isotype control (Bioxcell) were intraperitoneally administrated into cancer animals one day prior to sepsis induction.

CLP surgery

Cecal ligation and puncture (CLP) was subjected to cancer animals to induce polymicrobial sepsis. In general, cancer animal was anesthetized with anesthetize and the 50%-60% of cecum was ligated and punctured twice with a 25-gauge needle (29). Pain relief medication (0.1 mg/kg buprenex, McKesson Medical) was administered before the surgery. After surgery, septic animals received subcutaneous 1 ml saline at 0 hours and 4 doses of antibiotics (50 mg/kg ceftriaxone and 35 mg/kg metronidazole) at 0, 12, 24, and 36 hours. Sham surgery was performed as the control.

Flow cytometry and intracellular cytokine staining

Antibodies for flow cytometry were purchased from the following companies: BioLegend: CD8 (53-6.7), CD4 (GK1.5), CD28 (E18), CD44 (IM7), PD-1 (29F1A12), TIGIT (IG9), Tim-3 (RMT3-23), eBioscience Thermo Fisher Scientific: 2B4 (eBio244F4), Thy1.1 (HIS51), and LIVE/DEAD™ Fixable Aqua Dead Cell Stain. BD Pharmingen: CD3 (500A2), CD45 (30-F11), CD160 (CNX46-3). For intracellular cytokine staining, splenocytes were harvested and stimulated with 30 ng/ml PMA and 400 ng/ml ionomycin in the presence of GolgiStop for 5 h at 37℃. The following antibodies were used for cytokine staining: IFN-γ (XMG1.2), TNF (MP6-XT22), IL-2(JES6-5H4). CellEventTM Caspase-3/7 Green Flow Cytometry Assay Kit (Catalog: C10427) was used for Caspase 3/7 staining. Absolute cell counts were obtained using CountBrightTM Absolute Counting Beads (Catalog: C36950). BD LSRII and Fortessa are used for collecting data and analysis of flow cytometry data was performed using Flowjo and Cytobank.

Serum cytokine measurement

For serum cytokine detection, animals were bled 24 hours post-surgery to collect 25 μL serum. Cytokine levels were measured using BD™ Cytometric Bead Array (CBA) Mouse Inflammation

Kits and Bio-Plex Pro™ Mouse Cytokine Assays. Assays were done following the manufacturer's protocol found on the company websites.

4.6 Figures and Figure Legends

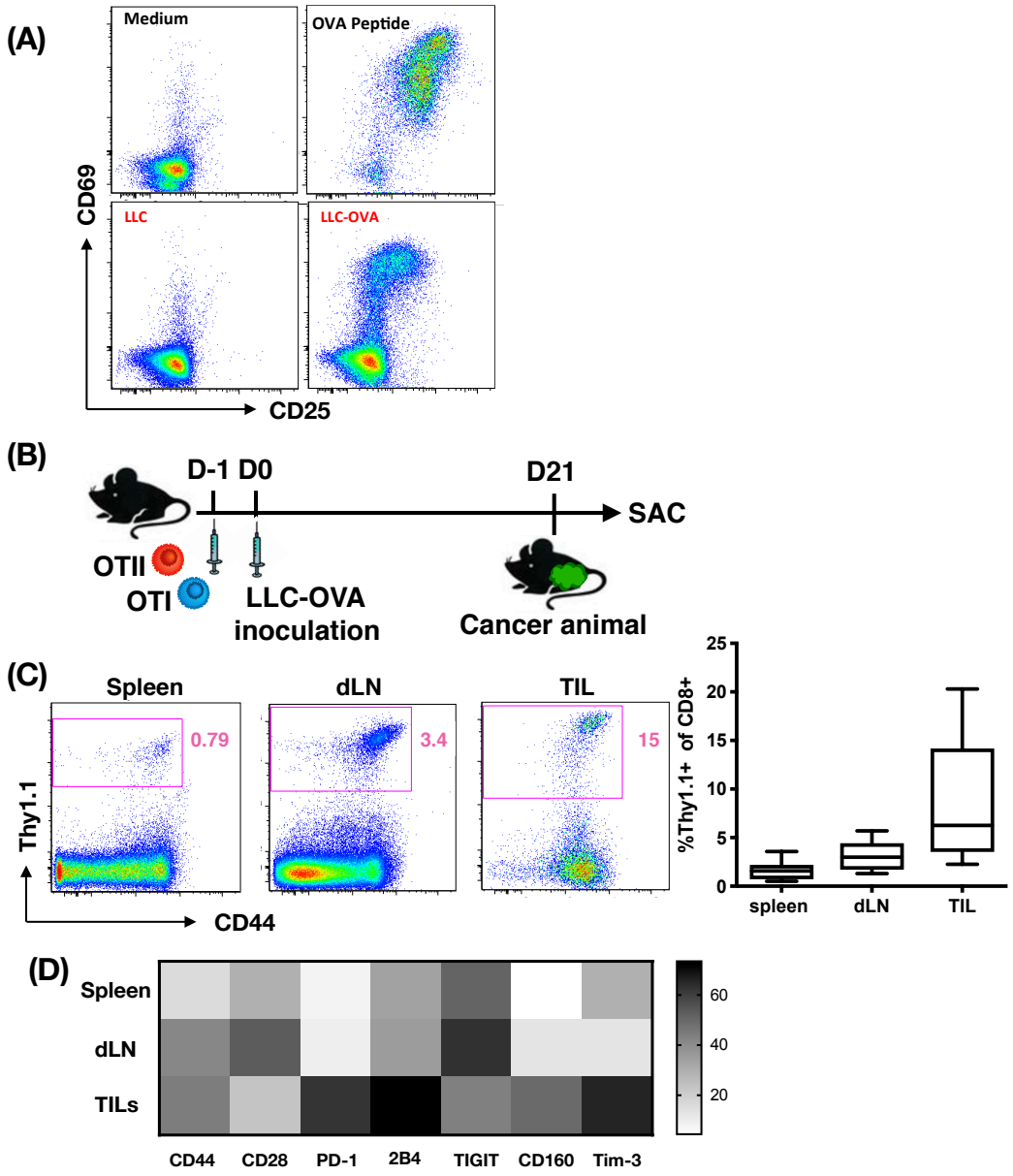

Figure 4.1 LLC-OVA activates OTI cells in vitro and in vivo

(A) OTI splenocytes were co-culture with LLC or LLC-OVA for four days. Surface markers CD25 and CD69 were stained for OTI activation. Representative counterplots (gated on $CD3^{+}CD8^{+}Thy1.1^{+}$) in each condition are shown. (B) The experiment timeline for LLC-OVA cancer animals. OTI and OTII cells were adoptively transferred into animals. Next, LLC-OVA

was inoculated on animal thigh for three weeks. After three weeks, OTI cells from different locations were harvested and assessed the phenotypes. (C) Representative counterplots (gated on Live cells and $CD45^+CD3^+CD8^+$) for the percentage of OTI cells in total $CD8^+$ T cells from different locations. The summary part of $Thy1.1^+$ in $CD8^+$ from different locations is shown. (D) The phenotype of OTI cells from different locations. The MFI of surface marker was transformed into heatmap graph.

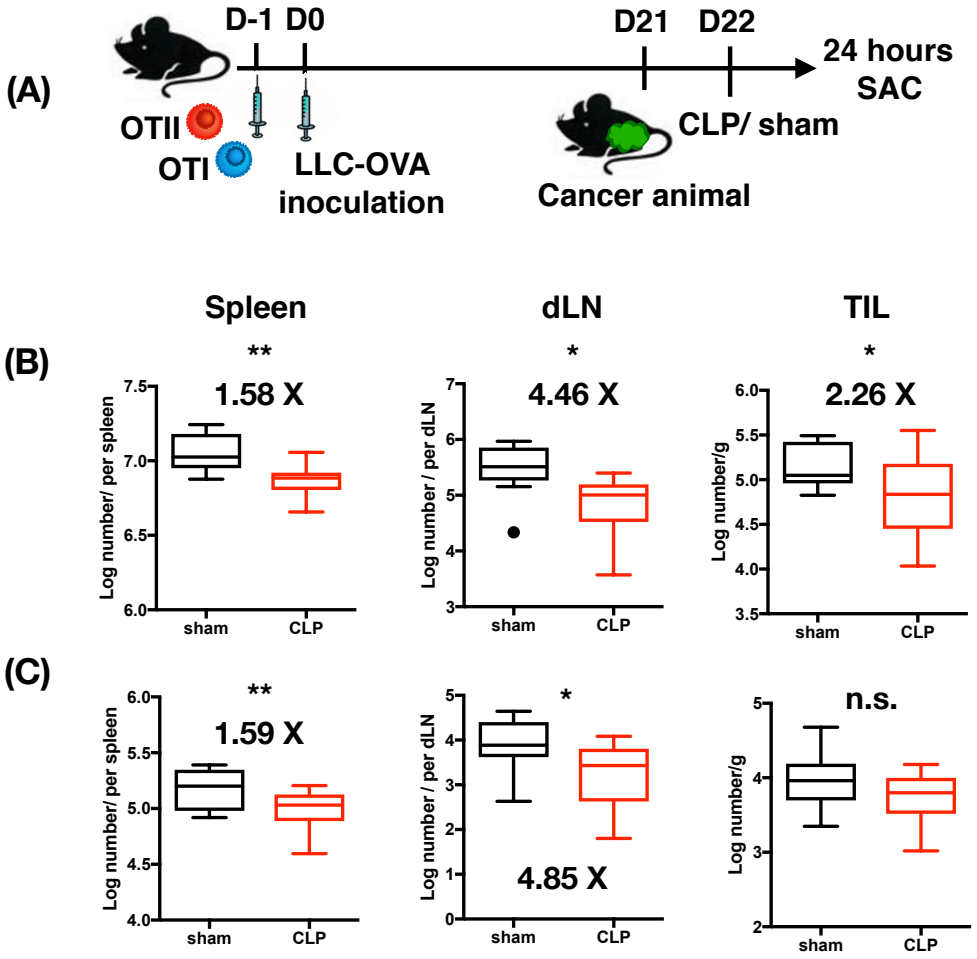

Figure 4.2 Sepsis induced tumor-specific T cell lost in the circulation but not in tumor tissue

(A) The experiment timeline for LLC-OVA cancer animals and the CLP surgery. LLC-OVA cancer animals were generated by previously decried. CLP surgery was performed to induce polymicrobial sepsis on animals and sham surgery was performed as control. After 24 hours, animals were sacrificed, and absolute T numbers were determined. (B) The absolute number of endogenous $CD8^+$ T cells ($CD3^+CD8^+Thy1.1^-$) in different locations were determined and the summary figures are shown. (C) The absolute number of tumor-specific OTI cells ($CD3^+CD8^+Thy1.1^+$) in different locations were determined and the summary figures are shown.

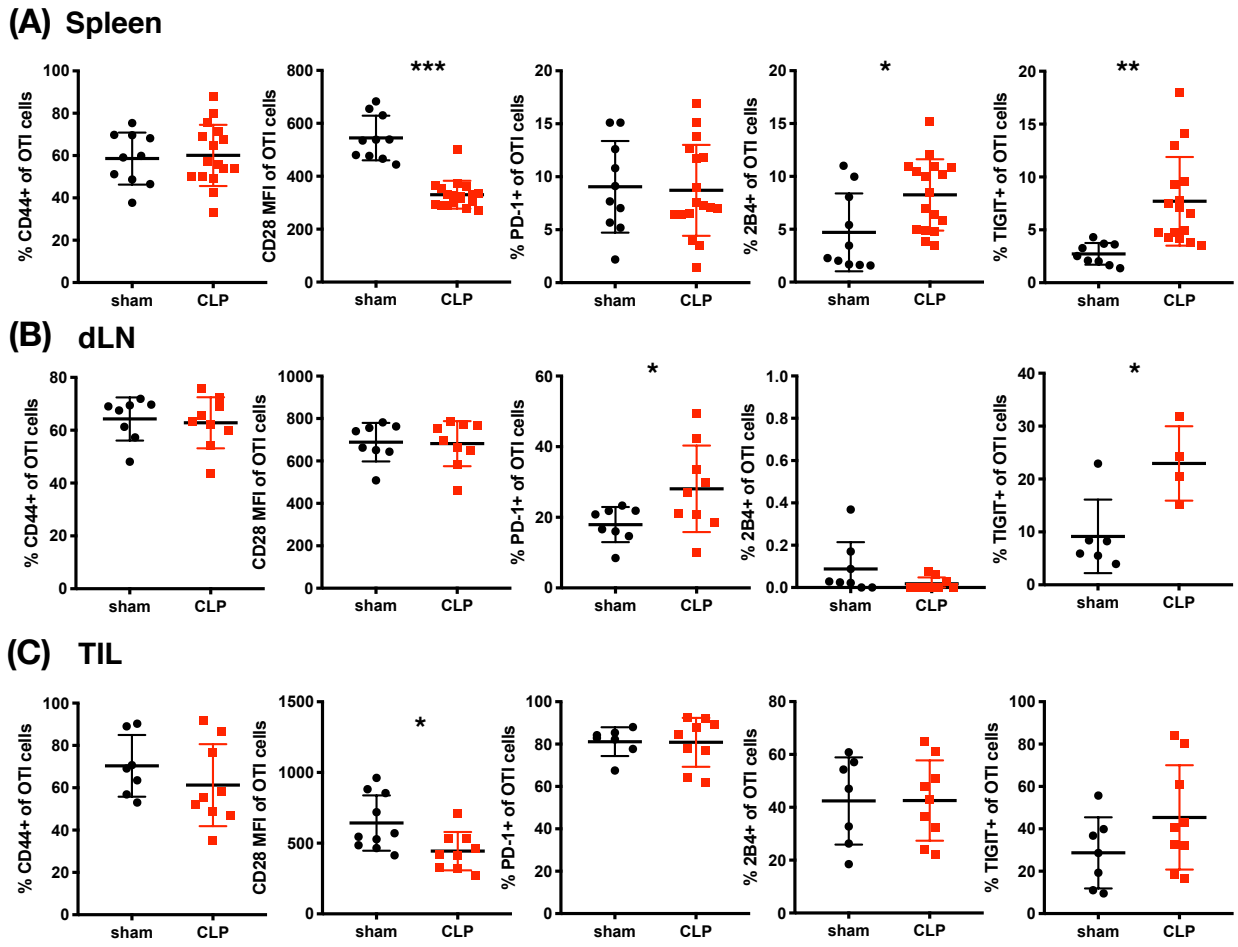

Figure 4.3 Increased co-inhibitory receptors on tumor-specific T cells were found in spleen and dLN, but not in tumor tissue

LLC-OVA cancer animals were sacrificed at 24 hours post CLP. The phenotype of OTI ($CD3^+CD8^+Thy1.1^+$) cells in sham and CLP mice were determined. (A) The summary figures of different surface markers for tumor-specific OTI cells in the spleen are shown. (B) The summary figures of different surface markers for tumor-specific OTI cells in the dLN are shown. (C) The summary figures of different surface markers for tumor-specific OTI cells in the TILs are shown.

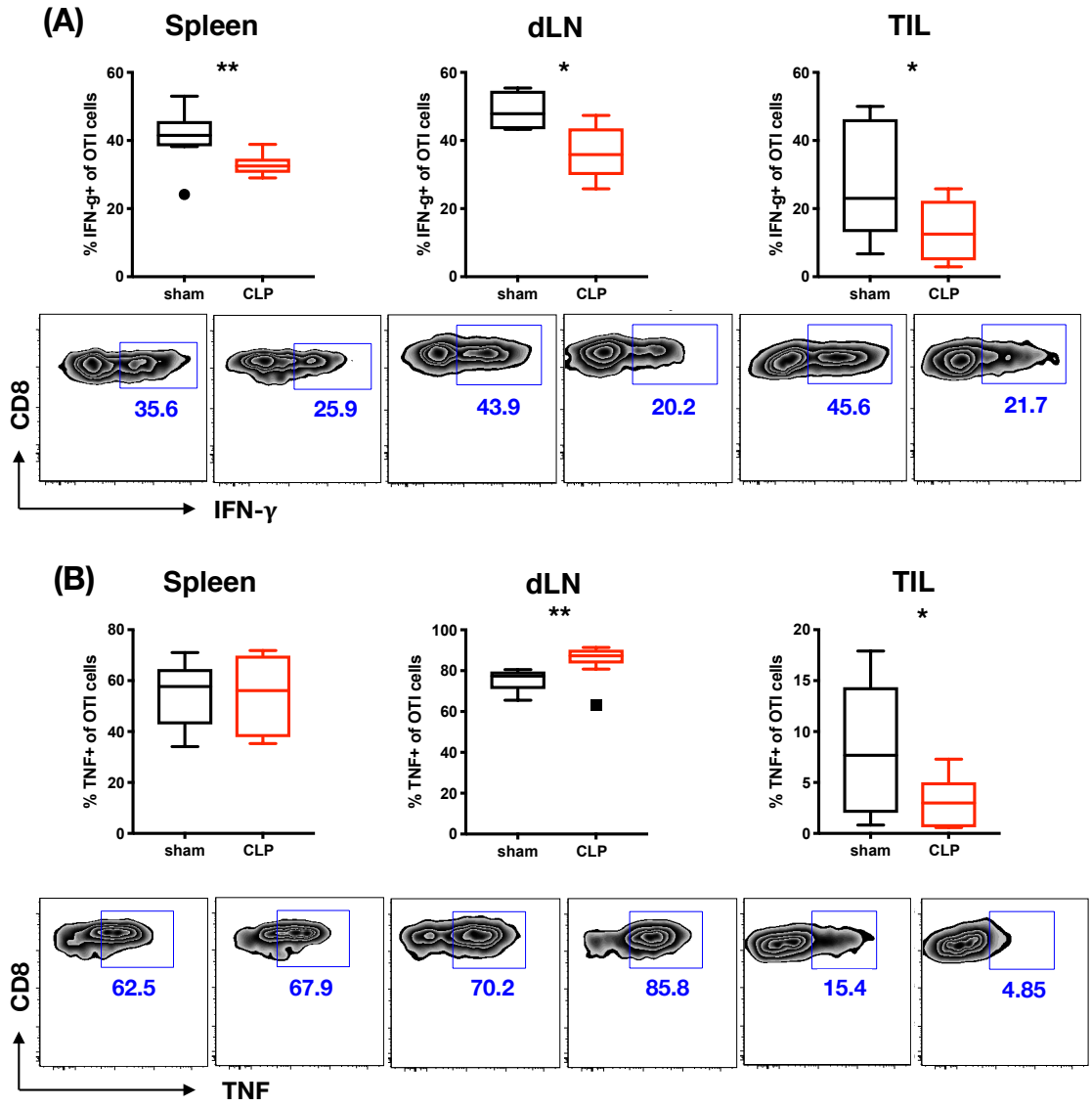

Figure 4.4 Sepsis impaired IFN-γ secretion of tumor-specific T cells in different locations

LLC-OVA cancer animals were sacrificed at 24 hours post-surgery. The splencytoes were collected and stimulated with PMA and Ionomycine for cytokine analysis. (A) The summary figures and representative flow figures for percentage of IFN-γ^+ in tumor-specific OTI cells ($CD3^+CD8^+Thy1.1^+$) in different locations. (B) The summary figures and representative flow figures for percentage of TNF^+ in tumor-specific OTI cells ($CD3^+CD8^+Thy1.1^+$) in different locations.

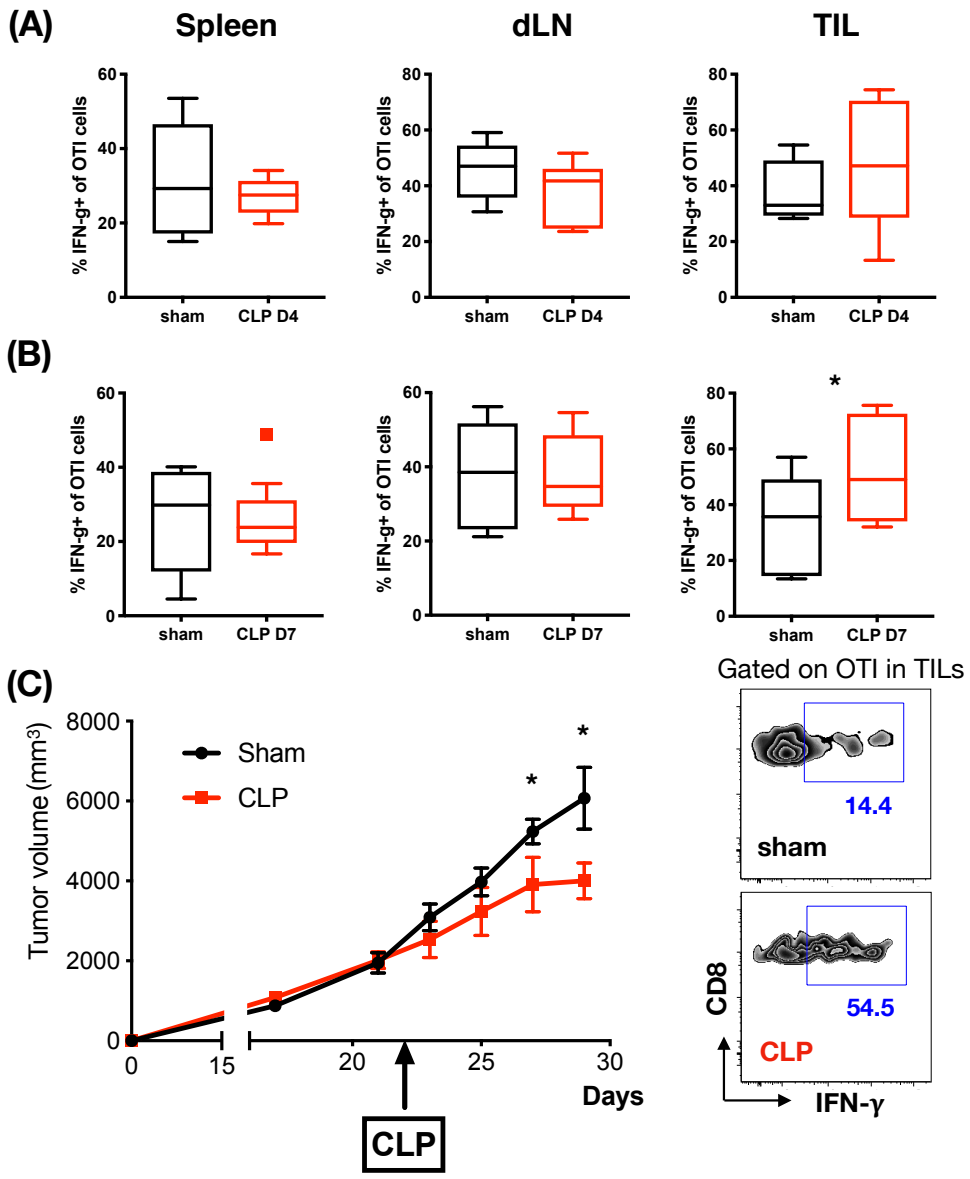

Figure 4.5 IFN-γ secretion of tumor-specific T cells recovered at later stage of sepsis and resulted in reduced tumor growth in CLP animals

(A) LLC-OVA cancer animals were sacrificed at day 4 post-surgery. The splencytoes were collected and stimulated with PMA and Ionomycine. The summary figures for percentage of IFN-γ^+ in tumor-specific OTI cells ($CD3^+CD8^+Thy1.1^+$) in different locations. (B) LLC-OVA cancer animals were sacrificed at day 7 post-surgery. The splencytoes were collected and stimulated with

PMA and Ionomycine. The summary figures for percentage of IFN-γ^+ in tumor-specific OTI cells ($CD3^+CD8^+Thy1.1^+$) in different locations. Representative flow figures showed IFN-γ staining for OTI cells in TILs at Day 7 post-surgery. (C) The tumor volume of LLC-OVA cancer animals was measured after surgery and the summary figure of tumor volume at different time points is shown.

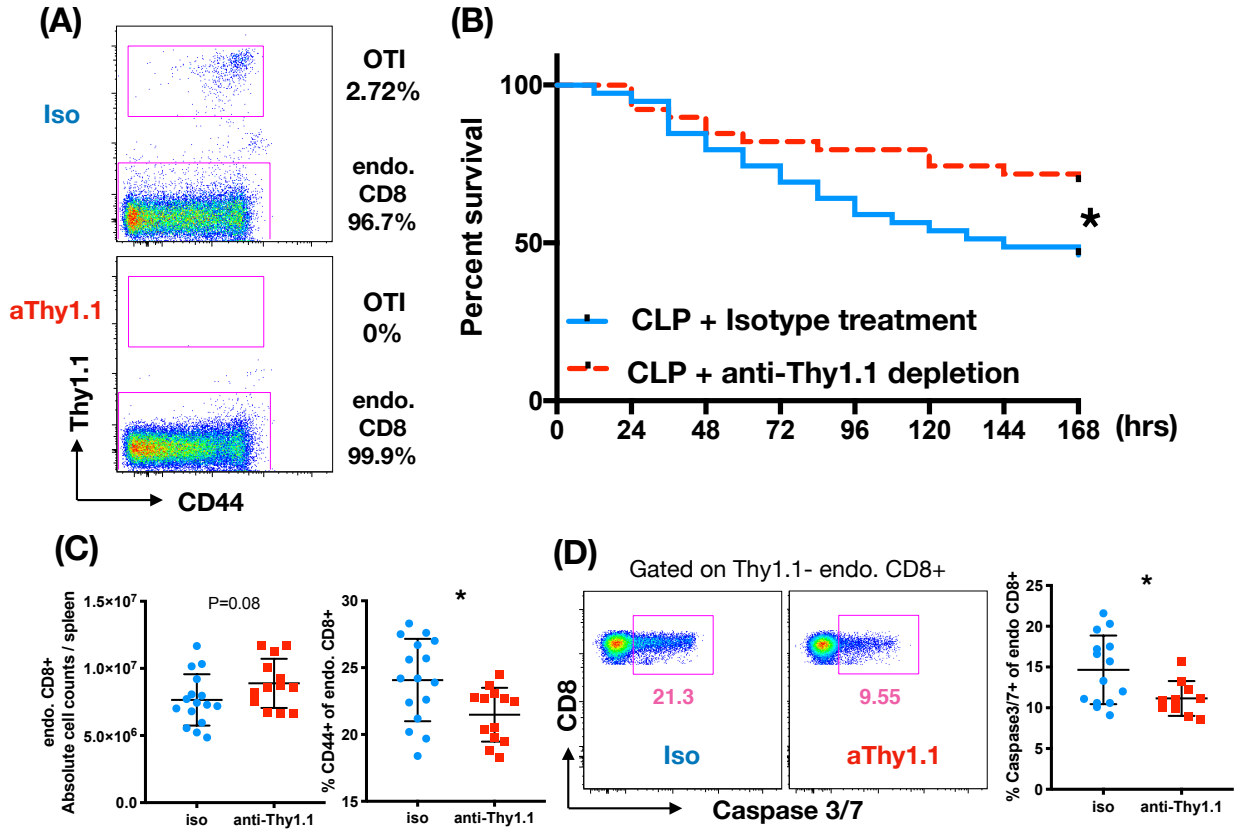

Figure 4.6 Tumor-specific T cells contributed to sepsis mortality by disturbing endogenous CD8$^+$ T cells cellular apoptosis during sepsis

(A) Representative flow figures showed the anti-Thy1.1 depletion of tumor-specific T cells and the gating strategy for endogenous CD8$^+$ T cells. (B) Anti-Thy1.1 depletion antibody or isotype control were given at one day prior to CLP, the survival curve was followed for 7 days post-surgery. (C) The animals were sacrificed at 48 hours post CLP and splencytoes were harvested for surface and caspase3/7 staining. Summary figures showed the absolute number of endogenous CD8$^+$ T cells, and percentage of CD44$^+$ in endogenous CD8$^+$ T cells (gated on CD3$^+$CD8$^+$Thy1.1$^-$). (D) Representative flow figures showed the caspase3/7 staining in endogenous CD8$^+$ T cells (gated on CD3$^+$CD8$^+$Thy1.1$^-$) in two groups. The summary figure of caspase3/7 staining in endogenous CD8$^+$ T cells is shown.

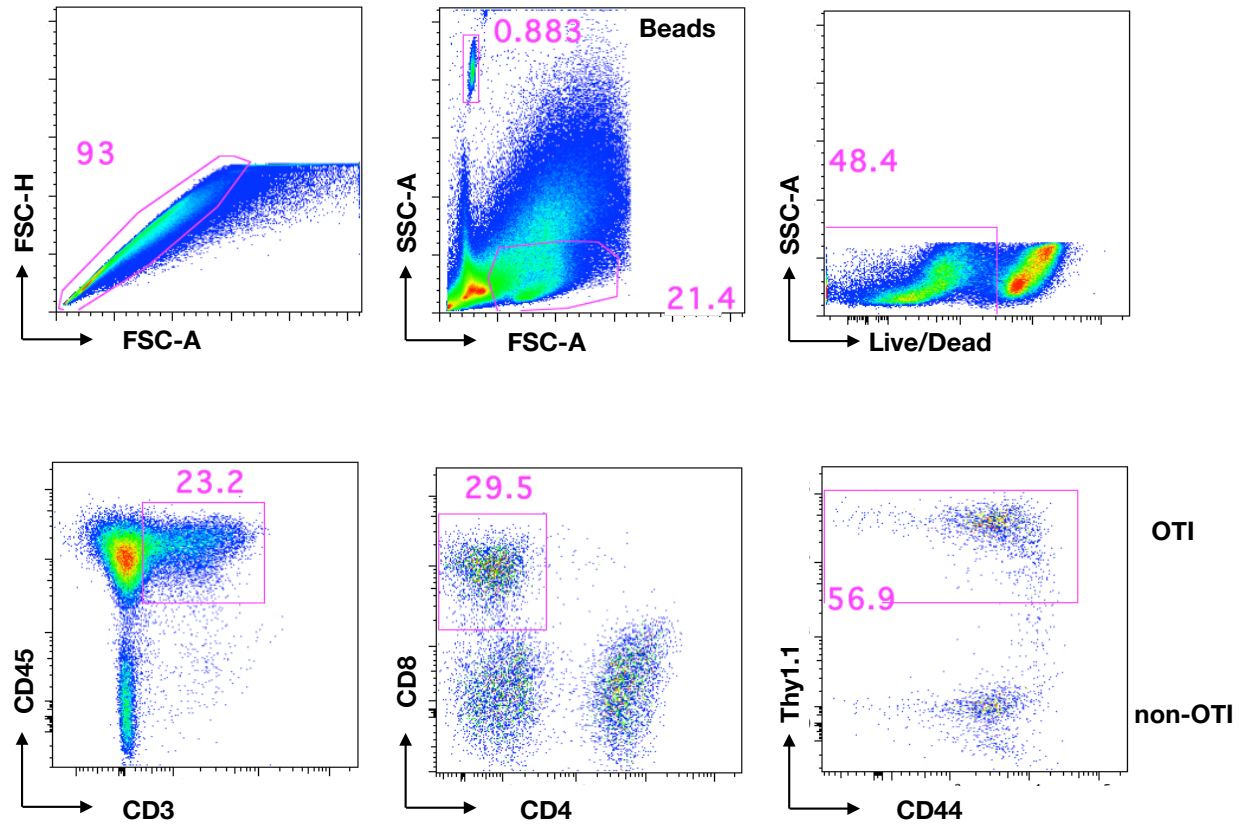

Figure 4.7 Gating strategy for tumor specific OTI T cells.

Representative flow figures showed the OTI gating strategy in TILs. Singlet and lymphocytes gate were applied first, and then Live^{+}CD3^{+}CD45^{+}CD8^{+} cells were gated. OTI cells were defined as CD8^{+}Thy1.1^{+} and non-OTI cells (endogenous CD8^{+} T cells) were defined as CD8^{+}Thy1.1^{-}.

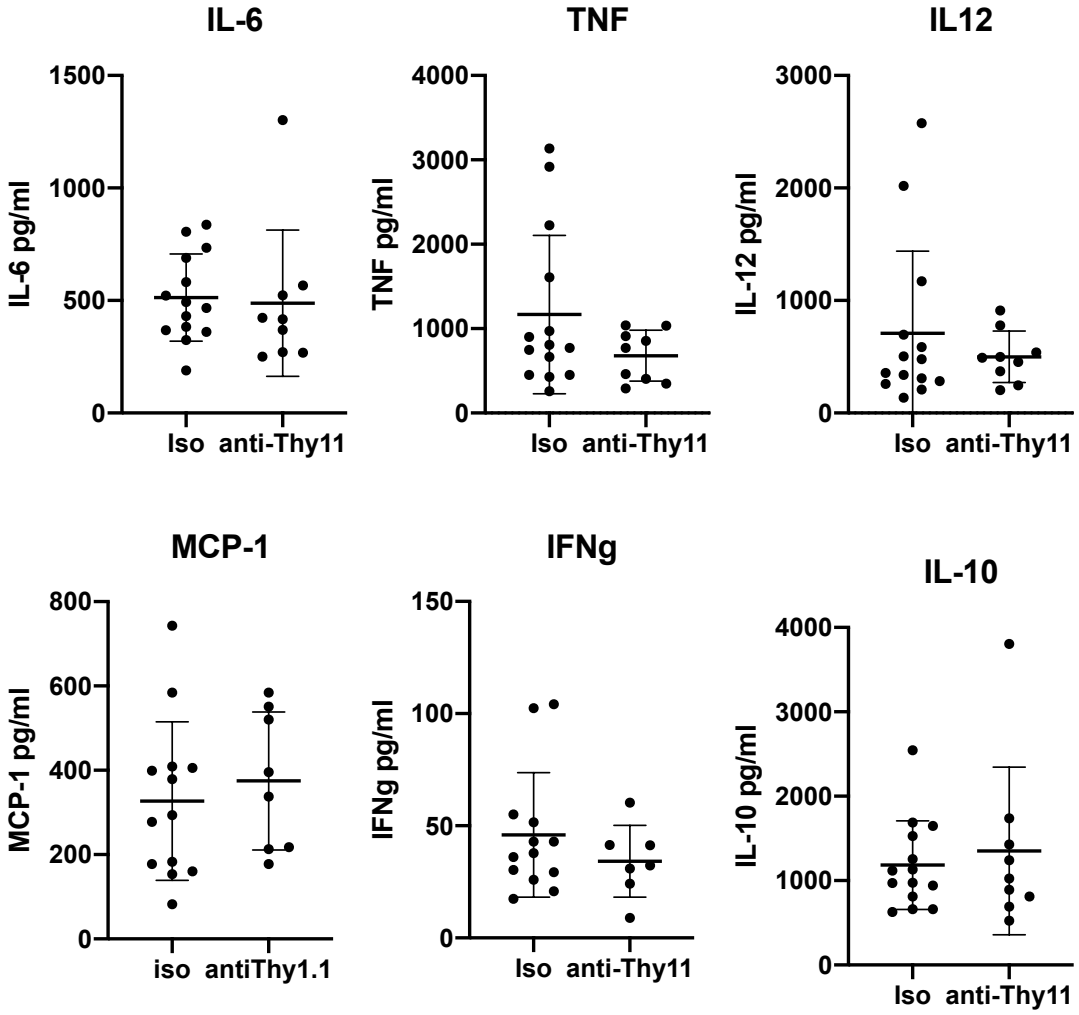

Figure 4.8 The serum cytokine in cancer septic animals at 24 hours-post CLP.

LLC-OVA cancer animals treated with anti-Thy1.1 or isotype control were subjected to CLP and bled at 24 hours post-CLP. The serum was collected and measured the inflammatory cytokine. Data in this figure were generated by Ching-wen Chen and Oami Takehiko.

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Chapter 5. Discussion

5.1 Discussion

The summary figure for the dissertation is in Figure 1. In Chapter 2 study, we showed that both septic animals and human septic patients exhibited increased 2B4 on $CD4^+$ T cells at the early stages of sepsis. Importantly, genetic deficiency of 2B4 or pharmacologic inhibition of 2B4 significantly increased survival in a murine polymicrobial sepsis model. Specific conditional knockout of 2B4 on $CD4^+$ T cells results in improved survival and reduced immune dysregulation during sepsis. Our findings illuminate a novel role for 2B4 coinhibitory signaling on $CD4^+$ T cells in contributing to sepsis-induced lymphocyte dysfunction and mediating sepsis mortality. Next, we aimed to study immune dysfunction during sepsis in animals with pre-existing cancer, a common morbidity in septic patients. In Chapter 3 study, we determined that PD-1 blockade exhibited no therapeutic effect on cancer sepsis survival, though it has been shown to improve sepsis survival in previously health septic animals. The mechanisms of diminished efficacy of PD-1 blockade in cancer septic animals involve the reduction in both the quality and quantity of PD-1^+ cells after sepsis. Specifically, the number of PD-$1^+$$CXCR5^+$ responder cells and their CD28 expression are both decreased during sepsis. We next showed that blockade of 2B4 in cancer septic animals modulated T cell co-signaling molecule expression and resulted in improved sepsis survival. In Chapter 4 project, we utilized tumor-specific TCR transgenic T cells in the pre-existing cancer sepsis model to study the impact of sepsis on tumor antigen-specific $CD8^+$ T cell responses. We found that sepsis impacts anti-tumor T cells differently based on the T cell location. Anti-tumor OT-I cells located in the tumor tissue exhibited less sepsis-induced dysfunction compared to OT-I cells in dLN and spleen, while cytokine secretion of tumor-specific T cells was impaired in all locations at early stages of sepsis. Importantly, we found that tumor-specific T cells partially contributed to sepsis mortality in cancer septic animals by affecting endogenous $CD8^+$ T cell

apoptosis. This novel research provides insight into how sepsis shapes anti-tumor immunity. In sum, my work suggests distinct co-inhibitory receptors signaling on T cells are involved in sepsis-induced immune dysfunction. Moreover, my work also helps depict the interplay between antigen-specific T cell responses and immune pathogenesis during sepsis (Figure 1).

Co-inhibitory receptor signaling on exhausted T cells is complicated and requires re-evaluation in different disease scenarios. Most of our understanding of co-inhibitory receptors has been gleaned by studying exhausted T cells in chronic virus infection and tumor models, which depend on $CD8^+$ T cells. However, in sepsis, the mechanisms that lead to T cell exhaustion and the functions of co-inhibitory receptors may be different from chronic virus infections. For instance, in chronic infection models or cancer models, T cell exhaustion is found on antigen-specific T cells due to persistent high antigen levels in the environment, and the exhausted profiles require a certain time to develop. About 3%-5% of total $CD8^+$ T cells in LCMV C1-13 infected mouse develop into exhausted after 30 days post infection. In contrast, the exhausted phenotype during sepsis are observed on systemic T cells at early time points and continually develop during the later stage of sepsis. Systemic T cell apoptosis and impaired functions are found as early as 24 hours post-CLP in septic animals in our model (Chapter 2, figure 2) or within 3 days of the onset of infection in septic patients. It is believed that the extensive cell apoptosis, cytokine storm, and reduced innate functions all contributed to T cell exhaustion in sepsis, which are distinct mechanisms than chronic virus infection. In addition to the differential mechanisms contributing to T cell exhaustion, the same co-inhibitory receptor may have different functions in different disease scenarios. Take PD-1 as an example, PD-1 blockade in previously healthy animals has been shown to improve sepsis survival in several animal models, and PD-1/PD-L1 blockade has entered clinical trials with encouraging results for septic patients (1). However, in the model of

sepsis comorbidity with pre-existing cancer, PD-1 blockade failed to improve sepsis survival. We found that PD-1 is already expressed on cancer lymphocytes prior to sepsis and maintained a similar level after CLP (Chapter 3, Figure 2). Notably, we observed the same result on the tumor-specific T cells (Chapter 4, Figure 3), indicating that PD-1 is not the primary inhibitory receptor to represent the exhausted status during cancer sepsis and checkpoint blockade should be considered carefully in the criteria for future clinical cancer sepsis trials. On the other hand, we identified that 2B4 and other co-inhibitory molecules, such as TIGIT (Zhang and Ford, unpublished data) are the co-inhibitory molecules mediating T cell dysfunction in cancer septic model. Our results suggest that different co-inhibitory molecules play distinct and non-redundant roles in inducing T cell dysfunction.

In our study, we found that 2B4 blockade improves T cell functions, supported by evidence in this dissertation. First, $2B4^{-/-}$ animals exhibited more IFN-γ secreting $CD4^+$ T cells and fewer apoptotic $CD4^+$ T cells after sepsis (Chapter 2, figure 2). Next, the specific chimera animals also exhibited increased IFN-γ secreting $CD4^+$ T cells and increase macrophage activation (Chapter 2, Figure 3). Third, in cancer septic animals, 2B4 blockade leads to increased CD48 expression and reduced PD-1 and CTLA4 expression during sepsis (Chapter 3, Figure 8). However, the full mechanisms behind how 2B4 blockade improves T cell functions remain ambiguous. 2B4 is a member of the CD2 family of proteins; 2B4 shares the same ligand CD48 in the mouse with CD2 (m2B4 has a 9-fold higher affinity to mCD48 than mCD2 (2)). In humans, CD2 binds to CD58 but has lower affinity for CD48 (3). One possible hypothesis we have developed is that 2B4 competes with CD2 for ligation of CD48 during sepsis (Figure 2). After sepsis, post-mortem and animal studies demonstrated that the expression of the co-stimulatory molecule CD28 on T cells is extensively diminished. It is possible that the activation and survival signaling of T cells may

heavily rely on other co-stimulatory molecules, such as CD2. The elevated 2B4 expression during early sepsis might result in competition for ligation of CD48 with CD2, function to reduce the co-stimulatory signals on T cells, and further lead to T cell dysfunction. Direct blockade of 2B4 on 2B4-expressing cells reduced the negative signaling in T cells. On the other hand, blockade of 2B4 not only increases total T cell CD48 expression (Chapter 3, Figure 8) and but also releases the occupation of CD2 for other T cells; therefore, both $2B4^+$ and $2B4^-$ T cells may exhibit increased CD2-CD48 signals, which could promote T cell survival and reduce other co-inhibitory receptor expressions (Figure 2).

One interesting finding is that the expression of 2B4 on human T cells has distinct patterns relative to the laboratory mouse. About 50% of $CD8^+$ T cells in human expressed 2B4, but only 5% of mouse T cells expressed 2B4 at baseline. In contrast, the 2B4 expression on $CD4^+$ T cells is similar between human and mouse at baseline. We posit that the differences in $CD8^+$ T cells stem from differential immune memory conditions in human and mouse. Laboratory animals are housed under specific pathogen-free conditions that limited the exposure to infection; as such the memory $CD8^+$ T cells compartment comprises only 15% of total $CD8^+$ T cells. In contrast, humans acquire memory $CD8^+$ T cells populations following occasional exposure to infectious agents. In support of this idea, we found that 2B4 is highly expressed on human memory $CD8^+$ T cells and its expression on total T cells is positively correlated with the age of patients (data not shown). In addition, in a memory mice model that animals are sequentially infected with LCMV and *Listeria* to generate memory immune compartments, the 2B4 expression on total $CD8^+$ T cells is increased after different pathogens infections (data not shown). In sum, these data suggest that 2B4 on $CD8^+$ T cells may be reflective of the memory status of the host. Of note, although 2B4 is highly expressed on memory $CD8^+$ T cells compared to $CD4^+$ T cells, genetically interrupting 2B4-CD48

in previous studies showed more impact on $CD4^+$ T cells than $CD8^+$ T cells. One research published by Dr. Arlene H. Sharpe and her colleagues demonstrated that $2B4^{-/-}$ mice exhibited enhanced humoral autoimmunity and increased $CD4^+$ T cell activation but not $CD8^+$ T cells. Furthermore, $CD48^{-/-}$ (2B4 ligand) mice also have marked defect in $CD4^+$ T cell activation (4). Our finding that specifically knocking out 2B4 on $CD4^+$ T cells improved sepsis survival, but not $CD8^+$ T cells, also supports this idea.

Our preliminary result indicated that $Foxp3^+$ Treg cells do not express 2B4 on their surface (data not shown). However, we found that blockade of 2B4 decreases regulatory T cells (Treg) percentage and Treg activation (Foxp3 MFI and CTLA4 MFI) (Chapter 3, Figure 8). Therefore, we hypothesized that the reduction of Treg cells may represent a result of improved immune function and is not the direct effect of 2B4 blockade. This hypothesis warrants further investigation. In addition to $CD4^+$ T cells, other immune compartments could contribute to the survival benefits observed in $2B4^{-/-}$ animals in the CLP model. One interesting population is intra-epithelial lymphocytes (IELs) in the gut. IELs play critical roles in the primary immune response against infection; however, few literatures have described the function of IELs during sepsis. 2B4 is highly expressed on IEL populations and $2B4^{-/-}$ animals have significantly reduced IEL numbers in the gut (5). Therefore, our preliminary hypothesis is that the reduced number of $2B4^{-/-}$ IELs contributes to the survival benefits observed in $2B4^{-/-}$ animals by reducing inflammation in the gut. $2B4^{-/-}$ animals will be an ideal model to dissect how immune cells interact with gut epithelial cells during sepsis. Noticeably, distinct 2B4 expression patterns were observed on T cells in different organs in the mouse. 2B4 expression is undetectable in peripheral lymph nodes (Chapter 4, Figure 3), mesenteric lymph nodes and bone marrow (data not shown). In contrast, 2B4 is expressed on

memory T cells in spleen and tumor tissue, indicating that 2B4 might function more than a co-inhibitory receptor on memory T cells.

One fundamental question we are interested in addressing is to understand the mechanism underlying the aggravated sepsis mortality in pre-existing cancer animals compared to previously healthy animals. Some mechanisms have been proposed in our group; here we will discuss the immune hyper-activation in cancer septic animals. Our preliminary data on in vivo 2-NBDG (a fluorescent glucose analog that has been used for measuring glucose uptake) staining showed that prior to CLP, cancer animals $CD8^+$ T cells showed an increase 2-NBDG uptake at baseline compared to previously healthy animals (Figure 3A). Next, Seahorse XF glycolysis Stress assay that measures the capacity of the glycolytic pathway was performed on $CD8^+$ T cells from previously healthy animals and cancer animals. Results showed that both glycolysis activity and glycolytic capacity are higher on $CD8^+$ T cells from cancer animals at baseline (Figure 3B). In addition, the increased glycolysis function on $CD8^+$ T cells is also found in cancer septic animals compared to previously healthy septic animals (Figure 3C). The metabolism profiles suggest that cancer lymphocytes exhibit effector-like phenotypes at baseline and heavily depend on glycolysis for cellular metabolisms. During sepsis, the effector-like phenotypes of lymphocytes may exaggerate hyper-inflammation in the host and may also deprive other cells of nutrients needed for survival. The hyperactivation of lymphocyte in cancer animals may contribute to sepsis mortality is supported by several findings. First, depletion of tumor-specific T cells improved cancer septic survival and decreased other $CD8^+$ T cells cellular apoptosis (Chapter 4, Figure 6). These results imply that selectively removing activated T cells may be beneficial to the host during sepsis. It is possible that survival/growth factors (such as IL-2, IL-7 and IL-15 for T cells) are limited during sepsis, and that activated tumor-specific T cells consume these resources at a cost to other T cells.

Second, our group previously showed that genetically preventing lymphocyte apoptosis by over-expressing the anti-apoptotic protein Bcl-2 or knocking-out pro-apoptotic protein Bim was not beneficial but harmful in cancer septic survivals. The unanticipated results are in contrast to the finding in previously healthy septic animals from others but support the idea that hyperactivation of lymphocytes is harmful in cancer sepsis. In the same study, the authors showed that over-expressing Bcl-2 in lymphocytes in cancer septic animals led to increased TNF and IFN-γ in bronchoalveolar lavage (BAL) and decreased IL-10 secretion from splenocytes, which reinforced the idea that hyper-activation in cancer animals contributes to sepsis mortality.

The balance of co-stimulatory and co-inhibitory molecules is critical in determining T cell function during infection. With increased attention on the immunosuppressive stage of sepsis, reversing sepsis-induced immune dysfunction has become a significant direction of sepsis therapies. Our research adds one potential checkpoint target in sepsis therapy and illustrates several new mechanisms of immune pathogenesis in cancer sepsis.

5.2 Figures and Figure Legends

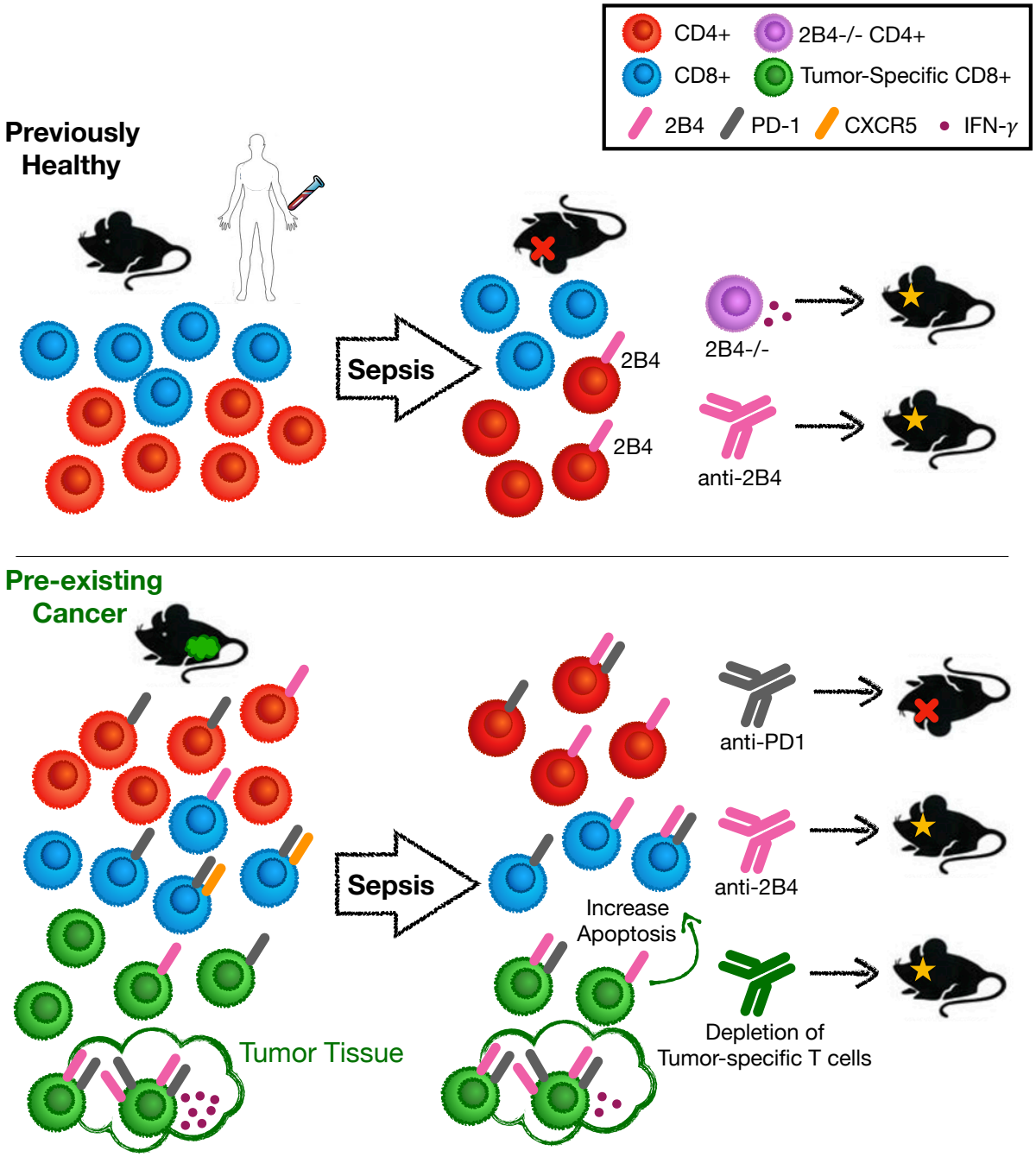

Figure 5.1 Model of the role of 2B4 and checkpoint blockade during sepsis in the absence and presence of pre-existing cancer

In previously healthy human and animals, we found increased 2B4 expression on $CD4^+$ T cells after sepsis. Genetically knocking-out 2B4 on $CD4^+$ T cells and 2B4 blockade both improve sepsis survival. In pre-existing cancer animals, we showed that PD-1 blockade fails to improve sepsis survival due to a decreased number of PD-1^+CXCR5^+ responder cells. Finally, we demonstrated that tumor-specific T cells contribute to sepsis mortality by increasing $CD8^+$ T cell apoptosis.

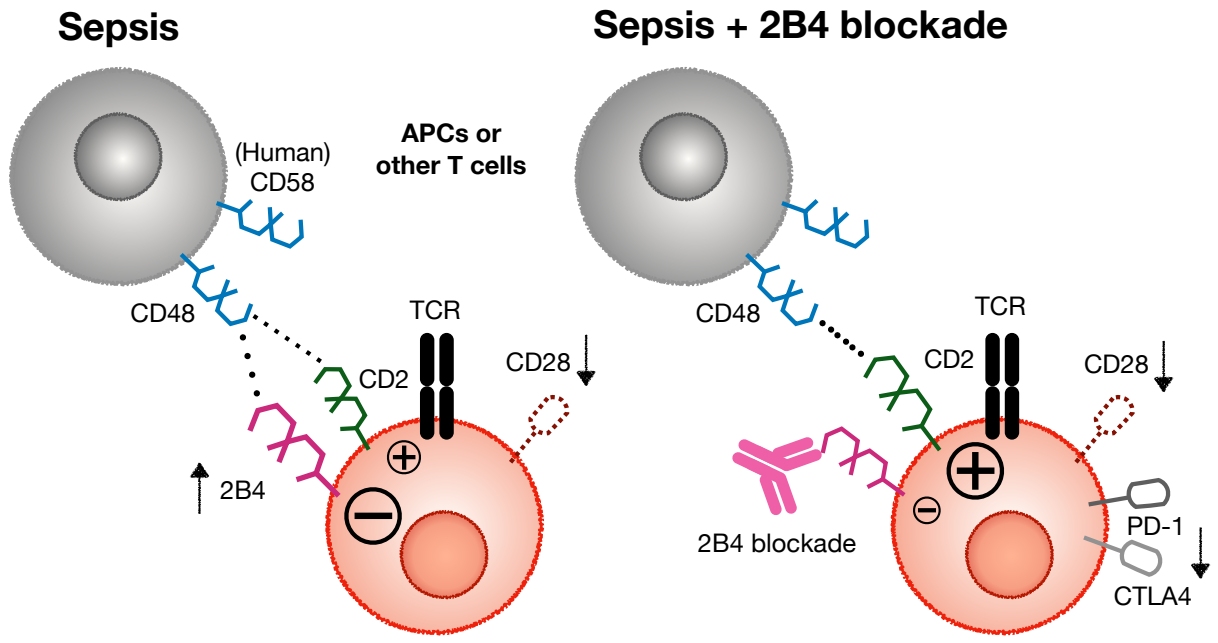

Figure 5.2 Blockade of 2B4 increases CD2-CD48 co-stimulatory signaling and results in decreased expression of PD-1 and CTLA-4

During sepsis, CD28 expression on T cells is decreased extensively and other co-stimulatory signaling (CD2-CD48) may become essential for T cell activation. 2B4 binds to CD48 and competes with CD2 during sepsis. Blockade of 2B4 enhances CD2-CD48 interaction and decreases PD-1 and CTLA-4 expression on T cells.

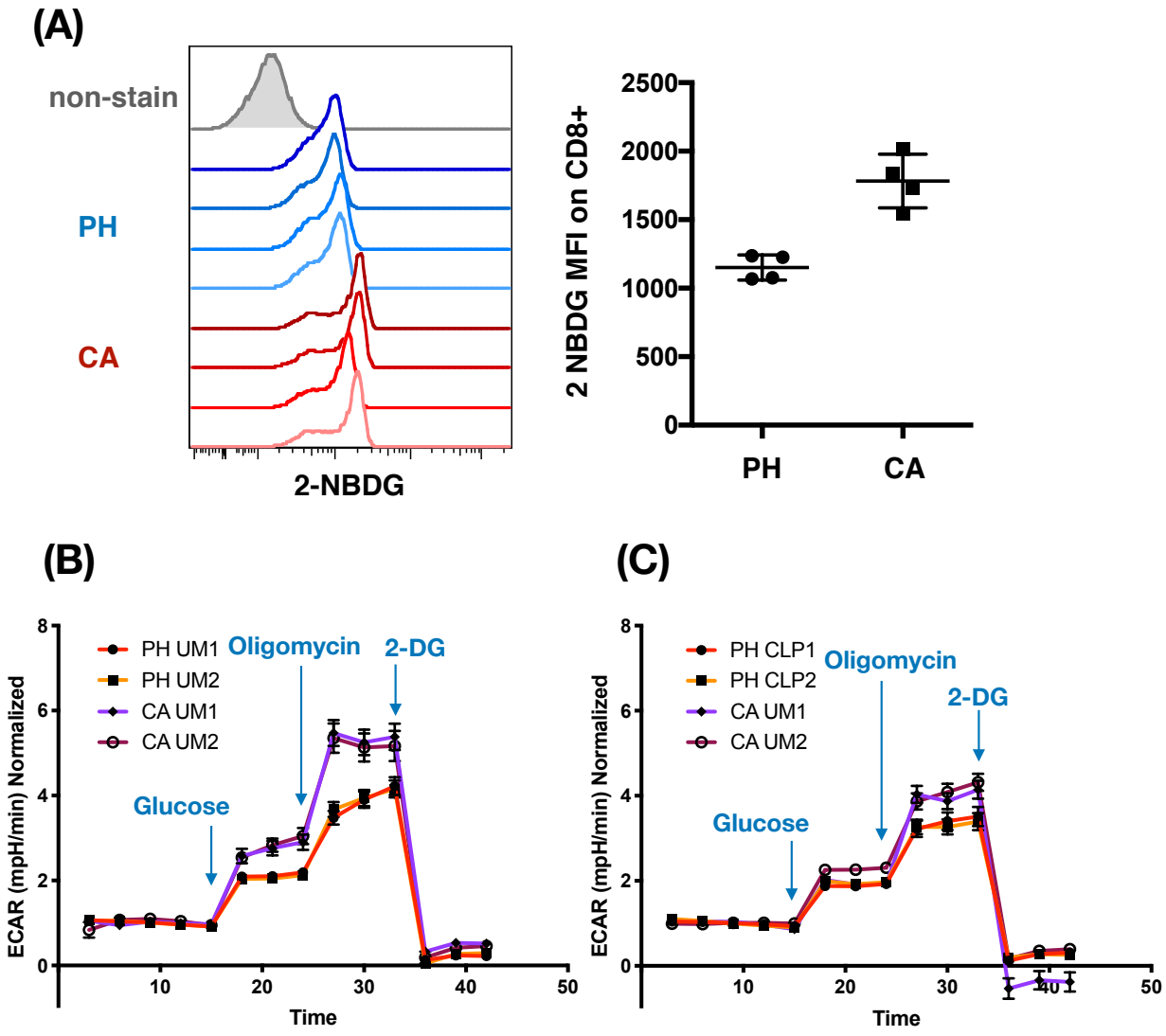

Figure 5.3 Cancer lymphocytes exhibited increased glycolysis.

(A) 2-NBDG was intravenous (IV) injected into cancer animals and previously healthy animals. Animals were sacrificed after one hour and splenocytes were analyzed. The histogram of 2-NBDG staining on $CD8^+$ T cells and summary figure are shown. (B) Seahorse Extracellular Flux Bioanalyzer glycolysis metabolism assay is performed on 300,000 cells of $CD8^+$ T cells from unmanipulated previously healthy animals or unmanipulated cancer animals. The normalized ECAR (extracellular acidification rate) profile is displayed. In the Seahorse glycolysis metabolism assay, the cells were cultured in glucose-free medium and measured the basic ECAR readings

without glycolysis. After 15 minutes, final concentration 10 mM of glucose was added into wells to measure the normal glycolysis function. At 24 minutes time point, oligomycin (final concentration 1 μM) was added to measure the glycolytic capacity in the cells. Oligomycin specifically inhibits mitochondria's ATP synthase and increases dependence on glycolysis, which allows us to measure the maximum capacity for glycolysis in cells. Finally, 2-deoxyglucose (2-DG, final concentration 50 mM) was added to reveal the glycolytic reserve in the cells. Glycolytic reserve can be calculated by glycolytic capacity minus normal glycolysis. 2-deoxyglucose is a derivative of glucose and functions as a competitive inhibitor with glucose to inhibits hexokinase in the first step of glycolysis. 2-deoxyglucose shuts down the glycolysis and shows the values of non-glycolytic acidification. (C) Seahorse glycolysis metabolism assay is performed on 300,000 cells of $CD8^+$ T cells from previously healthy septic animals or cancer septic animals. The normalized ECAR profile is displayed.

5.3 References

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