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IL-27 REGULATES TIGIT ON MEMORY T CELLS DURING SEPSIS

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Abstract

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By Kristen Nicole Morrow

Sepsis is a leading cause of morbidity and mortality worldwide. While most patients survive the first 30 days, long-term mortality is high due to immunosuppression. This immunosuppression is linked to heightened levels of immunoregulatory cytokines. The cytokine IL-27 is of particular interest, as it induces the co-inhibitory molecules TIGIT and PD-1 on $CD4^+$ and $CD8^+$ T cells during cancer and chronic viral infection. IL-27 is also linked to mortality in mouse models of sepsis. Therefore, our work examined IL-27's impact on T cells, particularly memory T cells, which prevent secondary infections after sepsis. We used the murine model of cecal ligation and puncture (CLP) to model sepsis induced by ruptured appendicitis in humans.

We found that most T cells express the IL-27 receptor (IL-27Rα) following CLP. However, memory T cells expressing IL-27Rα are significantly reduced in number and frequency compared to naïve T cells. Further analysis revealed that IL-27Rα associates with TIGIT expression on memory $CD4^+$, but not $CD8^+$, T cells during sepsis. Surprisingly, IL-27 was not associated with PD-1 expression in either T cell population. The induction of TIGIT was not associated with alterations in cellular apoptosis and was instead associated with increased proliferation of IL-27Rα$^+$ T cells one day following CLP.

Although IL-27 was previously reported to regulate TIGIT on $FoxP3^+$ regulatory T cells, the frequency of regulatory T cells was unaltered by IL-27 signaling. While TIGIT expression was not associated with alterations in IFNγ production, memory $CD4^+$ T cells expressing TIGIT had a reduced capacity to produce TNF. Ultimately, the IL-27 induced differences in memory T cells were not associated with changes in sepsis mortality – genetic abrogation of IL-27 signaling and blockade of IL-27 did not lead to any improvements.

These findings suggest that the induction of co-inhibitory molecules by IL-27 is disease-state and context-dependent. Further studies are needed to determine the factors that regulate IL-27's function during sepsis compared to other disease states.

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TABLE OF CONTENTS

TABLE OF CONTENTS ..... *i*

LIST OF FIGURES ..... *iii*

CHAPTER 1: INTRODUCTION *1*

I. AN OVERVIEW OF SEPSIS **1**

Treating sepsis 3

Early clinical trials for sepsis 4

Recent clinical trials for sepsis 6

Modeling sepsis in mice 7

II. THE DYSFUNCTIONAL IMMUNE RESPONSE TO SEPSIS **8**

Innate immune response to sepsis 9

Adaptive immune response to sepsis 11

The cytokine response to sepsis 14

III. IL-27 AND SEPSIS **16**

The role of IL-27 in sepsis pathophysiology 17

Interactions between IL-27 and other cytokines relevant to sepsis 18

IL-27 and IL-17 19

IL-27 and IL-33 20

IV. IL-27 AND T CELL FUNCTION **21**

IL-27 and Treg 21

IL-27 and memory T cells 22

IL-27 and co-inhibitory markers 22

V. FIGURES **24**

CHAPTER 2: THE IL-27 RECEPTOR REGULATES TIGIT ON MEMORY CD4⁺ T CELLS DURING SEPSIS *26*

I. ABSTRACT **26**

II. INTRODUCTION **26**

III. METHODS **29**

IV. RESULTS **33**

V. DISCUSSION **40**

VI. FIGURES **48**

CHAPTER 3: CONCLUSION *65*

References *75*

LIST OF FIGURES

Figure 1.1: The Proposed Role of the IL-17, IL-27, and IL-33 Axis During Sepsis..... 24

Figure 1.2: T cell dysfunction induced by IL-27 signaling during chronic viral infections and cancer 25

Figure 2.1: Sepsis results in a reduction in the frequency and number of IL-27Rα^{+} T cells.... 48

Figure 2.2: Gating strategy for IL-27Rα on CD4^{+} and CD8^{+} T cells..... 49

Figure 2.3: IL-27Rα^{-} CD44^{lo} and CD44^{hi} numbers are unchanged following sepsis..... 50

Figure 2.4: Gating strategy for PD-1 and TIGIT on CD4^{+} and CD8^{+} T cells 51

Figure 2.5: IL-27Rα expression is associated with an increased frequency of TIGIT expression on memory CD4^{+} T cells during sepsis 52

Figure 2.6: IL-27 signaling is not associated with CD44^{hi} T cell apoptosis in septic mice.....54

Figure 2.7: IL-27 signaling is associated with reduced proliferation of CD44^{hi} CD8^{+} T cells in septic mice 55

Figure 2.8: *Il27ra*$^{-/-}$ septic mice have a reduced frequency of TIGIT^{+} memory CD4^{+} T cells 57

Figure 2.9: IL-27 signaling does not impact the frequency of Tregs during sepsis..... 58

Figure 2.10: Memory CD4^{+} T cells in *Il27ra*$^{-/-}$ mice exhibit impaired production of IFN-γ at baseline and during sepsis 59

Figure 2.11: Circulating IFN*γ* is unchanged in *Il27ra*$^{-/-}$ vs wild type mice following CLP.....61

Figure 2.12: TIGIT is not associated with the impairments observed in cytokine production 62

Figure 2.13: IL-27 signaling does not impact the survival of septic mice 64

Figure 3.1 Impact of IL-27 signaling during chronic viral infections and cancer versus sepsis74

CHAPTER 1: INTRODUCTION

I. AN OVERVIEW OF SEPSIS

The word "sepsis" was coined by Grecian writers centuries before any understanding of germ theory existed in the medical community (Funk, Parrillo et al. 2009, Opal 2011). At the time, Hippocrates and others believed that sepsis was a state of decay (the Greek word "sepo" means "I rot") in the colon, and that enemas could reverse this decay and prevent death (Funk, Parrillo et al. 2009, Opal 2011). In 1837, the French physician Pierre Piorry coined the word "septicemia", combining the word "sepsis" with the Greek word "aima" (blood) (Cavaillon and Chretien 2019). While Piorry thought that the blood in septic patients was altered by a foreign contaminant, it wasn't until 1882 that scientists linked micro-organisms to sepsis (Cavaillon and Chretien 2019). In 1886, Van Arsdale argued that sepsis is caused by a very small number of bacteria in the blood—a number so small that it is often impossible to culture (Van Arsdale 1886). However, disagreement over the nature of sepsis persisted for many years, and it wasn't until 1991 that the first definition of sepsis was published by a committee of experts from the American College of Chest Physicians and Society of Critical Care Medicine (Bone, Balk et al. 1992).

At the time, there was a general idea that sepsis was a profoundly inflammatory state. Even though this state, which they termed systemic inflammatory response syndrome (SIRS) could occur in the absence of infection, the committee felt that SIRS was a necessary component of sepsis. In order to distinguish sepsis from other causes of inflammation, the committee decided that sepsis can only be suspected if SIRS was associated with a recognizable infection (Bone, Balk et al. 1992). The formal definition stated that "sepsis is the systemic inflammatory response to infection" associated with

two or more of the following: alterations in body temperature, elevated heart rate, elevated respiratory rate, altered white blood cell count (Bone, Balk et al. 1992).

In 2001 another group of experts convened to revisit the 1991 definition of sepsis (Levy, Fink et al. 2003). Although the definition of sepsis was not changed, the revised guidelines greatly expanded the diagnostic criteria that clinicians should consider when evaluating patients with suspected sepsis (Levy, Fink et al. 2003). In light of numerous advances that have occurred since 2001, the definition of sepsis was again revisited in 2016 (Singer, Deutschman et al. 2016). The task force removed the focus on inflammation and eliminated mention of SIRS, changing the definition of sepsis to the "life threatening organ dysfunction caused by a dysregulated host response to infection" (Singer, Deutschman et al. 2016). The task force put forward the quick Sequential Organ Failure Assessment (qSOFA) to identify adult patients at risk for poor outcomes due to suspected infections (Singer, Deutschman et al. 2016). The qSOFA criteria identifies patients at highest risk as those with a respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure less than or equal to 100 mm Hg) (Singer, Deutschman et al. 2016).

Based on the most recent definition of sepsis, there were an estimated 48.9 million cases of sepsis in 2017 worldwide with 11 million sepsis-related deaths (Rudd, Johnson et al. 2020). Sepsis-related mortality accounted for 19.7% of worldwide deaths that year, with the highest incidence and mortality occurring in low- and middle-income countries (Rudd, Johnson et al. 2020). However, these numbers represent a 18.8% decrease in sepsis cases between 1990 and 2017 (Rudd, Johnson et al. 2020). While these numbers suggest there has been an improvement in treating sepsis, no clinical trials have been successful in the past 30 years (Opal, Dellinger et al. 2014, Prescott, Calfee et al. 2016).

Therefore, improvements in survival are likely caused by improved recognition and diagnosis of sepsis and better adherence to treatment bundles (further described below).

Treating sepsis

Although the definition of sepsis has undergone many revisions over the past 30 years, the treatment of sepsis has remained much the same. For much of the past 30 years, the standard of care was to administer early, goal-directed therapy (EGDT) (Rivers, Nguyen et al. 2001, Dhooria, Sehgal et al. 2017). This therapy most commonly consisted of intravenous fluids, vasopressors, and red blood cell transfusions to meet pre-specified targets for blood pressure, oxygen saturation, and hemoglobin levels. While early studies showed that EGDT reduced 30 day mortality in patients with septic shock, this was tempered by later studies and not found to be generalizable to the wider population of sepsis patients (Investigators, Group et al. 2014, Investigators, Yealy et al. 2014, Angus, Barnato et al. 2015, Mouncey, Osborn et al. 2015, Investigators, Rowan et al. 2017).

More recently, the standard of care has simplified to prioritize the early administration of broad-spectrum antibiotics and intravenous fluids (Rhodes, Evans et al. 2017). This is based on studies that observed better patient recovery when antibiotics and fluids are started quickly (Ferrer, Artigas et al. 2009, Leisman, Goldman et al. 2017, Seymour, Gesten et al. 2017, Kuttab, Lykins et al. 2019). Even a small delay of one hour in administration of antibiotics leads to a measurable increase in mortality (Kumar, Roberts et al. 2006, Ferrer, Martin-Loeches et al. 2014). Due to these studies, the current recommendation is to begin fluid resuscitation and antibiotic therapy within one hour of hospital presentation or sepsis diagnosis (Levy, Evans et al. 2018).

Other than antibiotic therapy, patients receive supportive care as needed to

compensate for organ dysfunction or failure. Ventilators compensate for low circulating oxygen levels, enteral tubes provide nutrients, and dialysis replaces kidney filtration. Due to these innovations, the in-hospital mortality rate is significantly lower than mortality in the months to years following sepsis (Quartin, Schein et al. 1997, Davis, He et al. 2014, Linder, Guh et al. 2014, Wang, Szychowski et al. 2014, Prescott, Osterholzer et al. 2016). For patients whose endpoint is death at the hospital, the most common reasons are refractory shock and withdrawal of care (Vincent, Nelson et al. 2011, Moskowitz, Omar et al. 2017).

Early clinical trials for sepsis

Initial therapeutic development for sepsis focused on limiting inflammation (Freeman and Natanson 1995, Freeman and Natanson 2000). In particular, early clinical trials tested broadly acting corticosteroids and therapies specific for endotoxin, TNF, IL-1, platelet-activating factor, and nitric oxide (Freeman and Natanson 1995, Freeman and Natanson 2000).

High dose corticosteroids were among the earliest clinical trials for sepsis, and used under the assumption that excess inflammation is detrimental to sepsis survival (Freeman and Natanson 1995). The results of the first two clinical trials were published in 1987, with one trial finding that high-dose glucocorticoid therapy did not reduce 14 day mortality (Veterans Administration Systemic Sepsis Cooperative Study 1987) and the other concluding that steroids caused a significant increase in 14 day sepsis mortality due to secondary infection (Bone, Fisher et al. 1987). A 1995 meta-analysis found that there was no overall positive effect of steroids, and no differences between low- and high-dose corticosteroids (Lefering and Neugebauer 1995).

Other early clinical trials targeted endotoxin – lipopolysaccharides produced by gram negative bacteria that can trigger an over-active immune response (Greenman, Schein et al. 1991, Ziegler, Fisher et al. 1991, Bone, Balk et al. 1995). Pre-clinical studies indicated that therapies blocking endotoxin prevented death in septic mice with gram-negative bacteremia, and the first two studies in humans suggested similar results (Greenman, Schein et al. 1991, Ziegler, Fisher et al. 1991). A few years later, another large clinical trial cast doubt on these findings, as it was not associated with a reduction in sepsis mortality (Bone, Balk et al. 1995). The last nail in the coffin came in 2000, when a follow up study was halted early due to a lack of efficacy (Angus, Birmingham et al. 2000).

Inflammatory cytokines were another obvious target, and many early trials tested anti-TNF and anti-IL-1 therapies. TNF appeared to be a promising candidate for sepsis treatment due to its early induction and association with Although the first sepsis clinical trial targeting TNF did not observe a decrease in mortality between placebo treated and TNF blockade treated patients, there was a trend toward reduced mortality 28 days after infusion (Abraham, Wunderink et al. 1995). In a larger follow up phase III trial, anti-TNF had no effect on 28 day mortality or on the incidence or resolution of organ dysfunction in septic patients (Abraham, Laterre et al. 2001). Similarly, administration of anti-IL-1 failed to improve 28 day survival in sepsis patients, resulting in early termination of the phase III trial (Opal, Fisher et al. 1997).

Other molecules tied to inflammation during sepsis such as the lipid mediator platelet-activating factor, and the vasoactive nitric oxide, were also assessed in early clinical trials. The two earliest studies of platelet-activating factor suggested that it was safe and significantly decreased mortality compared to placebo treatment (Dhainaut, Tenaillon et al. 1994, Schuster, Metzler et al. 2003). However, a much larger study found

that platelet-activating factor did not alter the risk of 28 day mortality in sepsis patients (Opal, Laterre et al. 2004). A clinical trial studying a nitric oxide synthase inhibitor was met with even worse results, significantly increasing mortality in treated patients compared to placebo (59% vs 49%, respectively) (Lopez, Lorente et al. 2004).

Collectively, these early studies indicate that inflammatory molecules/pathways are not the best sepsis therapeutic targets. Therefore, most clinical trials since this time have been focused on reducing and reversing patient immunosuppression.

Recent clinical trials for sepsis

While early administration of antibiotics and fluids have improved in-hospital patient outcomes, mortality remains high weeks to months after hospital discharge due to immunosuppression. This highlights the need for additional treatments to reverse immunosuppression. Two immunotherapies have shown promise for sepsis treatment in recent clinical trials. The IRIS-7 trial used recombinant human IL-7 in a small group of patients with septic shock and severe lymphopenia (Francois, Jeannet et al. 2018). Treatment with IL-7 increased absolute lymphocyte numbers and circulating T cells for several weeks after administration; T cell activation and proliferation were also enhanced (Francois, Jeannet et al. 2018). PD-L1 blockade may also improve immune function, as higher doses were associated with increased monocyte HLA-DR expression (Hotchkiss, Colston et al. 2019).

Despite the recent promise of these early stage clinical trials, recent late stage clinical trials have been marred by a lack of success (Opal, Dellinger et al. 2014). Part of the difficulty in sepsis clinical trials is the heterogeneity among patients with sepsis (Prescott, Calfee et al. 2016, Leligdowicz and Matthay 2019). Therapeutic effect may differ

in patient subgroups, confounding the interpretation of clinical trials where it appears there is no clinical benefit from a therapy (Opal, Dellinger et al. 2014, Prescott, Calfee et al. 2016). A recent re-analysis of several clinical trials showed considerable within-trial variations in the baseline risk of death between patients (Santhakumaran, Gordon et al. 2019). When split based on baseline risk of death, there was not enough statistical power to determine if the therapies were effective within individual subgroups (Santhakumaran, Gordon et al. 2019). Another recent retrospective study identified four subgroups of sepsis patients that significantly differed in their clinical response to fluid resuscitation (Zhang, Zhang et al. 2018). In order to routinely allow for subgroup analyses, many more sepsis patients would need to be recruited into clinical trials than is currently done (Opal, Dellinger et al. 2014, Prescott, Calfee et al. 2016).

Modeling sepsis in mice

Prior to reaching clinical trials, therapeutic candidates for sepsis are evaluated in animal models of sepsis. Choice of animal model greatly affects the perceived success of a therapy, without necessary relevance to what will be seen in humans (Opal, Dellinger et al. 2014).

To facilitate the study of cell and organ functions over the course of sepsis, and test new compounds prior to clinical trials, animal models were created that mimicked human sepsis in a standardized way. One of the earliest models of sepsis relies on the injection of bacterial lipopolysaccharide (LPS) or heat killed bacteria to induce endotoxemia. Although this model induces inflammation and organ dysfunction, these changes are not physiologically relevant to human sepsis (Villa, Sartor et al. 1995, Remick, Newcomb et al. 2000). In response, other models relying on live bacterial infection were created.

Cecal ligation and puncture (CLP) was developed in 1983 to model human ruptured appendicitis (Baker, Chaudry et al. 1983). In this procedure, part of the cecum is tied off to restrict blood flow and is punctured by a small needle to allow for the release of stool inside the peritoneum. The immune system will then mount a dysregulated response to the microorganisms in the stool, ultimately causing the mouse to develop organ dysfunction (and therefore sepsis) (Baker, Chaudry et al. 1983, Villa, Sartor et al. 1995, Remick, Newcomb et al. 2000).

When compared with the injection of LPS, CLP induces more complex cytokine kinetics (Villa, Sartor et al. 1995, Remick, Newcomb et al. 2000). While both models result in an increase in circulating TNF, IL-1, and IL-6, these cytokines increase (and then decrease) more rapidly and to a greater extent in mice treated with LPS than in mice that have undergone CLP or in human sepsis patients (Villa, Sartor et al. 1995, Remick, Newcomb et al. 2000).

II. THE DYSFUNCTIONAL IMMUNE RESPONSE TO SEPSIS

During a normal infection, there are multiple levels of defense that prevent the spread of harmful microorganisms throughout the body. The first level of defense is anatomic barriers such as the intestinal epithelium and other mucosal surfaces. Cells within these barriers produce antimicrobial peptides to further protect against microbial invasion. Microbes that withstand these barriers may be killed or incapacitated by complement, granulocytes, or dendritic cells. If not, dendritic cells can then recruit innate leukocytes. Cells of the innate immune system can then present antigens from the microorganism to T and B cells, triggering an even more powerful response. At the same time, regulatory cells limit tissue damage and promote wound repair.

During sepsis, many of these aspects of the immune response become dysregulated, resulting in organ dysfunction and potentially death (Boomer, To et al. 2011, Slotwinski, Sarnecka et al. 2015, Danahy, Strother et al. 2016, Delano and Ward 2016, Jensen, Sjaastad et al. 2018, Ammer-Herrmenau, Kulkarni et al. 2019). Particularly common immune alterations among sepsis patients are dysregulated cytokine production and the apoptosis of a large number of lymphocytes (Gogos, Kotsaki et al. 2010).

Based on cytokine kinetics during sepsis, the immune response was originally thought to proceed through two distinct phases: an initial hyper-inflammatory state followed by a state of profound immunosuppression associated with lymphocyte impairment (Oberholzer, Oberholzer et al. 2001). Further studies revealed that these phases only reflect what is happening in the circulation of patients (Ayala, Herdon et al. 1996, Cavaillon, Adib-Conquy et al. 2001, Cavaillon and Annane 2006, Cavaillon and Adib-Conquy 2007) and even then only in patients that are immunocompetent (Kalil and Opal 2015). A more appropriate understanding is that both pro- and anti-inflammatory cytokines and immune cells are induced by sepsis, but these responses are unregulated and lead to long-lasting immunosuppression (Boomer, To et al. 2011, Boomer, Shuherk-Shaffer et al. 2012, Osuchowski, Craciun et al. 2012, Roquilly, McWilliam et al. 2017).

Innate immune response to sepsis

The impact of sepsis on the innate immune system is highlighted by studies in which a secondary infection is induced shortly after the original septic insult. A reduction in neutrophil number and function and increase in monocyte recruitment and function are associated with worsened mortality to *Pseudomonas aeruginosa* infection and improved survival to *Listeria monocytogenes* (Delano, Thayer et al. 2011). Impairments

in macrophage and dendritic cell function during sepsis pneumonia are also associated with prolonged immunosuppression after sepsis and susceptibility to secondary pneumonia (Roquilly, McWilliam et al. 2017).

In a mouse model of CLP-induced sepsis, recruitment of neutrophils to the site of infection (peritoneum) within six hours is associated with a significant improvement in bacterial clearance by 24 hours and improved 28 day survival (Craciun, Schuller et al. 2010). Bacterial clearance depends on the formation of neutrophil extracellular traps (NETs) (Meng, Paunel-Gorgulu et al. 2012) following neutrophil activation by plasma ATP (Sumi, Woehrle et al. 2014). Although NETs result in lung edema and injury following CLP (Luo, Zhang et al. 2014), neutrophils can also produce IL-10 to exert an anti-inflammatory influence during sepsis (Ocuin, Bamboat et al. 2011).

While neutrophils are more or less functional during sepsis, dendritic cell (DC) numbers and function are significantly altered by sepsis. DCs are depleted in the spleens of sepsis patients (Hotchkiss, Tinsley et al. 2002) and in the lymph nodes of septic mice (Efron, Martins et al. 2004). In addition, sepsis results in the loss of resident DC in the bone marrow while inducing the differentiation of DC precursors into regulatory DCs that impair Th1 priming and NK function (Pastille, Didovic et al. 2011). Sepsis induced alterations in DCs similarly impair the priming of $CD8^+$ T cells (Strother, Danahy et al. 2016).

Neutrophils and dendritic cells are major players in the immune response to sepsis, but other cell populations are also impacted by sepsis and contribute to patient outcomes. While macrophages are not depleted in sepsis patients (Hotchkiss, Tinsley et al. 2002), their dysfunction is linked to worsened sepsis survival (Csoka, Nemeth et al. 2018, Patoli, Mignotte et al. 2020). Sepsis impairs the number and function of NK cells,

leaving the host more vulnerable to secondary infections (Jensen, Winborn et al. 2018). Even mast cells play a role in the immune response to sepsis, controlling the migration of immature neutrophils (Malaviya, Ikeda et al. 1996), improving neutrophil killing (Sutherland, Olsen et al. 2008), and helping to limit cellular apoptosis (Ramos, Pena et al. 2010).

Adaptive immune response to sepsis

The adaptive immune system's response to sepsis is also disordered; the extent of this disorder determines whether the original septic insult is cleared and the extent to which long term immunosuppression develops. B cells are vital for the early production of IFN-inducible chemokines and inflammatory cytokines that mediate survival and bacterial clearance during polymicrobial sepsis (Kelly-Scumpia, Scumpia et al. 2011). The IgA, IgG1, and IgM antibodies produced by B cells are also important, as a reduction in circulating antibodies of these subtypes at sepsis onset is associated with increased risk of death (Bermejo-Martin, Rodriguez-Fernandez et al. 2014, Krautz, Maier et al. 2018). The utility for IgA in peritonitis-induced sepsis can be partly explained by the finding that Proteobacteria-targeting IgA is protective against polymicrobial sepsis induced by CLP (Wilmore, Gaudette et al. 2018).

However, sepsis induces long-term impairments in primary B cell responses characterized by a reduction in both B cell maturation and class switching (Mohr, Polz et al. 2012, Sjaastad, Condotta et al. 2018, Duan, Jiao et al. 2020). These impairments are associated with a numerical reduction in circulating T follicular cells (Tfh), suggesting that fewer T cells are available to help in these processes (Sjaastad, Condotta et al. 2018, Duan, Jiao et al. 2020). However, these alterations are not associated with a reduction in

the number of follicles or in the size of the germinal center areas or B cell areas in the spleen or lymph node (Mohr, Polz et al. 2012). In addition, B cells are protected within mice with artificially increased numbers of memory T cells to better reflect the frequency found in humans (Taylor, Brewer et al. 2020). Collectively, these results suggest that sepsis-induced impairment of the B cell response is more limited in previously immunocompetent humans compared to mice.

In addition to the reduction in Tfh cell numbers during sepsis, the number and effector function of other $CD4^+$ T cells is impacted (Hotchkiss, Osmon et al. 2005, Unsinger, Herndon et al. 2006, Cabrera-Perez, Condotta et al. 2015, Ammer-Herrmenau, Kulkarni et al. 2019). Even after numerical recovery of $CD4^+$ T cells occurs, alterations in the naïve and memory $CD4^+$ T cell compartment persist that limit the immune responses to secondary infections (Cabrera-Perez, Condotta et al. 2015, Ammer-Herrmenau, Kulkarni et al. 2019, Xie, Chen et al. 2019). Interestingly, the bone marrow is the primary site of memory $CD4^+$ T cell homing during sepsis, and memory $CD4^+$ T cells in the bone marrow have increased proliferative ability compared cells in the spleen (Skirecki, Swacha et al. 2020).

The importance of functional $CD4^+$ T cells in sepsis is illustrated by the increase in mortality in $CD4^+$ knockout (but not $CD8^+$ knockout) mice in the first 30 hours following CLP (Martignoni, Tschop et al. 2008). This may be due to the anti-apoptotic impact of $CD4^+$ T cells on gut epithelium cells, as this was previously found to be protective following polymicrobial sepsis (Stromberg, Woolsey et al. 2009). $CD4^+$ T cell dysfunction during sepsis is linked to the expression of co-inhibitory molecules, including PD-1, LAG-3, and 2B4 (Chen, Mittal et al. 2017, Ramonell, Zhang et al. 2017, Jensen, Sjaastad et al. 2018, Niu, Zhou et al. 2019). Treatment with anti-2B4 or anti-PD-1 increases survival and

improves $CD4^+$ function following CLP-induced polymicrobial sepsis (Chang, Svabek et al. 2014, Chen, Mittal et al. 2017).

Early studies suggested that splenic $CD4^+$ $CD25^+$ T regulatory (Treg) cells effectively suppressed $CD4^+$ $CD25^-$ effector T cell proliferation (Scumpia, Delano et al. 2006). However, antibody depletion of total $CD4^+$ or $CD4^+$ $CD25^+$ cells did not alter survival; there was also no difference between CD25 and IL-10 knockout mice and wild type mice in terms of survival from CLP (Scumpia, Delano et al. 2006, Wisnoski, Chung et al. 2007). As CD25 is expressed on the surface of all activated T cells, it is unclear whether the studies using CD25 to define Treg were truly able to discern the impact of Treg compared to other cells.

More recent studies using the Treg transcription factor FoxP3 to define Tregs have found that Tregs contribute to recovery from CLP-induced sepsis and limit the inflammatory response (Kuhlhorn, Rath et al. 2013, Tatura, Zeschnigk et al. 2015). While Tregs reach the site of infection within one day of the septic insult, these cells decrease to baseline levels by seven days after infection (Nascimento, Alves-Filho et al. 2010). Similar changes occurred in the draining lymph nodes, whereas the frequency and number of $FoxP3^+$ Treg was elevated in the spleen and thymus up to 15 days after CLP compared to naïve mice (Nascimento, Alves-Filho et al. 2010). $FoxP3^+$ Treg were found to increase susceptibility to secondary infection with the opportunistic pathogen *Legionella pneumophilia* (Nascimento, Alves-Filho et al. 2010).

Similar to $CD4^+$ T cells, $CD8^+$ T cells are lost following sepsis (Unsinger, Herndon et al. 2006, Duong, Condotta et al. 2014). Effector function, sensitivity to antigen, and antigen-driven proliferation of memory $CD8^+$ T cells is also impaired during sepsis (Duong, Condotta et al. 2014, Danahy, Strother et al. 2016). The impairments in $CD8^+$ T

cell populations lead to significantly increased susceptibility to secondary viral infections and reactivation of previously latent viruses (Condotta, Khan et al. 2015, Choi, Kim et al. 2017, Xie, Crepeau et al. 2019). Interestingly, some tumor-specific responses are reinvigorated during sepsis (Danahy, Jensen et al. 2019) while others become more impaired (Danahy, Kurup et al. 2019). Even in the absence of cognate antigen, the sepsis environment allows some $CD8^+$ T cells to gain anti-tumor effector capabilities through bystander activation (Danahy, Berton et al. 2020).

The loss of memory $CD8^+$ T cells during sepsis has been associated with the induction of apoptosis by the co-inhibitory molecule 2B4 (Xie, Chen et al. 2019). In septic animals latently infected with a gamma herpesvirus (gHV), gHV-specific memory $CD8^+$ T cells upregulate the co-inhibitory marker 2B4 and are reduced in number (Xie, Crepeau et al. 2019). This corresponds to increased gHV viral load, and suggests that memory $CD8^+$ T cell impairment drives viral reactivation during sepsis (Xie, Crepeau et al. 2019). Viral reactivation is common in sepsis patients (Walton, Muenzer et al. 2014), so the 2B4-associated dysfunction in the memory $CD8^+$ T cell response may explain this phenomenon (Walton, Muenzer et al. 2014, Choi, Kim et al. 2017).

The cytokine response to sepsis

Another aspect of the immune system that significantly impacts sepsis mortality is dysregulation of the cytokine response. Pro- and anti- inflammatory cytokines are released shortly after sepsis onset (Tamayo, Fernandez et al. 2011, Cazalis, Lepape et al. 2014) and continue to be released in tandem throughout the course of the illness (Andaluz-Ojeda, Bobillo et al. 2012, Osuchowski, Craciun et al. 2012, Frencken, van Vught et al. 2017). Evidence now exists that pro- and anti-inflammatory cytokines are released

simultaneously, refuting the previously held paradigm of systemic inflammatory response syndrome (SIRS) preceding a compensatory anti-inflammatory response syndrome (CARS) (Osuchowski, Welch et al. 2006, Tamayo, Fernandez et al. 2011, Osuchowski, Craciun et al. 2012). Within six hours after CLP, the proinflammatory cytokines IL-6, TNF, and IL-1 and the anti-inflammatory cytokine IL-10 are released in significant amounts (Osuchowski, Welch et al. 2006). Although these cytokines are released at the earliest time points, many other cytokines are upregulated and play a major role in the immune response to sepsis, and ultimately in mortality.

Mice lacking the cytokine IL-17 have significantly increased mortality following CLP that correlates with higher bacteremia at 12 hours (Ogiku, Kono et al. 2012). IL-17$^{-/-}$ mice also have significantly impaired IgA production during sepsis (Ramakrishnan, Zhang et al. 2019). The impact of IL-17 on sepsis mortality depends on the microbe that initiated the infection. Using a bacterial pneumonia model, Ritchie et al. found that the role of IL-17 in sepsis is highly dependent on the encapsulation status of the infecting bacterium (Ritchie, Ritchie et al. 2018). IL-17 was beneficial during infections caused by minimally encapsulated bacteria, but significantly increased lung pathology and mortality if the infectious organism was heavily encapsulated (Ritchie, Ritchie et al. 2018). The authors concluded that this was due to the accumulation of neutrophils unable to phagocytose the bacteria (Ritchie, Ritchie et al. 2018). In conjunction with IL-23 signaling, IL-17 increases the recruitment of neutrophils and their accumulation in the lung following CLP, partially explaining the inflammation seen in the lung following polymicrobial sepsis originating in other tissues (Cauvi, Williams et al. 2014). IL-17 has also been linked to the development of acute kidney injury in septic patients and animal models (Maravitsa, Adamopoulou et al. 2016). Given these findings, it is not surprising

that multiple groups have reported that the neutralization of IL-17 improves survival (Li, Zhang et al. 2012, Wynn, Wilson et al. 2016).

A 2010 study by Alves-Filho et al. found that the administration of IL-33 significantly improves survival in mice with CLP-induced sepsis (Alves-Filho, Sonego et al. 2010). IL-33 improves neutrophil migration to the site of infection (Alves-Filho, Sonego et al. 2010), resulting in improvements in bacterial clearance, and is associated with a reduction in lymphocyte apoptosis. Despite being linked to improvements in survival early after sepsis onset, IL-33 signaling may not always be beneficial. IL-33 is implicated in the induction and maintenance of immunosuppression during sepsis through the induction of Tregs (Nascimento, Melo et al. 2017). Nascimento et al. found that this occurs through the production of IL-4 and IL-13 by ILC2s that receive IL-33 signaling (Nascimento, Melo et al. 2017). The IL-4 and IL-13 then drives the proliferation of IL-10 producing macrophages and ultimately an expansion in Treg numbers (Nascimento, Melo et al. 2017).

III. IL-27 AND SEPSIS

IL-27 is another cytokine that has been found to increase in the plasma of septic patients (Batten, Li et al. 2006, Colgan and Rothman 2006, Stumhofer, Laurence et al. 2006, Diveu, McGeachy et al. 2009, Scicluna and van der Poll 2012, Wong, Cvijanovich et al. 2012, Wong, Lindsell et al. 2013, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015, Gao, Yang et al. 2016, He, Du et al. 2017, He, Zhang et al. 2018) and in diverse models of sepsis in mice (Wirtz, Tubbe et al. 2006, Nelson, Tolbert et al. 2010, Bosmann, Russkamp et al. 2014, Cao, Xu et al. 2014, Gwyer Findlay, Villegas-Mendez et al. 2014). The blockade of the p28 subunit of IL-27 (Bosmann, Russkamp et al. 2014) or depletion of IL-27 using

a soluble and recombinant IL-27Rα (Wirtz, Tubbe et al. 2006) significantly reduces mortality in the cecal ligation and puncture (CLP) model of sepsis and is associated with reduced bacterial burden in the tissues and blood.

The role of IL-27 in sepsis pathophysiology

Originally thought to be pro-inflammatory, there is now consensus that IL-27 is a potent immunosuppressant. It is composed of an alpha subunit (IL-27p28, also known as IL-30) and EBI3 (shared with IL-35) (Pflanz, Timans et al. 2002). IL-27 binds to the IL-27 receptor alpha (IL-27Rα, also known as WSX-1) and gp130 and is primarily produced by dendritic cells (DCs), monocytes and macrophages (Pflanz, Timans et al. 2002). The lymphocyte populations that respond to the presence of IL-27 or one of its subunits are T cells, natural killer (NK) cells, natural killer T (NKT) cells, and DCs (Villarino, Larkin et al. 2005, Matta, Raimondi et al. 2012, Mascanfroni, Yeste et al. 2013, Sowrirajan, Saito et al. 2017, Harker, Wong et al. 2018, Wehrens, Wong et al. 2018). This allows IL-27 to have wide ranging effects on cells of both the innate and adaptive immune response in addition to autocrine effects.

In septic patients and in murine models of sepsis, the plasma concentration of IL-27 significantly increases (Wirtz, Tubbe et al. 2006, O'Dwyer, Mankan et al. 2008, Nelson, Tolbert et al. 2010, Rinchai, Khaenam et al. 2012), briefly causing it to be considered as a potential diagnostic biomarker in adults (Wong, Lindsell et al. 2013, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015) and children (Wong, Cvijanovich et al. 2012, He, Du et al. 2017). However, these results have not been consistently replicated in humans, limiting its current therapeutic potential. In mice, the results are more consistent and indicate a clear role for IL-27 in the pathology of sepsis and critical illness. When the p28

subunit is neutralized or the IL-27Rα is blocked, mortality is significantly decreased in both CLP and endotoxemia (Wirtz, Tubbe et al. 2006, Bosmann, Russkamp et al. 2014, Cao, Xu et al. 2014).

In a study by Cao et al., mice lacking the IL-27Rα were resistant to a secondary bacterial infection caused by *Pseudomonas aeruginosa* following CLP in a manner dependent on alveolar macrophages and neutrophils (Cao, Xu et al. 2014). Specifically, the neutrophils and alveolar macrophages in these mice had a significantly improved ability to kill *P. aeruginosa* upon phagocytosis (Cao, Xu et al. 2014). Similarly, Bossman et al. observed that the oxidative burst of macrophages was improved upon the elimination of IL-27 signaling, and determined that IL-10 limits the production of IL-27p28 *in vivo* following CLP (Bosmann, Russkamp et al. 2014). In addition, this study found that the cells primarily responsible for the production of IL-27p28 in the CLP model of sepsis are splenic macrophages (Bosmann, Russkamp et al. 2014). However, a more recent study has found conflicting evidence that indicates a protective role for p28 during sepsis (Yan, Mitra et al. 2016). In this study, the administration of the p28 subunit or its overproduction through genetic therapy led to a reduction in mortality during sepsis directly linked to the reduction in NKT cell production of inflammatory cytokines (Yan, Mitra et al. 2016).

Interactions between IL-27 and other cytokines relevant to sepsis

The ability for lymphocytes to recognize and respond to slight changes in their environment makes the immune system very adaptable and ensures that the balance between inflammatory and immunosuppressive responses is fine-tuned. While the ability of lymphocytes to respond so readily to their surroundings is beneficial from an

evolutionary point of view, it makes it significantly harder to elucidate the role of individual cytokines. The individual and combined actions of the cytokines in the IL-17, IL-27, and IL-33 axis are summarized in **Figure 1.1**.

IL-27 and IL-17

By limiting the differentiation of naïve $CD4^+$ T cells into Th17 cells, IL-27 is able to attenuate experimental autoimmune encephalomyelitis and rheumatoid arthritis (Batten, Li et al. 2006, Stumhofer, Laurence et al. 2006, Diveu, McGeachy et al. 2009, El-behi, Ciric et al. 2009, Murugaiyan, Mittal et al. 2009, Liu and Rohowsky-Kochan 2011, Hirahara, Ghoreschi et al. 2012, Fitzgerald, Fonseca-Kelly et al. 2013, Moon, Park et al. 2013, Wang, Li et al. 2013). Similarly, IL-27 signaling prevents the development of neurological damage during chronic *Toxoplasma gondii* infection (Stumhofer, Laurence et al. 2006) and reduces tissue damage during RSV infection (de Almeida Nagata, Demoor et al. 2014). Further research has shown that STAT1 signaling (which IL-27 induces) inhibits the expression of the transcription factor RORγt, necessary for Th17 differentiation, while promoting the induction of the protein suppressor of cytokine signaling 1 (SOCS1) (Batten, Li et al. 2006, Diveu, McGeachy et al. 2009, Liu and Rohowsky-Kochan 2011, Peters, Fowler et al. 2015). This leads to the suppression of IL-22 production by Th17 cells, impairing antimicrobial defenses in the epithelium (Liu and Rohowsky-Kochan 2011, Wang, Li et al. 2013). In addition to its direct effects on T cells, IL-27 can also inhibit Th17 differentiation by inhibiting the production of the Th17-polarizing cytokines IL-1β, IL-6, and IL-23 by DCs (Murugaiyan, Mittal et al. 2009). In contrast, T cells that have already committed to the Th17 lineage are not directly inhibited by IL-27 signaling (El-behi, Ciric et al. 2009, El-Behi, Dai et al. 2014). Instead, inhibition

occurs indirectly through the induction of Tr1 (Murugaiyan, Mittal et al. 2009) and the expression of co-inhibitory receptors and their ligands (Hirahara, Ghoreschi et al. 2012, Moon, Park et al. 2013).

IL-27 and IL-33

While IL-27 signaling promotes type 1 immune responses and directly limits type 17 immunity, it also serves as a negative regulator of type 2 responses by interfering with IL-33 signaling. The first paper to describe this phenomenon utilized *in vitro* experiments which showed that IL-27 reduced type 2 cytokine production in bone marrow cells exposed to IL-33, including IL-5, IL-13, and GM-CSF (Duerr, McCarthy et al. 2016). For IL-5, this effect was dependent on STAT1 signaling, as STAT1 knockout bone marrow cells were not impacted by the presence of IL-27 (Duerr, McCarthy et al. 2016). Moro et al. confirmed these findings *in vivo* using STAT1 knockout mice, and revealed that while IL-27 reduces type 2 cytokine production by ILC2 cells, it does not affect cytokine production in Th2 cells (Moro, Kabata et al. 2016). Another recent study reported that the administration of IL-27 limits IL-33 induced ILC2 accumulation and activation in the lungs, liver, spleen, and mesenteric lymph node *in vivo* (McHedlidze, Kindermann et al. 2016). The administration of IL-27 also led to the overrepresentation of IL-27R$\alpha^{-/-}$ cells in chimeric mice (McHedlidze, Kindermann et al. 2016). While not specifically addressing IL-27, another murine study found that STAT1 signaling induced by infection with respiratory syncytial virus is sufficient to reduce the production of IL-33 (Stier, Goleniewska et al. 2017). These studies collectively show that a major function of IL-27 is

to negatively regulate the type 2 immune response, specifically ILC2 cells, in a manner that is dependent on STAT1 signaling.

IV. IL-27 AND T CELL FUNCTION

With IL-27 strongly suspected to play a major role in sepsis outcomes, it is important to consider the various ways IL-27 impacts the function of T cells. IL-27 promotes the differentiation of Th1 cells and it is also a potent inducer of type 1 Treg (Tr1) cells (Awasthi, Carrier et al. 2007). While Tr1 cells produce IFN-γ, they also produce large quantities of IL-10 and have potent suppressive functions (Battaglia, Gregori et al. 2006). In addition to the induction of this cell population, IL-27 signaling leads to an increase in co-inhibitory molecule expression on T cells following chronic antigen exposure and during cancer (Chihara, Madi et al. 2018).

As T cell dysfunction and exhaustion is associated with the development of immunosuppression during sepsis and ultimately worsened survival (Jensen, Sjaastad et al. 2018, Thampy, Remy et al. 2018, Ammer-Herrmenau, Kulkarni et al. 2019, Xie, Crepeau et al. 2019), IL-27 could be an effective therapeutic target.

IL-27 and Treg

Tregs are necessary to limit immune-mediated damage to the body but if unchecked can leave the host more susceptible to infectious insults. IL-27 was found to induce the formation of Treg through the induction of the co-stimulatory receptor ICOS and the transcription factor c-Maf (Awasthi, Carrier et al. 2007, Pot, Jin et al. 2009). Although these initial studies looked at Treg lacking FoxP3 expression, IL-27 is also important to the functioning of FoxP3-expressing Treg. Through the induction of CD39,

IL-27 suppresses the $CD8^+$ T cell response to cancer, resulting in enhanced tumor growth (Park, Ryu et al. 2019). Through the induction of LAG-3, IL-27 is responsible for the ability of FoxP3 Treg to alleviate colitis, autoimmunity, and allergic airway inflammation (Do, Visperas et al. 2016, Do, Kim et al. 2017, Kim, Le et al. 2019, Nguyen, Jang et al. 2019).

IL-27 and memory T cells

Another possible factor for IL-27 to mediate sepsis patient outcomes is through its effect on the development of memory T cells. Memory $CD4^+$ and $CD8^+$ T cells have high levels of IL-27Rα expression, suggesting that they are (or were) highly responsive to its effects (Villarino, Larkin et al. 2005). Indeed, IL-27 has been found to enhance the survival of activated tumor-specific $CD8^+$ T cells, programming them into memory precursors that contribute to tumor rejection (Liu, Liu et al. 2013). In addition, IL-27 signaling regulates memory $CD4^+$ T cells to suppress the inflammatory response during malaria infection (Gwyer Findlay, Villegas-Mendez et al. 2014). Circulating DC production of IL-27 is also necessary to induce the formation of protective memory T cells following subunit vaccination (Pennock, Gapin et al. 2014, Kilgore, Pennock et al. 2020).

IL-27 and co-inhibitory markers

A third way that IL-27 may impact T cell function during sepsis is through the upregulation of co-inhibitory markers. In addition to inducing the co-inhibitory marker LAG-3 on Treg, IL-27 signaling upregulates other co-inhibitory markers on $CD4^+$ and $CD8^+$ T cells (Do, Visperas et al. 2016, Kim, Le et al. 2019). Through the induction of NFIL3, IL-27 signaling induces Tim-3 expression and IL-10 production in $CD4^+$ T cells,

leading to their impairment in resolving tumors (Zhu, Sakuishi et al. 2015). In combination with TCR signaling in vitro, IL-27 leads to increased T cell expression of LAG-3, CTLA-4, and TIGIT; interestingly PD-L1 can be upregulated from IL-27 signaling alone (DeLong, O'Hara Hall et al. 2019). Under conditions of overexpression of IL-27 in mice, PD-L1, LAG-3, TIGIT, and Tim-3 were all upregulated on T cells (DeLong, O'Hara Hall et al. 2019). In the contexts of chronic viral infections and cancer, IL-27 was again linked to the induction of PD-1, Tim-3, LAG-3, and TIGIT, but not CTLA-4 or PD-L1 (summarized in **Figure 1.2**) (Chihara, Madi et al. 2018). These co-inhibitory markers appeared to be linked in a gene program regulated by the transcription factors PRDM1 and c-Maf (Chihara, Madi et al. 2018).

Based on these studies, we hypothesized that IL-27 signaling could be responsible for the induction of co-inhibitory markers on memory T cells during sepsis, contributing to their dysfunction. In this study we have focused on evaluating the link between IL-27 signaling and the induction of PD-1 and TIGIT expression on memory T cells in the context of polymicrobial sepsis induced by CLP.

V. FIGURES

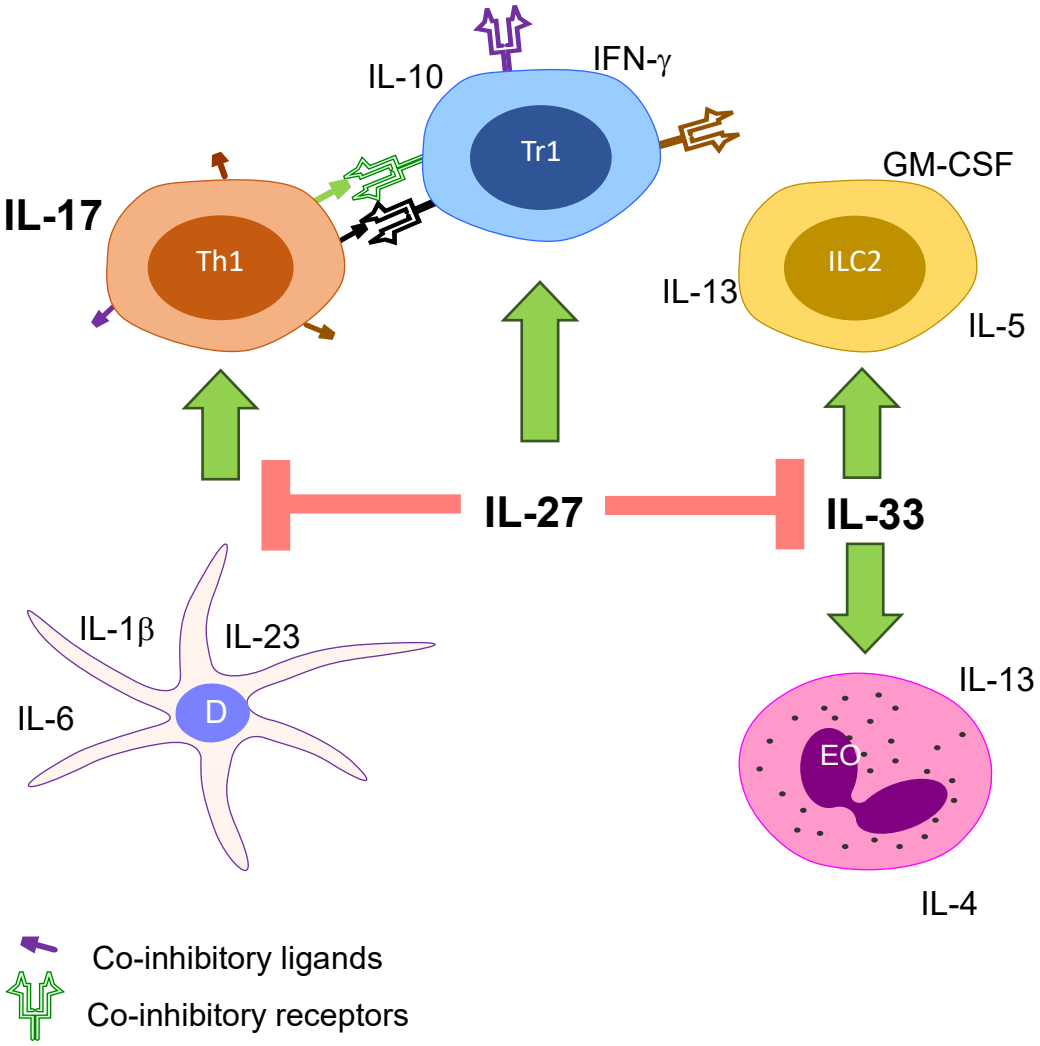

Figure 2.1: The Proposed Role of the IL-17, IL-27, and IL-33 Axis During Sepsis

Th17 cells that receive co-inhibitory signaling from IL-27 induced Tr1 cells have inhibited production of IL-17. Differentiation of naïve T cells into Th17 cells is also inhibited by IL-27 through the modulation of DC cytokine production. ILC2 cells and EOs expansion are also inhibited through the action of IL-27 signaling on IL-33. Abbreviations: Th17, T helper type 17 cell; Tr1, T regulatory type 1 cell; ILC2, innate lymphocyte type 2 cell, DC, dendritic cell; EO, eosinophil.

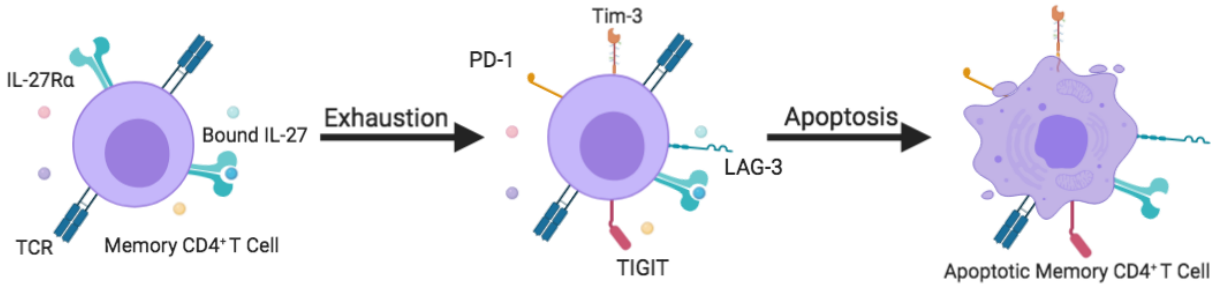

Figure 1.2: T cell dysfunction induced by IL-27 signaling during chronic viral infections and cancer

After T cells receive signaling from IL-27 through IL-27Rα and gp130, they upregulate the expression of the co-inhibitory markers PD-1, Tim-3, LAG-3, and TIGIT in a TCR independent manner. These markers make T cells exhausted and trigger them to undergo programmed cell death (apoptosis). The apoptosis of the T cells leads to impairments in resolving the infection and clearing tumors.

CHAPTER 2: THE IL-27 RECEPTOR REGULATES TIGIT ON MEMORY CD4$^+$ T CELLS DURING SEPSIS

I. ABSTRACT

Sepsis is a leading cause of morbidity and mortality associated with significant impairment in memory T cells. These changes include the upregulation of co-inhibitory markers, a decrease in functionality, and an increase in apoptosis. Due to recent studies describing IL-27 regulation of TIGIT and PD-1, we assessed whether IL-27 impacts these co-inhibitory molecules in sepsis. Based on these data, we hypothesized that IL-27 was responsible for T cell dysfunction during sepsis. Using the cecal ligation and puncture (CLP) sepsis model, we found that IL-27Rα was associated with the upregulation of TIGIT on memory CD4$^+$ T cells following CLP. However, IL-27 was not associated with sepsis mortality.

II. INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction resulting from the body's dysregulated response to infection (Singer, Deutschman et al. 2016). It is a significant source of morbidity and mortality worldwide—in 2017 alone, it is estimated that 11 million people died from sepsis, accounting for 20% of all deaths that year (Rudd, Johnson et al. 2020). Early recognition of sepsis and rapid initiation of supportive care is effective at reducing mortality in the early stages of sepsis (Ferrer, Artigas et al. 2009, Seymour, Gesten et al. 2017), but a substantial proportion of sepsis patients die between 31 days and 2 years (Prescott, Osterholzer et al. 2016). This mortality is partially attributed to a

long-term dysfunction in the immune system that begins shortly after sepsis onset but persists for many months following recovery (Boomer, To et al. 2011).

The immune dysfunction caused by sepsis is likely multi-faceted and represents many changes in both the adaptive and innate immune systems. Current evidence points to changes in the T cell repertoire that lead to adaptive immune system dysfunction. We have previously published data describing alterations in the T cell compartment following sepsis. We have found that there are significant reductions in the number of bulk $CD4^+$ and $CD8^+$ T cells following the cecal ligation and puncture (CLP) model of sepsis (Chen, Mittal et al. 2017, Ramonell, Zhang et al. 2017). Within the $CD4^+$ T cell compartment, both naïve and memory $CD4^+$ T cell numbers are significantly reduced following CLP. While the numbers of naïve $CD8^+$ T cells are similar between CLP and sham-surgery mice at all time points, the number of memory $CD8^+$ T cells is significantly reduced following sepsis (Serbanescu, Ramonell et al. 2016, Xie, Chen et al. 2019). One day following CLP, the number of memory $CD8^+$ T cells is reduced by 44%, and remains significantly reduced until three days after surgery (Serbanescu, Ramonell et al. 2016). For instance, the expression of co-inhibitory markers such as TIGIT and PD-1 are highly upregulated on both naïve and memory T cells and are linked to T cell apoptosis (Boomer, Shuherk-Shaffer et al. 2012, Chen, Mittal et al. 2017, Sjaastad, Condotta et al. 2018, Ammer-Herrmenau, Kulkarni et al. 2019, Xie, Chen et al. 2019, Xie, Crepeau et al. 2019).

In the past few years, the immunosuppressive cytokine IL-27 has been linked to the induction of TIGIT and PD-1 expression on T cells during cancer (Chihara, Madi et al. 2018) and toxoplasmosis (DeLong, O'Hara Hall et al. 2019). Furthermore, IL-27 was found to inhibit memory T cell recall responses during secondary malaria infection (Gwyer Findlay, Villegas-Mendez et al. 2014). Based on these findings, we hypothesized

that IL-27 signaling could be responsible for the upregulation of TIGIT and PD-1 on memory T cells during sepsis and ultimately for sepsis mortality. Previous studies have observed that the level of serum IL-27 increases following sepsis in mouse models and in human septic patients (Wirtz, Tubbe et al. 2006, Nelson, Tolbert et al. 2010, Wong, Cvijanovich et al. 2012, Wong, Lindsell et al. 2013, Bosmann, Strobl et al. 2014, Cao, Xu et al. 2014, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015, Gao, Yang et al. 2016, Yan, Mitra et al. 2016). Produced by antigen presenting cells (Pflanz, Timans et al. 2002), IL-27 primarily regulates the effector response of dendritic cells and T cells during infections (Villarino, Hibbert et al. 2003, Yoshimura, Takeda et al. 2006, Guzzo, Ayer et al. 2012, Clement, Marsden et al. 2016, Patin, Jones et al. 2016, Sowrirajan, Saito et al. 2017, Wehrens, Wong et al. 2018). It is well established that memory T cells express particularly high levels of the IL-27 receptor (IL-27Rα) (Villarino, Larkin et al. 2005).

To determine whether IL-27 signaling is responsible for co-inhibitory molecule expression on memory T cells during sepsis, we analyzed the phenotype and function of IL-27Rα$^+$ on memory T cells following cecal ligation and puncture (CLP). We found that IL-27 receptor expression was associated with TIGIT (but not PD-1) expression on memory CD4$^+$ T cells and their impaired production of IFNγ. However, there was no difference in sepsis mortality in the presence vs. absence of IL-27 signaling. These results suggest that IL-27Rα signaling induces the upregulation of TIGIT on memory CD4$^+$ T cells during sepsis but does not play a role in sepsis mortality in this model.

III. **METHODS**

Animals

Six to 12-week-old male and female gender matched mice (mean weight 20g) were used for all experiments. The wild type mice used for T cell phenotyping experiments were either C57BL/6J or C57BL/6NJ mice obtained from Jackson Laboratories (Bar Harbor, ME). Transgenic *Il27ra*$^{-/-}$ mice on a mixed C57BL/6NJ and 6NTac background were a gift from Dr. Jacob Kohlmeier (Emory University, Atlanta, Georgia; animals were originally obtained from Jackson Laboratories, Bar Harbor, ME). C57BL/6NJ (Jackson Laboratories, Bar Harbor, ME) mice were used as controls in experiments with transgenic *Il27ra*$^{-/-}$ mice. Mice obtained from external sources were acclimated for at least 72 hours prior to being used in experiments. Mice were randomly allocated to receive either sham surgery or cecal ligation and puncture (CLP, details below) and were either sacrificed between 24 and 96 hours after surgery or followed for 7 days to determine survival. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee (Protocol 201700361.RM001-El-N). Mice were housed in specific pathogen free conditions with a 12-hour light cycle.

Cecal Ligation and Puncture (CLP)

Cecal ligation and puncture was performed as previously described (Chen et al., 2017, Chen et al., 2019). Surgeries were performed between the hours of 9am and 2pm to minimize the confounding effects of circadian rhythm. Prior to surgery, 0.1 mg/kg of buprenorphine (McKesson Medical, San Francisco, CA) was administered to each mouse subcutaneously (s.c.) to minimize suffering. Ophthalmic eye gel was administered to

prevent corneal ulceration during surgery and abdominal fur was shaved to minimize risk of wound contamination. The anesthetic depth necessary for surgery was induced with inhaled isoflurane (3% in 100% O_2) and reduced to 2% after the surgical plane was reached. After skin disinfection, a midline incision was made, and the cecum was exteriorized. For mice in the control group (receiving sham surgery), the cecum was then replaced in the abdominal cavity, and the abdominal wall closed with 4-0 silk thread. If mice underwent cecal ligation and puncture, approximately 75% of the cecal length was ligated with nylon thread, before being punctured with a 25-gauge needle through and through. A small amount of stool was then gently expelled before the cecum was returned to the abdominal cavity. The abdominal wall was closed with 4-0 silk suture and the skin closed with veterinary glue. Immediately after surgery, 1mL of sterile saline was administered s.c. for fluid resuscitation in addition to 50 mg/kg ceftriaxone (Acros Organics, Morris Plains, NJ) and 35 mg/kg metronidazole (Sigma-Aldrich, St. Louis, MO) for pathogen control**.** Animals were then placed in a new cage on a warming pad and monitored for recovery. Following recovery, the cages were returned to their housing room. Antibiotics (same as above) were administered every 12 hours for the first 48 hours following surgery. All mice were monitored twice a day for the duration of the experiments and weighed every other day. Any mouse that lost 25% body weight or appeared moribund was humanely euthanized by asphyxiation with CO_2 or exsanguination after exposure to a high concentration of isoflurane followed by cervical dislocation. Moribund animals were defined by a) major organ failure or medical conditions unresponsive to treatment, b) surgical complications unresponsive to immediate intervention or c) clinical or behavioral signs unresponsive to appropriate intervention persisting for 24 hours. In experiments using neutralizing IL-27p28 antibody (clone: MM27.7B1, BioXCell), 500 µg

was administered into the intraperitoneal cavity before abdominal wall closure following CLP or sham surgery.

ELISAs

Plasma samples were obtained via terminal heart puncture or saphenous vein collection into tubes containing EDTA. After centrifugation, the plasma layer was collected and cryopreserved until analysis. Samples were diluted using 1x PBS and assessed for IL-27 concentration using an anti-IL-27p28 ELISA kit (Invitrogen, Carlsbad, CA) or IFNγ concentration using an anti-IFNγ ELISA kit (Invitrogen, Carlsbad, CA) following manufacturer instructions. Results were analyzed using Four Parameter Logistic Regression.

Flow cytometry

On days 1-4 following CLP, mice from each group were randomly chosen for sacrifice. Spleens were harvested and strained through a 70 μM nylon filter before washing with cold 1x PBS through centrifugation. Splenocytes were subsequently resuspended in PBS and 2 million cells were used for staining. Prior to staining with target antibodies, all samples were stained with TruStain FcX anti-mouse CD16/32 (BioLegend, San Diego, CA) following manufacturer instructions. Cells were then stained for surface markers and incubated on ice for 25 minutes. After staining, samples were washed with MACS Buffer and resuspended in CountBright Absolute Counting Beads (Thermo Fisher Scientific, Waltham, MA) according to manufacturer instructions. The antibodies used for T cell exhaustion phenotyping are as follows: TIGIT-BV421 (Clone 1G9, BD), Live/Dead Aqua (Invitrogen), NK1.1-BV650 (clone PK136, BioLegend), CD44 on PerCP

Cy 5.5 (clone IM7, BioLegend) or BUV737 (clone IM7, BD), IL-27Rα-PE (clone 2918, BD), PD-1-APC/Cy7 (clone 29F.1A12, BioLegend), CD4-BUV395 (clone GK1.5, BD), CD3e-BUV496 (clone 145-2C11, BD), and CD8a on BUV737 or BUV805 (both clone 53-6.7, BD). For caspase 3/7 staining, surface staining was done as described above with the following markers: CD4-BUV395 (clone GK1.5, BD), CD3e-BUV496 (clone 145-2C11, BD), CD8a on BUV737 (clone 53-6.7, BD), NK1.1-BV650 (clone PK136, BioLegend), and IL-27Rα-PE (clone 2918, BD).Cells were then resuspended in 1x PBS and Caspase-3/7 stain according to manufacturer instructions (CellEvent Caspase-3/7 Green Detection Reagent, Invitrogen, Carlsbad, CA). Samples were incubated for one hour at 37□ C and caspase 3/7 staining was immediately detected. For intracellular cytokine staining (ICCS), splenocytes were stimulated for 4 hours with 20 ng/mL PMA (Sigma-Aldrich) and 0.75 μg/mL Ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD). Samples were subsequently surface stained as described above with CD4-BUV395 (clone GK1.5, BD), CD3e-BUV496 (clone 145-2C11, BD), CD8a on BUV737 (clone 53-6.7, BD), IL-27Rα-PE (clone 2918, BD). Following surface staining, the cells were fixed and permeabilized according to manufacturer's instructions (BD Fixation/Permeabilization Solution Kit). Cells were then stained with TNFα-APC and IFNγ-A700. For Treg and Ki67 (proliferation) staining, extracellular staining was done as above using CD4-BUV395 (clone GK1.5, BD), CD3e-BUV496 (clone 145-2C11, BD), CD8a on BUV737 (clone 53-6.7, BD), PD-1-APC/Cy7 (clone 29F.1A12, BioLegend), TIGIT-BV421 (Clone 1G9, BD), Live/Dead Aqua (Invitrogen), NK1.1-BV650 (clone PK136, BioLegend), and CD44-BUV737 (clone IM7, BD). Following extracellular staining, cells were fixed and permeabilized using the Foxp3/transcription staining buffer kit (eBioscience) according to manufacturer instructions. Cells were then stained with FoxP3-APC (clone FJK-16s,

eBioscience) and Ki67-AF700 (clone 16A8, BioLegend). All samples were run on a LSRFortessa (BD Biosciences, San Jose, CA). All flow cytometric data was analyzed using FlowJo version 10.2 (BD, Ashland, OR). The FlowAI plugin (Monaco et al., 2016) found on FlowJo Exchange (www.flowjo.com/exchange) was used in sample pre-processing to eliminate artifacts caused by variable flow rate before analysis.

Statistics

All statistical analysis was performed using Prism 8.3.1 (GraphPad, San Diego, CA). Kruskal-Wallis tests with Dunn's multiple comparisons test was used when comparing within a single group longitudinally. When comparing between two groups at multiple time points, two-way ANOVA was used with Sidak's multiple comparison test. Survival curves were assessed using a Log-rank (Mantel-Cox) Test. Results are reported as the mean of each group ± SD. P-values ≤ 0.05 were considered statistically significant.

IV. **RESULTS**

Sepsis increases plasma IL-27 but is associated with a reduction in the frequency and number of IL-27Ra$^+$ CD4$^+$ and CD8$^+$ T cells

Although plasma IL-27 has been previously shown to increase at early time points during sepsis, it is not known how long systemic IL-27 remains elevated (Wirtz, Tubbe et al. 2006, Nelson, Tolbert et al. 2010, Wong, Cvijanovich et al. 2012, Wong, Lindsell et al. 2013, Bosmann, Strobl et al. 2014, Cao, Xu et al. 2014, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015, Gao, Yang et al. 2016, Yan, Mitra et al. 2016). To address this, mice underwent cecal ligation and puncture (CLP) or sham surgery (see Transparent Methods) and plasma was collected on days 1, 3, and 5 following surgery. Animals that underwent

CLP surgery had increased plasma IL-27p28 between days 1 and 5 compared to mice that underwent sham surgery (**Fig. 2.1A**). Although systemic IL-27p28 was increased following CLP, it was unclear what proportion of T cells were able to express the IL-27 receptor (IL-27Rα) following sepsis. Multi-color flow cytometry was used to determine the frequency and number of IL-27Rα expressing T cells in the spleens of CLP and sham mice. A high frequency of $CD4^+$ and $CD8^+$ T cells in unmanipulated mice expressed IL-27Rα (**Fig. 2.2**). After CLP, the frequency of IL-27Rα$^+$ cells among $CD44^{lo}$ naïve $CD4^+$ T cells was reduced compared to sham controls on days 2 and 4 (**Fig. 2.1B-C**). In contrast, the frequency of IL-27Rα$^+$ $CD44^{hi}$ memory $CD4^+$ T cells was decreased at day 2 following CLP but increased back to sham levels on days 3 and 4 (**Fig. 2.1B-C**). We found that the frequency of IL-27Rα$^+$ $CD44^{lo}$ and $CD44^{hi}$ T cells decreased following CLP. Because sepsis can also result in lymphopenia, we also assessed absolute numbers of cells. We found that absolute numbers of IL-27Rα$^+$ $CD44^{lo}$ and $CD44^{hi}$ $CD4^+$ T cells were also significantly reduced after CLP, with a greater reduction in the $CD44^{hi}$ population (**Fig. 2.1D**). In the $CD8^+$ T cell compartment, there was no significant difference in the frequency of IL-27Rα$^+$ $CD44^{lo}$ or $CD44^{hi}$ $CD8^+$ T cells after CLP (**Fig. 2.1E-F**). However, the absolute cell number of IL-27Rα$^+$ $CD44^{hi}$ (but not $CD44^{lo}$) $CD8^+$ T cells was reduced on days 2 and 3 after CLP (**Fig. 2.1G**). The absolute number of IL-27Rα$^+$ $CD44^{hi}$ T cells decreased more than the absolute number of IL-27Rα$^+$ $CD44^{lo}$ T cells during sepsis. Moreover, neither the number of IL-27Rα$^-$ $CD44^{hi}$ nor IL-27Rα$^-$ $CD44^{lo}$ cells was decreased in either the $CD4^+$ and $CD8^+$ compartments following CLP (**Fig. 2.3**).

IL-27 signaling is associated with the upregulation of TIGIT on memory $CD4^+$ T cells in septic mice

Based on the literature previously showing that IL-27 signaling regulates the expression of TIGIT and PD-1 (Chihara, Madi et al. 2018, DeLong, O'Hara Hall et al. 2019), we sought to determine whether IL-27Rα expression was associated with the expression of these markers on memory T cells during sepsis. To do this, we assessed the frequency of the co-inhibitory molecules TIGIT and PD-1 (gating strategy shown in **Fig. 2.4**) in IL-27Rα$^+$ and IL-27Rα$^-$ CD44^{hi} CD4$^+$ T cells on days 1-4 following CLP. Assessment of TIGIT revealed an increased frequency of TIGIT$^+$ cells in the IL-27Rα$^+$ CD4$^+$ memory population relative to the IL-27Rα$^-$ CD4$^+$ memory population beginning at day 3 following CLP and continuing to day 4 (**Fig. 2.5A, B**). When comparing sham mice to CLP mice on days 1, 2, 3, and 4 following surgery, TIGIT expression was increased on IL-27Rα$^+$ memory CD4 T cells ($p=0.01$), with $p=0.004$ when comparing sham vs. day 3 post-CLP and $p=0.04$ when comparing sham vs. day 4 post-CLP (**Fig. 2.5A-B**). When making the same comparisons for IL-27Rα$^-$ memory CD4$^+$ T cells, the overall difference was not significant ($p=0.37$) and $p>0.99$ on day 3 and 4 after CLP. This suggests that sepsis increases the expression of TIGIT selectively on the IL-27Rα$^+$ cell population (**Fig. 2.5A-B**). However, we found no difference in the frequency of PD-1$^+$ cells between IL-27Rα$^+$ vs IL-27Rα$^-$ memory CD4$^+$ T cell populations (**Fig. 2.5A, C**). Assessment of the IL-27Rα$^+$ vs IL-27Rα$^-$ memory CD8$^+$ T cell populations showed no significant differences in the frequencies of TIGIT$^+$ (**Fig. 2.5D-E**) or PD-1$^+$ (**Fig. 2.5D, F**), T cells at any point following CLP. In contrast to the memory CD4$^+$ T cell compartment, memory CD8$^+$ T cells did not have a distinct population of TIGIT$^+$ or PD-1$^+$ cells (**Fig. 2.5E**). These results demonstrate that IL-27 receptor expression is associated with the expression of TIGIT on CD44^{hi} CD4$^+$ T cells.

IL-27 signaling is associated with reduced proliferation, but not apoptosis, of CD44^{hi} CD8^{+} T cells in septic mice

To determine whether IL-27 modulates the apoptosis or proliferation of T cells during sepsis, we next assessed whether the IL-27 signaling was associated with increased activity of the apoptotic proteases Caspase 3 and Caspase 7 or increased expression of the proliferation marker Ki67. We measured active Caspase 3/7 on memory T cells using flow cytometry and found that both CD44^{hi} CD4^{+} T cells in both wild type and *Il27ra*$^{-/-}$ mice exhibit similar frequencies of active caspase 3/7^{+} cells (**Fig. 2.6A**). In addition, the frequency of caspase 3/7^{+} apoptotic cells is similar between the CD44^{hi} CD8^{+} T cells of wild type vs. *Il27ra*$^{-/-}$ septic mice (**Fig. 2.6B**). The frequency of proliferating (Ki67^{+}) cells among CD4^{+} CD44^{hi} memory T cells was similar in IL-27Rα^{-} vs. IL-27Rα^{+} populations (**Fig. 2.7A-B**). We next looked specifically at the association between TIGIT expression and proliferation in the IL-27Rα^{-} and IL-27Rα^{+} populations. Among IL-27Rα^{-} memory CD4^{+} T cells, TIGIT^{+} cells exhibited reduced frequencies of proliferating cells compared to TIGIT^{-} cells in non-septic animals but similar frequencies following CLP (**Fig. 2.7C**). In contrast, among IL-27Rα^{+} memory CD4^{+} T cells, TIGIT^{+} cells proliferated more than TIGIT^{-} cells one day following CLP (**Fig. 2.7C**). In the CD44^{hi} memory CD8^{+} T cell compartment, IL-27Rα expression was associated with reduced proliferation one and two days after CLP (**Fig. 2.7D-E**). Among IL-27Rα^{-} cells, there was no difference in proliferation based on TIGIT expression at any timepoint analyzed. In contrast, among IL-27Rα^{+} T cells, TIGIT^{+} cells proliferated more than TIGIT^{-} T cells one day following CLP (**Fig. 2.7F**). These results indicate that the numerical reduction in IL-27Rα^{+} memory CD4^{+} T cells following CLP is unrelated to a deficit in proliferation, but that reduced proliferation may be responsible for the reduced numbers in the CD8^{+} T cell

compartment. In addition, TIGIT expression is associated with higher proliferation in IL-27Rα$^+$, but not IL-27Rα$^-$, memory T cells after CLP.

Septic mice lacking the IL-27Rα have a reduced frequency of TIGIT$^+$ memory CD4$^+$ T cells but a similar frequency of Treg compared to wild type mice

After identifying an association between IL-27Rα positivity and TIGIT expression on CD4$^+$ memory T cells, we assessed TIGIT and PD-1 expression on memory T cells in *Il27ra*$^{-/-}$ mice to determine whether deficiency of IL27Rα altered their expression. First, we compared the frequency of the co-inhibitory molecules TIGIT and PD-1 in the CD44^{hi} T cell compartment of the *Il27ra*$^{-/-}$ mice to wild type (WT) mice. We found that frequencies of TIGIT$^+$ cells among the memory CD4$^+$ T cell population increased following CLP in both WT and *Il27ra*$^{-/-}$ mice compared to the sham group (**Fig. 2.8A**). However, on days 1 and 2 after CLP, *Il27ra*$^{-/-}$ mice had a significantly reduced frequency of TIGIT$^+$ memory CD4$^+$ T cells compared to WT mice (**Fig. 2.8A**). We found that the frequency of PD-1$^+$ cells among CD44^{hi} CD4$^+$ T cells increased in both WT and *Il27ra*$^{-/-}$ mice following CLP compared to the sham group; however, there was no difference between WT vs. *Il27ra*$^{-/-}$ mice in the sham or CLP groups (**Fig. 2.8B**). In contrast to the memory CD4$^+$ T cell compartment, the memory CD8$^+$ T cell compartment of the WT vs. *Il27ra*$^{-/-}$ mice showed no difference in the frequency of TIGIT$^+$ cells (**Fig. 2.8C**) or PD-1$^+$ cells (**Fig. 2.8D**) among CD44^{hi} CD8$^+$ T cells. These results further support the idea that IL-27 signaling enhances TIGIT expression on CD4$^+$ memory T cells.

Previous studies have shown that IL-27 promotes the development and effector function of T regulatory cells (Tregs) (Moon, Park et al. 2013, Do, Kim et al. 2017,

Wehrens, Wong et al. 2018, Kim, Le et al. 2019, Nguyen, Jang et al. 2019). Since many Tregs express the co-inhibitory receptor TIGIT, we next looked to determine the frequency of Tregs in wild type and *Il27ra*$^{-/-}$ mice. The frequency of FoxP3^{+} Tregs significantly increased in both wild type and *Il27ra*$^{-/-}$ mice following CLP, but there was no difference in Treg frequency between these groups (**Fig. 2.9A-B**). Within the TIGIT^{+} CD4^{+} T cell population, *Il27ra*$^{-/-}$ mice had a higher frequency of FoxP3^{+} expression following CLP compared to mice than underwent sham surgery (**Fig. 2.9C**). However, the frequency of Tregs within the TIGIT^{+} population was unchanged in wild type septic mice (**Fig. 2.9C**).

Memory CD4^{+} T cells in Il27ra$^{-/-}$ mice exhibit impaired production of IFN-γ, but this is not linked to TIGIT expression

To assess whether the IL-27-driven signals during sepsis impair the production of effector cytokines, we measured IFN-γ and TNFα production by memory CD4^{+} and CD8^{+} T cells in wild type compared to *Il27ra*$^{-/-}$ mice following CLP-induced sepsis. *Il27ra*$^{-/-}$ mice exhibited a reduced frequency of IFN-γ-producing CD44^{hi} CD4^{+} T cells compared to WT in mice receiving sham surgery and day 1 after CLP-induced sepsis (**Fig. 2.10A-B**). The frequency of TNFα producing CD4^{+} cells was similar in sham and CLP mice (**Fig. 2.10A, C**). Assessment of the CD44^{hi} CD4^{+} T cells producing both IFN-γ and TNFα showed reduced frequencies of double positive cells in the *Il27ra*$^{-/-}$ mice compared to the WT mice following sham surgery, but this difference did not persist after CLP-induced sepsis (**Fig. 2.10D**). In the memory CD44^{hi} CD8^{+} T cell compartment, WT and *Il27ra*$^{-/-}$ mice exhibited similar frequencies of IFN-γ^{+} (**Fig. 2.10E-F**), TNFα^{+} (**Fig. 2.10E, G**), and IFN-γ^{+} TNFα^{+} (**Fig. 2.10E,H**) cells following CLP-induced sepsis. Circulating

(plasma) IFN-γ was unchanged between wild type vs. Il27ra$^{-/-}$ mice that underwent sham surgery and CLP (**Fig. 2.11**). Interestingly, circulating IFN-γ was reduced one and two days after CLP in wild type mice (**Fig. 2.11**).

To determine the impact of IL-27-induced TIGIT expression on the production of IFN-γ and TNFα, we assessed cytokine production in wild type IL-27Rα^{+} CD44^{hi} T cells. TIGIT expression was not associated with a difference in the frequency of IFNγ production by memory CD4^{+} T cells in non-septic mice that underwent sham surgery or in septic mice (**Fig. 2.12A-B**). However, the TIGIT^{-} memory CD4^{+} T cells in non-septic mice produced significantly more TNF than did TIGIT^{+} cells (**Fig. 2.12C**). The frequency of IFNγ^{+} TNF^{+} memory CD4^{+} T cells was also elevated in the TIGIT^{-} population of non-septic animals (**Fig. 2.12D**). In the memory CD8^{+} T cell compartment (**Fig. 2.12E**), TIGIT expression was not associated with the frequency of IFNγ^{+} (**Fig. 2.12F**), TNF^{+} (**Fig. 2.12G**), or IFNγ^{+} TNF^{+} (**Fig. 2.12H**) producing cells. These data indicate that TIGIT expression is not associated with the defect in IFN-γ production by CD44^{hi} CD4^{+} T cells in *Il27ra*$^{-/-}$ vs wild type mice.

IL-27 signaling does not impact sepsis mortality

Our previous results indicated that IL-27 signaling is associated with an increase in TIGIT-expressing CD4^{+} memory T cells during sepsis. To determine if these differences could result in a difference in sepsis survival, we first compared the survival of mice deficient in *Il27ra* to wild type mice. In this setting, there was also no difference in sepsis mortality between *Il27ra*$^{-/-}$ and wild type mice. (**Fig. 2.13A**). To confirm these results, we next used a monoclonal antibody specific to the p28 subunit of IL-27 (α-IL-27p28) to pharmacologically disrupt IL-27 signaling in wild type mice. Mice that received the αp28

mAb exhibited similar survival compared to saline treated mice over the course of 7 days following CLP (**Fig. 2.13B**). These results indicate that in a moderate mortality model of sepsis, IL-27 is not associated with worsened survival.

V. **DISCUSSION**

Based on previous reports investigating the role of IL-27 in cancer and chronic infections, we hypothesized that IL-27 signaling upregulates PD-1 and TIGIT on memory T cells during sepsis. While IL-27Rα was associated with increased TIGIT expression on memory $CD4^+$ T cells following sepsis, this change did not correspond with a difference in T cell apoptosis, effector function, or sepsis survival.

Our results indicate that plasma IL-27 is significantly elevated following CLP, in agreement with previous work (Wirtz, Tubbe et al. 2006, Nelson, Tolbert et al. 2010, Wong, Cvijanovich et al. 2012, Wong, Lindsell et al. 2013, Bosmann, Strobl et al. 2014, Cao, Xu et al. 2014, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015, Gao, Yang et al. 2016, Yan, Mitra et al. 2016). Due to previous studies linking IL-27 signaling to sepsis mortality, we sought to determine the impact of IL-27Rα signaling on memory T cells. Specifically, we sought to determine if there was an increase in IL-27Rα expression, or changes in T cell apoptosis or cytokine production due to IL-27Rα. We found that the number of IL-27Rα$^+$ $CD4^+$ and $CD8^+$ memory T cells decreased following CLP, but this was not associated with an increase in IL-27Rα expression, T cell apoptosis, or alterations in cytokine production.

The link we observed between IL-27Rα and the upregulation of TIGIT on $CD4^+$ T cells during sepsis is consistent with previous papers that established a similar link in the

setting of cancer and chronic infections (Chihara, Madi et al. 2018, DeLong, O'Hara Hall et al. 2019). In contrast to these studies, however, we did not find that IL-27 regulated TIGIT expression on $CD8^+$ T cells. This suggests that mechanisms other than IL-27 are responsible for the induction of TIGIT on $CD8^+$ T cells. Moreover, prior studies also reported a relationship between IL-27 and PD-1 expression on $CD4^+$ and $CD8^+$ T cells, which we also did not observe (Chihara, Madi et al. 2018, DeLong, O'Hara Hall et al. 2019). These studies highlight that the impact of IL-27 signaling on TIGIT expression may be disease state- and context-dependent.

Although our global knockout approach did not parse apart the cell-autonomous vs indirect effect of IL-27Rα signaling on TIGIT expression on $CD4^+$ memory T cells, previous studies have found that TIGIT expression is induced directly by IL-27 on T cells through the action of the transcription factors PRDM1 and c-Maf (Chihara, Madi et al. 2018). Another possibility is that IL-27 signaling on other cells subsequently results in the upregulation of TIGIT on $CD4^+$ T cells. As dendritic cells express IL-27Rα and also interact with T cells, it is possible that IL-27 signaling on DC may indirectly regulate TIGIT expression on T cells.

One possible explanation for the association between IL-27 signaling and TIGIT expression and the *lack* of association between IL-27 signaling and PD-1 expression is the difference in TCR requirements for their expression. DeLong et al. noted that TCR signaling is required *in vitro* for IL-27 to upregulate the expression of TIGIT, but that TCR signaling was not required for IL-27 to upregulate PD-L1 (DeLong, O'Hara Hall et al. 2019). This suggests that TCR signaling in the earliest stages of sepsis could play a role in the ability for IL-27 to upregulate TIGIT. In contrast, the factors regulating IL-27 dependent expression of PD-1 are not involved in the early stages of sepsis. First, there

are multiple pathways that regulate the transcription of coinhibitory molecules within T cells. While no literature exists specifically for TIGIT, Boss and colleagues have shown that *Pdcd1* expression is differentially regulated by the duration of antigen exposure (acute vs chronic) (Austin, Lu et al. 2014, Bally, Austin et al. 2016). In this sepsis model, we were only able to assess the T cell phenotype in settings of acute antigen exposure. It is possible that after longer periods of time, we would observe the changes noted in previous studies assessing the role of IL-27 in cancer and chronic infection. Despite little known information on the impact of antigen exposure and TIGIT, we observed an association between IL-27 signaling and TIGIT expression at early time points during sepsis. This indicates that acute antigen exposure allows a small but significant number of memory $CD4^+$ T cells to upregulate the co-inhibitory molecule TIGIT. Surprisingly, these effects did not extend to memory $CD8^+$ T cells, something that was previously seen in murine models of chronic infection and cancer. Since sepsis is primarily associated with the activation of $CD4^+$ compared to $CD8^+$ T cells, the difference in activation may explain why TIGIT was not upregulated on memory $CD8^+$ T cells (Patenaude, D'Elia et al. 2005, McDunn, Turnbull et al. 2006).

Previous studies have linked memory T cell apoptosis to sepsis mortality and the reactivation of latent viral infections in septic humans and mice. Due to the relationship between IL-27 signaling and TIGIT expression, we hypothesized that IL-27 is responsible for memory T cell apoptosis observed during sepsis. Surprisingly, we did not observe any difference in the frequency of apoptotic (Caspase $3/7^+$) memory T cells between wild type and *Il27ra*$^{-/-}$ mice despite the differences in TIGIT expression. These results are not necessarily surprising, as TIGIT is upregulated in a TCR signaling-dependent mechanism

(DeLong, O'Hara Hall et al. 2019), and TCR signaling does not lead to the T cell apoptosis observed during sepsis (Unsinger, Herndon et al. 2006).

In this study, we looked specifically at memory T cells due to the link between sepsis-impaired memory T cells and increased long term mortality (Duong, Condotta et al. 2014, Chen, Mittal et al. 2017, Xie, Crepeau et al. 2019). In previous studies, IL-27 limited the response of activated and memory T cells (Pflanz, Timans et al. 2002, Villarino, Hibbert et al. 2003, Yoshimura, Takeda et al. 2006, Gwyer Findlay, Villegas-Mendez et al. 2014). In line with these results, we found that the frequency of TIGIT^{+} memory CD4^{+} T cells in *Il27ra*$^{-/-}$ mice was significantly reduced one and two days after CLP. However, the frequency of TIGIT expressing memory CD4^{+} T cells in both wild type and *Il27ra*$^{-/-}$ mice were similar in mice that underwent sham surgery, suggesting that IL-27 is not required for the induction of TIGIT on memory T cells. In addition, the changes in TIGIT expression induced by sepsis were not associated with alterations in T cell effector function, as the reduction in IFNγ seen in *Il27ra*$^{-/-}$ memory CD4^{+} T cells seen after CLP was also present in mice that underwent sham surgery. These findings are consistent with previous studies that found IL-27 signaling-induced expression of IFNγ through the transcription factor *T-bet* (Villarino, Hibbert et al. 2003, Mayer, Mohrs et al. 2008). Interestingly, a more recent study found that IFNγ expression was no different in the *Il27ra*$^{-/-}$ mice with chronic LCMV infection (Harker, Wong et al. 2018), suggesting a disease-state and context-dependent role of IL-27 signaling in mediating IFNy production.

Multiple previous studies reported a significant increase in sepsis mortality caused by IL-27 signaling. Similar to the present study, these studies used pharmacological inhibitors of IL-27 signaling to measure the impact IL-27 has on sepsis survival (Wirtz,

Tubbe et al. 2006, Bosmann, Russkamp et al. 2014, Bosmann, Strobl et al. 2014). The other studies reported impressive decreases in sepsis mortality following pharmacological inhibition of IL-27 signaling, suggesting a major role for IL-27 in sepsis mortality. We did not observe a similar decrease in mortality following IL-27 blockade. This may be linked to significant differences in the severity of sepsis model used, as well as the reagents used. While the previous studies used a high mortality CLP (where the vast majority of mice do not survive), our model used a mild to moderate mortality in which 50% or more mice are expected to survive. Thus, our data do not negate the possible role for IL-27 in high-mortality models of sepsis. Another difference in our study compared to the previous studies are the reagents used to interrupt IL-27 signaling: previous studies used a polyclonal α-IL-27 antibody (Bosmann, Russkamp et al. 2014) or a soluble IL-27Rα (Wirtz, Tubbe et al. 2006). In contrast, we used a monoclonal α-IL-27 neutralizing antibody. Furthermore, our study compared the survival of *Il27ra*$^{-/-}$ mice to that of wild type mice using the cecal ligation and puncture model of sepsis. In contrast, a previous study comparing wild type to Il27ra$^{-/-}$ mice during sepsis used a model of severe endotoxemia (Bosmann, Strobl et al. 2014), which may not adequately replicate the inflammation and immunological response of septic patients (Opal, Dellinger et al. 2014, Osuchowski, Ayala et al. 2018).

Our results indicate that sepsis decreases the frequency and number of IL-27Rα expressing memory T cells. We hypothesized that the decrease in the IL-27Rα^{+} memory T cell population could occur independently from apoptosis via a TIGIT-mediated inhibition of T cell proliferation (Joller, Hafler et al. 2011). However, we did not observe a difference in the frequency of proliferating IL-27Rα^{-} vs. IL-27Rα^{+} memory CD4^{+} T cells, although TIGIT expression was associated with increased proliferation in IL-27Rα^{+} cells.

IL-27Rα$^+$ memory CD8$^+$ T cells had a lower frequency of proliferating cells compared to the IL-27Rα$^-$ population, but TIGIT expression was again associated with a higher frequency of proliferation. These data suggest that TIGIT does not inhibit memory T cell proliferation in septic mice. One possibility for the reduction in the IL-27Rα$^+$ memory T cell population is that these cells are leaving the circulation and going to the site of infection (Unsinger, McGlynn et al. 2010). Additionally, IL-27Rα expression was associated with an increase in the expression of TIGIT on CD4$^+$ memory T cells in septic mice. Differences in antigen chronicity, TCR signaling, and the cytokine milieu between sepsis and these models may explain this. Future studies investigating the role of each of these factors will be important to establish what is responsible for the upregulation of co-inhibitory markers during sepsis.

Limitations of the Study

Our results indicate that plasma IL-27 is significantly elevated following CLP, in agreement with previous work (Wirtz, Tubbe et al. 2006, Nelson, Tolbert et al. 2010, Wong, Cvijanovich et al. 2012, Wong, Lindsell et al. 2013, Bosmann, Strobl et al. 2014, Cao, Xu et al. 2014, Wong, Liu et al. 2014, Hanna, Berrens et al. 2015, Gao, Yang et al. 2016, Yan, Mitra et al. 2016). However, this increase did not correlate with an increase in the number of T cells expressing IL-27Rα. In fact, we found that both the frequency and number of CD44^{lo} naïve and CD44^{hi} memory CD4$^+$ T cells expressing IL-27Rα were reduced following CLP compared to sham mice. We further found that the number of CD44^{hi} CD8$^+$ T cells expressing IL-27Rα$^+$ decreased following sepsis. These findings are in line with published results showing that IL-27Rα can be shed from the cell surface of activated T cells through the action of metalloproteases (Dietrich, Candon et al. 2014).

Thus, increased metalloprotease-mediated shedding of cell surface IL-27Rα during sepsis might underlie this observation. The reduction in surface expression of IL-27Rα is in contrast to previous research on the role of IL-27 during toxoplasma infection (Villarino, Larkin et al. 2005). Villarino et al. found that mice infected with toxoplasma have a significantly higher frequency of both $CD4^+$ and $CD8^+$ T cells expressing IL-27Rα compared to uninfected mice (Villarino, Larkin et al. 2005). Since the immune response to a parasitic infection is very distinct from what occurs during sepsis, our results suggest that sepsis differentially regulates IL-27Rα expression.

Previous studies of co-inhibitory receptor expression on T cells following CLP have revealed that co-inhibitory receptor expression remains elevated far longer than expected from T cell activation and is associated with impairments in T cell effector function, and ultimately apoptosis. However, TIGIT may also be acting as an activation marker. We cannot draw conclusions about whether it was acting as an activator or suppressor of T cell function. In addition, the changes in the frequency of TIGIT expression on T cells in this study were quite small, and the biological significance of these changes is unclear. Few studies have assessed the impact of TIGIT on T cell responses in the context of sepsis, but previous studies in our laboratory have found a similar induction of TIGIT expression in the $CD4^+$ T cells of septic mice with solid tumors (Chen, Xue et al. 2019). However, future studies addressing the physiological impact of TIGIT on survival from sepsis are necessary to better understand the biological relevance of these findings.

Although T cell exhaustion is observed early after the onset of sepsis in both mouse models and human patients, it is unclear if the factors that regulate T cell exhaustion in early stages are the same that maintain T cell exhaustion in the weeks to months following sepsis resolution. Future studies that look at these factors will be necessary. Another

limitation of this study was the use of immunologically naïve mice, which have a lower frequency of memory T cells than do humans. We have previously published data indicating that mice sequentially infected with viral and bacterial infections have a memory compartment that better reflects what is observed in humans (Xie, Chen et al. 2019). While we failed to observe an association between IL-27 signaling and PD-1 expression in previously healthy septic mice, the findings in mice with pre-existing cancer may be different, since there is an association between IL-27 signaling and PD-1 expression in the setting of cancer. In line with this idea, previous research in our laboratories has found that cancer septic mice differ in the expression of co-inhibitory markers on their T cells compared to previously healthy septic mice (Fox, Robertson et al. 2010, Lyons, Mittal et al. 2016, Xie, Robertson et al. 2018, Chen, Xue et al. 2019).

VI. **FIGURES**

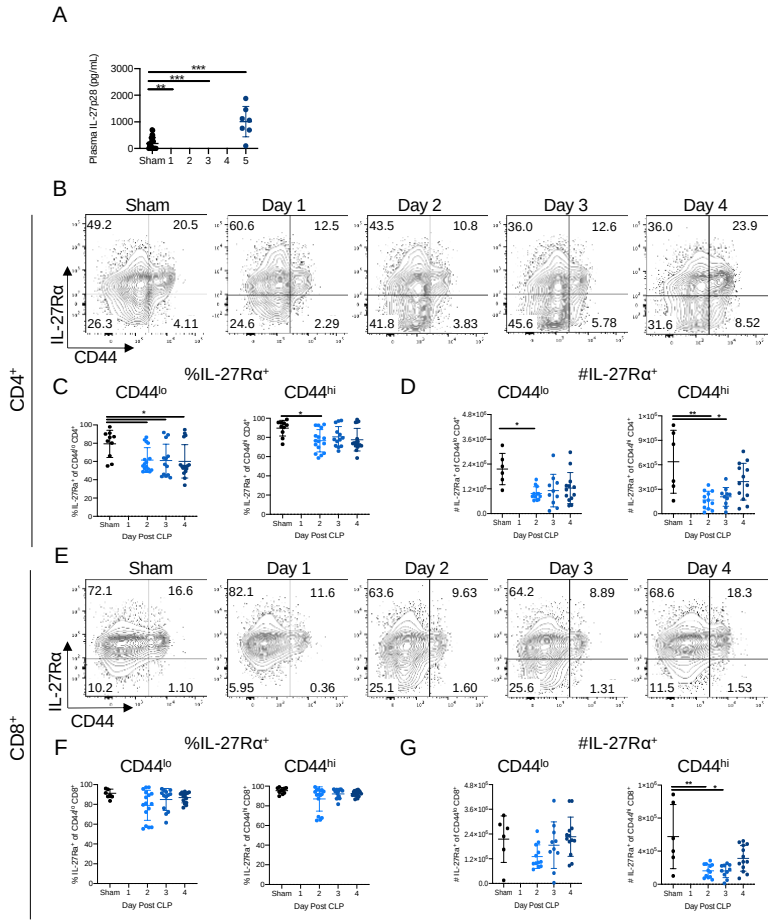

Figure 2.1: Sepsis results in a reduction in the frequency and number of IL-27Rα⁺ T cells

Following cecal ligation and puncture (CLP) or sham surgery (sham), animals were euthanized on the indicated days. Plasma was collected for IL-27p28 ELISAs on days 1 through 5 and spleens harvested for analysis by flow cytometry on days 1 through 4. **(A)** Concentration of IL-27p28 in the plasma of sham and CLP mice on days 1 through 5 following surgery. **(B)** Representative flow cytometric plots showing the frequency of IL-27Rα expressing $CD4^+$ T cells in sham and CLP mice on days 1-4 after surgery. **(C)** The frequency of $CD4^+$ $CD44^{lo}$ naïve (left) and $CD4^+$ $CD44^{hi}$ memory (right) T cells expressing the IL-27Rα in sham and CLP mice on days 1-4 after surgery. **(D)** The absolute number of $CD4^+$ $CD44^{lo}$ naïve (left) and $CD4^+$ $CD44^{hi}$ memory (right) T cells expressing the IL-27Rα in sham and CLP mice on days 1-4 after surgery. **(E)** Representative flow cytometric plots showing the frequency of IL-27Rα expressing $CD8^+$ T cells in sham and CLP mice on days 1-4 after surgery. **(F)** The frequency of $CD8^+$ $CD44^{lo}$ naïve (left) and $CD8^+$ $CD44^{hi}$ memory (right) T cells expressing the IL-27Rα in sham and CLP mice on days 1-4 after surgery. **(G)** The absolute number of $CD8^+$ $CD44^{lo}$ naïve (left) and $CD8^+$ $CD44^{hi}$ memory (right) T cells expressing the IL-27Rα in sham and CLP mice on days 1-4 after surgery. All summary data was pooled from 3 independent experiments, with n=7-18 mice per group. *p<0.05, **p<0.01, ***p<0.001.

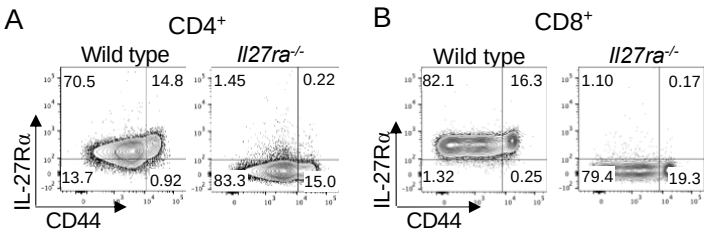

Figure 2.2: Gating strategy for IL-27Rα on $CD4^+$ and $CD8^+$ T cells (Related to Figure 2.1)

Splenocytes were obtained from wild type or *Il27ra*$^{-/-}$ mice and stained for IL-27Rα and CD44. (**A**) Representative flow cytometric plots showing IL-27Rα (y-axis) vs CD44 (x-axis) for $CD4^+$ T cells in wild type (left) and *Il27ra*$^{-/-}$ (right) mice. (**B**) Representative flow cytometric plots showing IL-27Rα (y-axis) vs CD44 (x-axis) for $CD4^+$ T cells in wild type (left) and *Il27ra*$^{-/-}$ (right) mice.

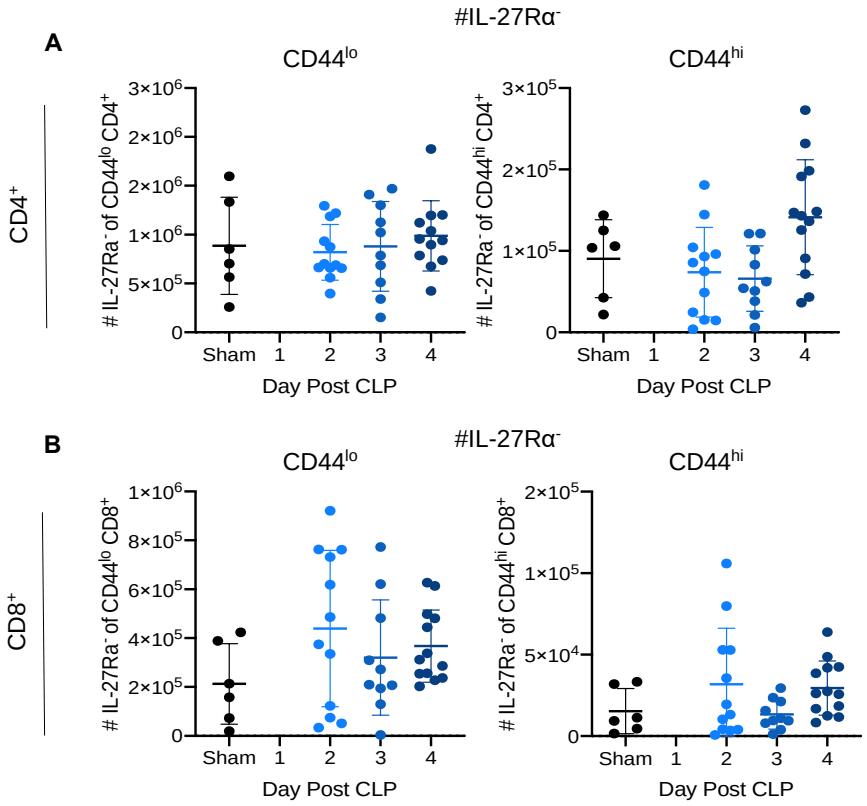

Figure 2.3: IL-27Rα^{-} CD44^{lo} and CD44^{hi} numbers are unchanged following sepsis (Related to Figure 2.1).

Following cecal ligation and puncture (CLP) or sham surgery (sham), animals were euthanized on the indicated days. Spleens were harvested for analysis by flow cytometry on days 1 through 4. **(A)** The absolute number of CD4^{+} CD44^{lo} naïve (left) and CD4^{+} CD44^{hi} memory (right) T cells *not* expressing IL-27Rα in sham and CLP mice on days 1-4 after surgery. **(B)** The absolute number of CD8^{+} CD44^{lo} naïve (left) and CD8^{+} CD44^{hi} memory (right) T cells *not* expressing IL-27Rα in sham and CLP mice on days 1-4 after surgery. All summary data was pooled from 3 independent experiments, with n=7-18 mice per group.

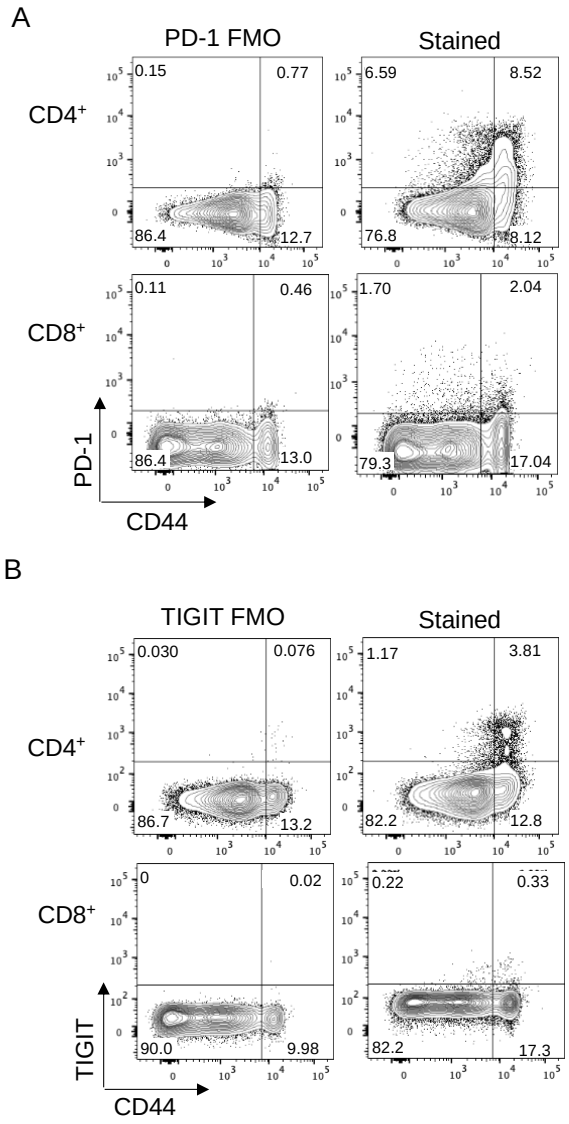

Figure 2.4: Gating strategy for PD-1 and TIGIT on $CD4^+$ and $CD8^+$ T cells
Splenocytes were obtained from wild type septic mice on day 4 following CLP and used for flow cytometric analysis. (**A**) Representative flow cytometric plots showing PD-1 (y-axis) vs CD44 (x-axis) using a PD-1 FMO (left) and stained (right) controls. $CD4^+$ T cells are shown in the top series and $CD8^+$ cells at bottom. **(B**) Representative flow cytometric plots showing TIGIT (y-axis) vs CD44 (x-axis) using a PD-1 FMO (left) and stained (right) controls. $CD4^+$ T cells are shown in the top series and $CD8^+$ cells at bottom.

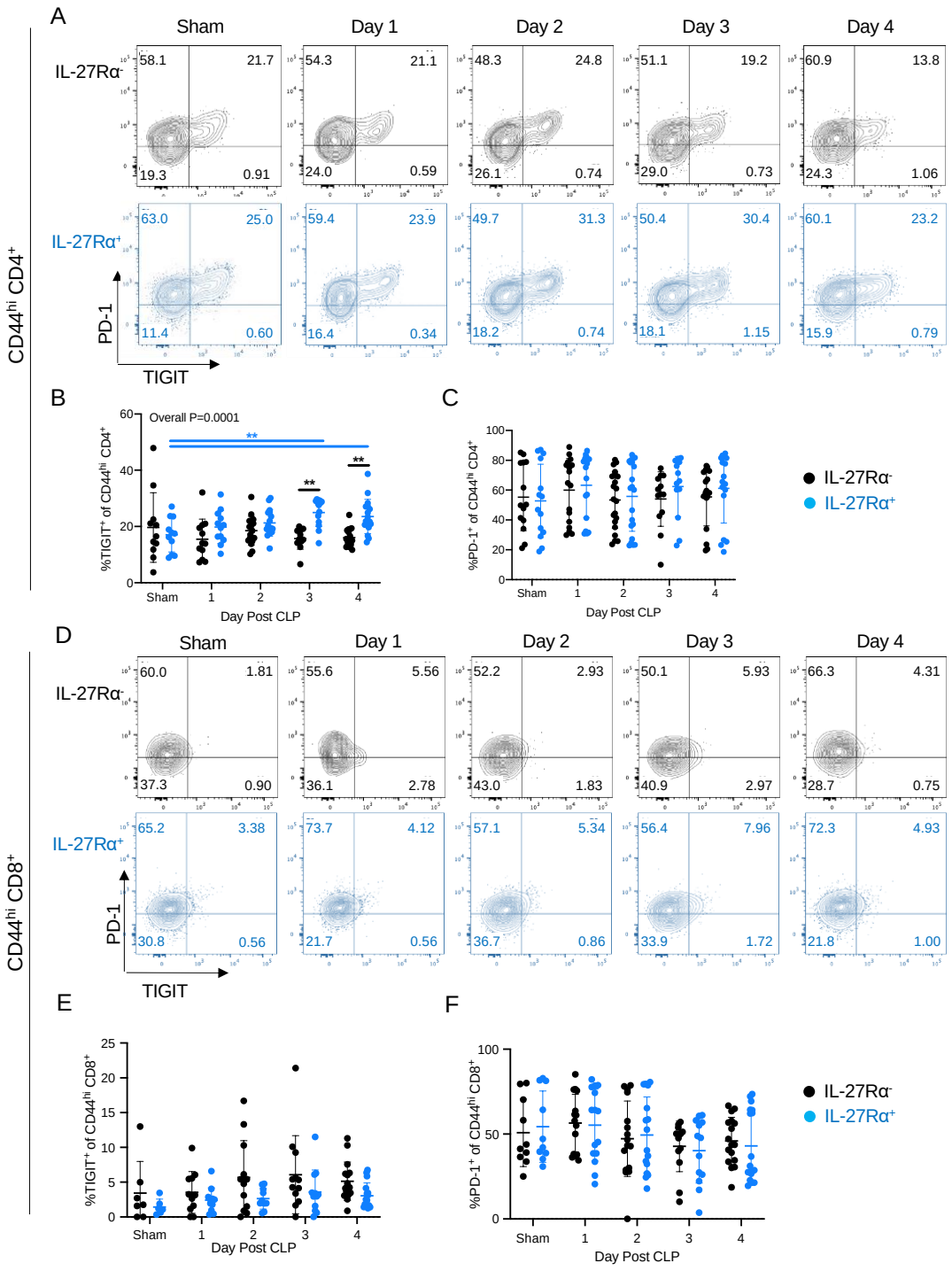

Figure 2.5: IL-27Rα expression is associated with an increased frequency of TIGIT expression on memory CD4⁺ T cells during sepsis. The gating strategy for PD-1 and TIGIT is shown in Figure 2.4.

(A) Representative flow cytometric plots showing PD-1 and TIGIT expression on CD44^{hi} memory CD4^{+} T cells lacking (black) or expressing (blue) IL-27Rα in wild type mice on days 1-4 after sham

surgery ("sham") or CLP. **(B)** The frequency of TIGIT$^+$ expressing IL-27Rα$^-$ (black) and IL-27Rα$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells in sham and CLP wild type mice on days 1-4 after surgery. **(C)** The frequency of PD-1$^+$ expressing IL-27Rα$^-$ (black) and IL-27Rα$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells in sham and CLP wild type mice on days 1-4 after surgery. **(D)** Representative flow cytometric plots showing TIGIT and PD-1 expression on CD44^{hi} memory CD8$^+$ T cells lacking (black) or expressing (blue) IL-27Rα in sham mice and CLP wild type mice on days 1-4 after surgery. **(E)** The frequency of TIGIT$^+$ expressing IL-27Rα$^-$ (black) and IL-27Rα$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells in sham and CLP wild type mice on days 1-4 after surgery. **(F)** The frequency of PD-1$^+$ expressing IL-27Rα$^-$ (black) and IL-27Rα$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells in sham and CLP wild type mice on days 1-4 after surgery. Data were pooled from 3 independent experiments, with n=7-17 mice per group. **p<0.01.

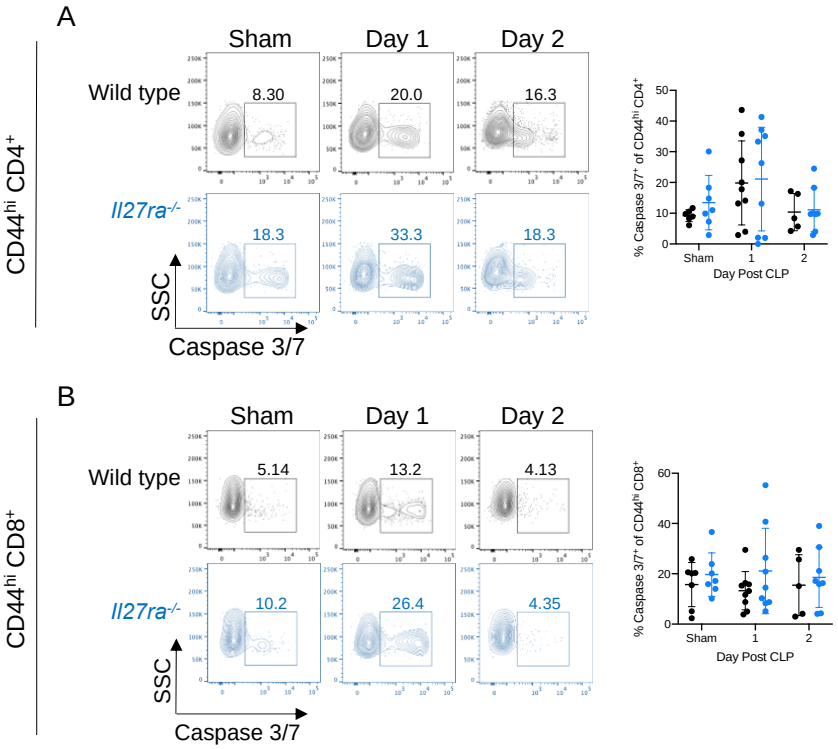

Figure 2.6: IL-27 signaling is not associated with CD44^{hi} T cell apoptosis in septic mice

(**A**) Representative flow cytometric plots showing Caspase 3/7 (x-axis) by SSC (y-axis) on CD44^{hi} memory CD4^{+} T cells in wild type and *Il27ra*$^{-/-}$ mice on days 1 and 2 after sham surgery ("sham") or CLP surgery (left). The frequency of apoptotic (Caspase 3/7^{+}) CD44^{hi} memory CD4^{+} T cells is summarized on the right. (**B**) Representative flow cytometric plots showing Caspase 3/7 (x-axis) by SSC (y-axis) on CD44^{hi} CD8^{+} T cells after sham or CLP surgery in wild type and *Il27ra*$^{-/-}$ mice on days 1 and 2 after surgery (left). The frequency of apoptotic (Caspase 3/7^{+}) CD44^{hi} memory CD8^{+} T cells is summarized on the right. Data are representative of two experiments with n=5-9 mice per group.

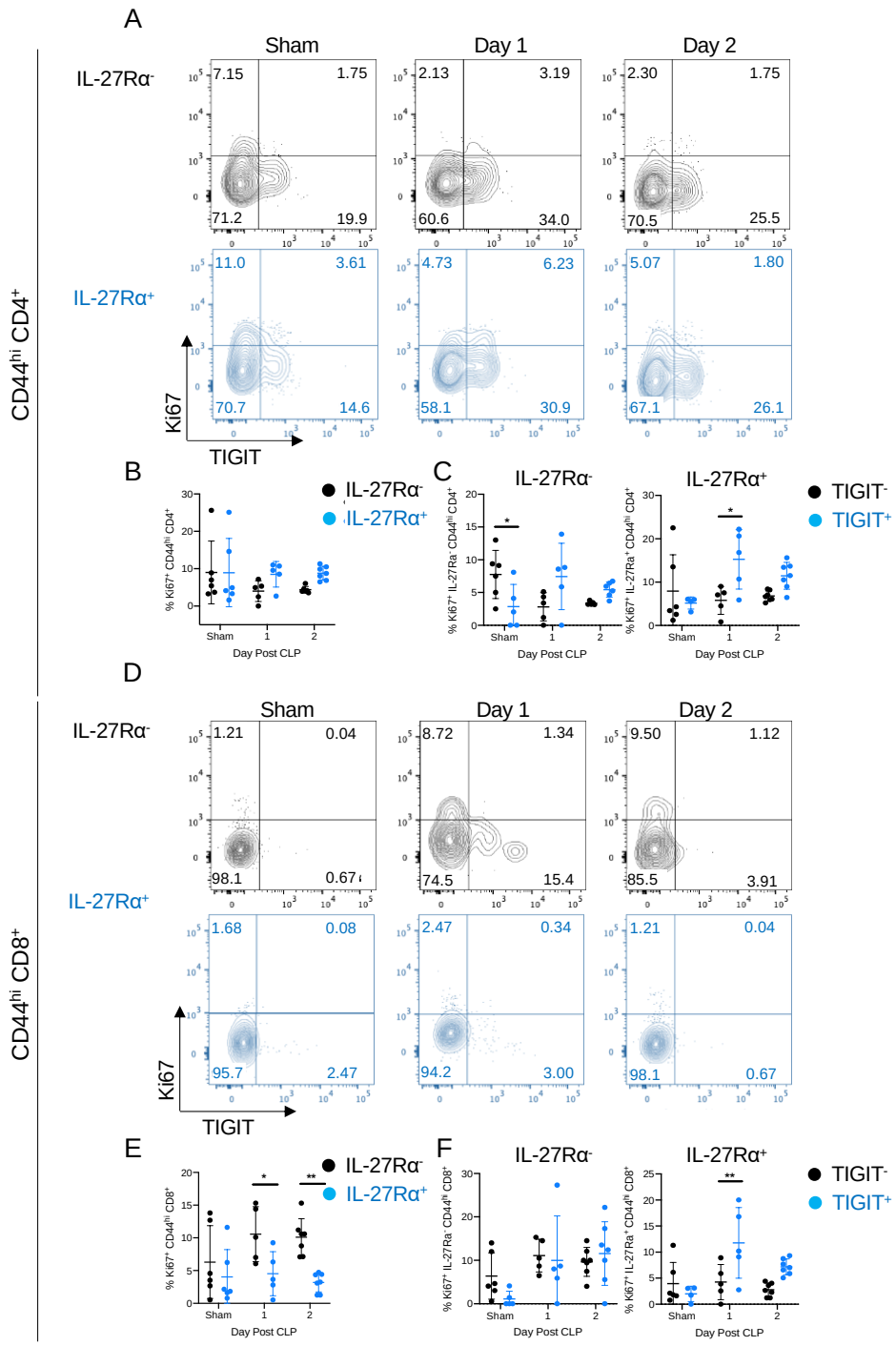

Figure 2.7: IL-27 signaling is associated with reduced proliferation of CD44^{hi} CD8^{+} T cells in septic mice

Wild type mice underwent sham ("sham") or CLP surgery and splenocytes were harvested one to two days later for flow cytometric analysis. (**A**) Representative flow cytometric plots showing TIGIT (x-axis) versus Ki67 (y-axis) expression in IL-27Rα^{-} (black) and IL-27Rα^{+} (blue) CD44^{hi} CD4^{+} T cells. (**B**) Summary graph showing the frequency of Ki67^{+} CD44^{hi} CD4^{+} T cells between

the IL-27Rα^{-} (black) and IL-27Rα^{+} (blue) populations. (**C**) Summary graphs showing the frequency of Ki67^{+} CD44^{hi} CD4^{+} T cells within TIGIT^{-} (black) and TIGIT^{+} (blue) cells of the IL-27Rα^{-} (left) and IL-27Rα^{+} (right) populations. (**D**) Representative flow cytometric plots showing TIGIT (x-axis) versus Ki67 (y-axis) expression in IL-27Rα^{-} (black) and IL-27Rα^{+} (blue) CD44^{hi} CD8^{+} T cells. (**E**) Summary graph showing the frequency of Ki67^{+} CD44^{hi} CD8^{+} T cells between the IL-27Rα^{-} (black) and IL-27Rα^{+} (blue) populations. (**F**) Summary graphs showing the frequency of Ki67^{+} CD44^{hi} CD8^{+} T cells within TIGIT^{-} (black) and TIGIT^{+} (blue) cells of the IL-27Rα^{-} (left) and IL-27Rα^{+} (right) populations. Data were pooled from two independent experiments with n=5-7 per group. *$p<0.05$, **$p<0.01$

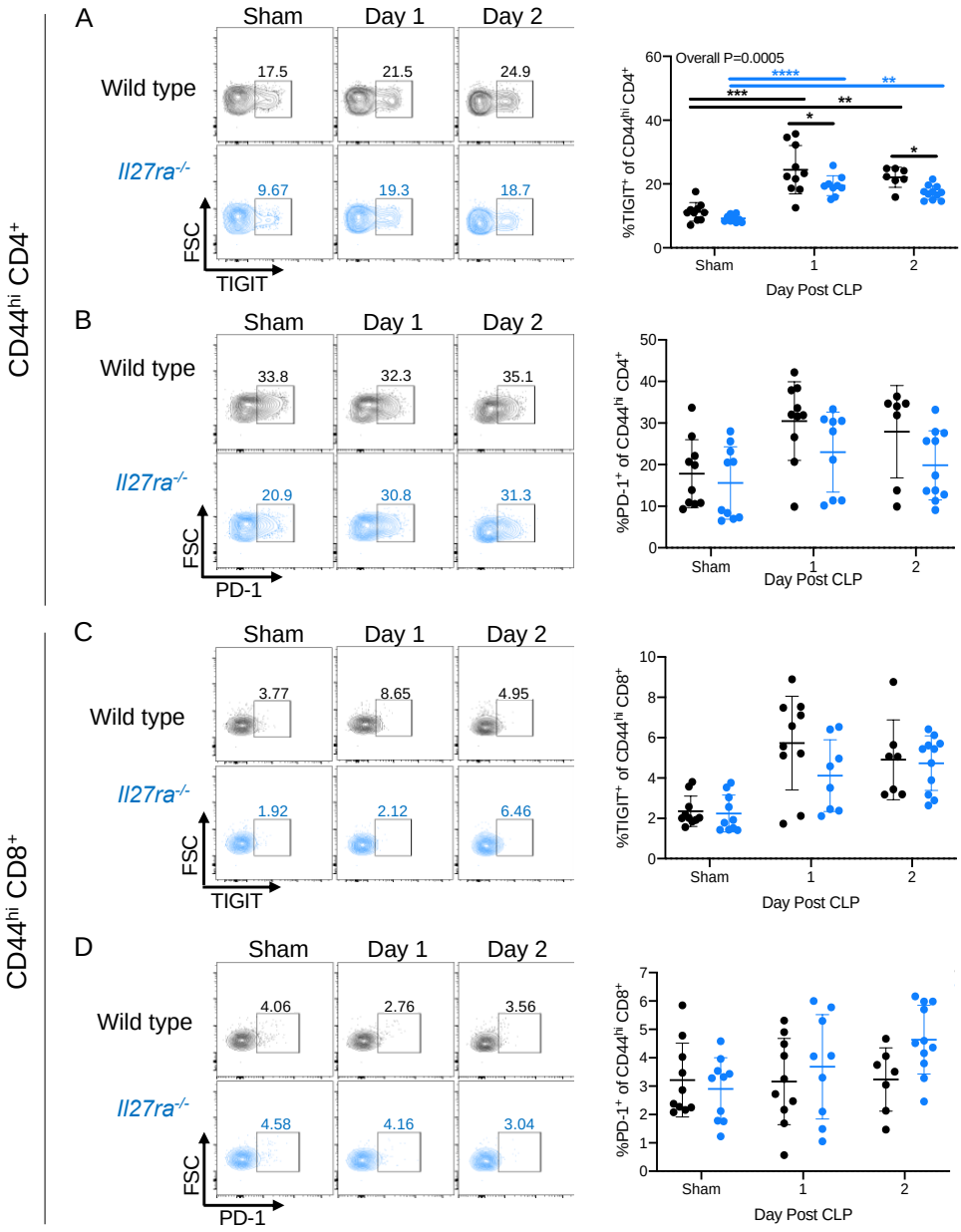

Figure 2.8: *Il27ra*$^{-/-}$ septic mice have a reduced frequency of TIGIT^{+} memory CD4^{+} T cells

Wild type and *Il27ra*$^{-/-}$ mice underwent sham surgery ("sham") or CLP surgery. On days 1 and 2 following surgery, splenocytes were collected and used for flow cytometric analysis. (**A**) Representative flow cytometric plots (left) and summary graphs (right) showing TIGIT expression (x-axis) in CD44^{hi} memory CD4^{+} T cells of wild type (top) and *Il27ra*$^{-/-}$ (bottom) mice. (**B**) Representative flow cytometric plots (left) and summary graphs (right) showing PD-1 expression (x-axis) in CD44^{hi} memory CD4^{+} T cells of wild type (top) and *Il27ra*$^{-/-}$ (bottom) mice. (**C**) Representative flow cytometric plots (left) showing TIGIT expression (x-axis) in CD44^{hi} memory CD8^{+} T cells of wild type (top) and *Il27ra*$^{-/-}$ (bottom) mice. (**D**) Representative flow cytometric plots (left) and summary graphs (right) showing PD-1 expression (x-axis) in CD44^{hi} memory CD8^{+} T cells of wild type (top) and *Il27ra*$^{-/-}$ (bottom) mice. Data were pooled from 2 independent experiments, with n=7-11 mice per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

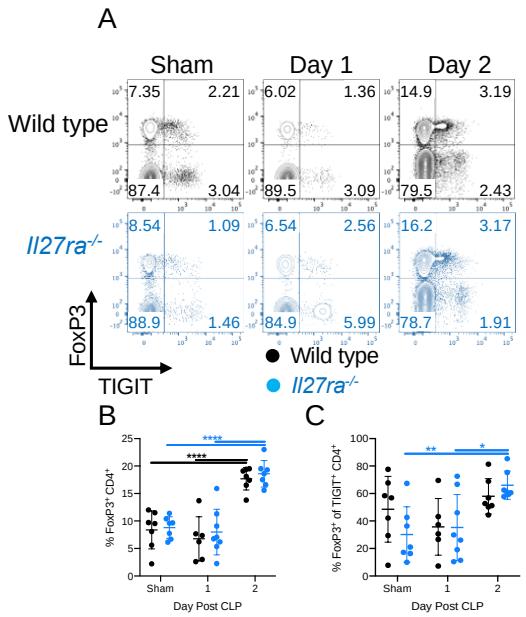

Figure 2.9: IL-27 signaling does not impact the frequency of Tregs during sepsis

One and two days after sham or CLP surgery on wild type and *Il27ra*$^{-/-}$ mice, splenocytes were collected and used for flow cytometric analysis. (**A**) Representative flow plots showing TIGIT (x-axis) vs FoxP3 (y-axis) in wild type (black) and *Il27ra*$^{-/-}$ (blue) mice that underwent sham surgery (sham) or CLP one or two days prior. (**B**) Summary graph showing the frequency of FoxP3^{+} CD4^{+} T cells within wild type (black) or *Il27ra*$^{-/-}$ (blue) mice following surgery. (**C**) Summary graph showing the frequency of FoxP3^{+} cells within the TIGIT^{+} CD4^{+} compartments of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following sham or CLP surgery. Data were pooled from two independent experiments with n=6-8 per group. *p<0.05, **p<0.01, ****p<0.0001.

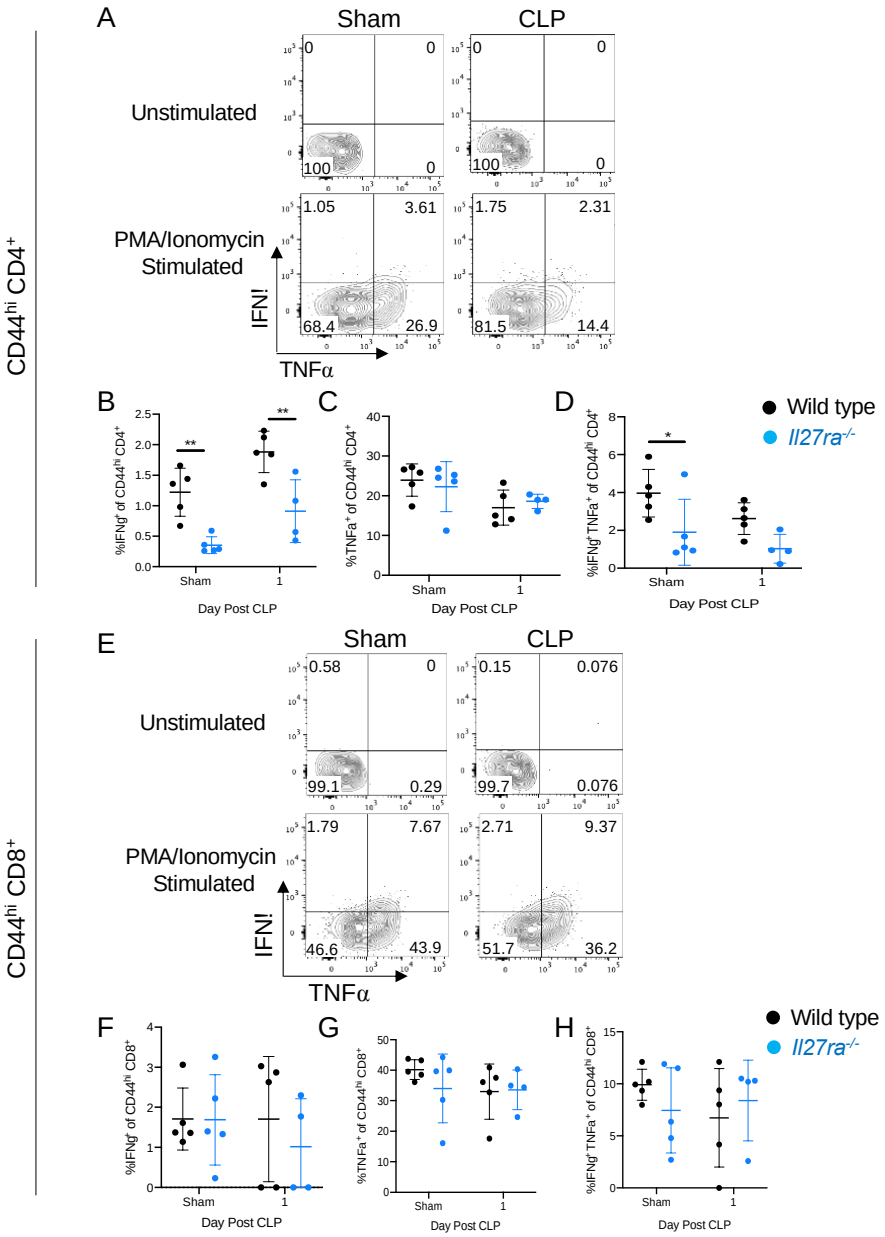

Figure 2.10: Memory CD4⁺ T cells in *Il27ra*$^{-/-}$ mice exhibit impaired production of IFN-γ at baseline and during sepsis

CLP and sham surgery ("sham") were performed on wild type and *Il27ra*$^{-/-}$ mice. One day after surgery, splenocytes were harvested for stimulation with PMA/Ionomycin or incubated without stimulation. Following incubation, samples were stained for IFN-γ and TNF-α and assessed by flow cytometry. (**A**) Representative flow cytometric plots of IFN-γ (y-axis) and TNF-α (x-axis) production by the CD44^{hi} memory CD4⁺ T cells of wild type sham (left) and wild type CLP mice (right) one day following surgery. The top row shows unstimulated controls and bottom row shows stimulated samples. (**B**) The frequency of IFN-γ producing CD44^{hi} memory CD4⁺ T cells of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. (**C**) The frequency of TNF-α producing CD44^{hi} memory CD4⁺ T cells of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. (**D**) The frequency of IFN-γ and TNF-α co-producing CD44^{hi} memory CD4⁺ T cells of wild

type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. (**E**) Representative flow cytometric plots of IFN-γ (y-axis) and TNF-α (x-axis) production by the CD44^{hi} memory CD8^{+} T cells of wild type sham (left) and wild type CLP mice (right) one day following surgery. The top row shows unstimulated controls and bottom row shows stimulated samples. (**F**) The frequency of IFN-γ producing CD44^{hi} memory CD8^{+} T cells of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. (**G**) The frequency of TNF-α producing CD44^{hi} memory CD8^{+} T cells of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. (**H**) The frequency of IFN-γ and TNF-α co-producing CD44^{hi} memory CD8^{+} T cells of wild type (black) and *Il27ra*$^{-/-}$ (blue) mice following surgery. Data are representative of one experiment with n=4-5 mice per group. *p<0.05, **p<0.01

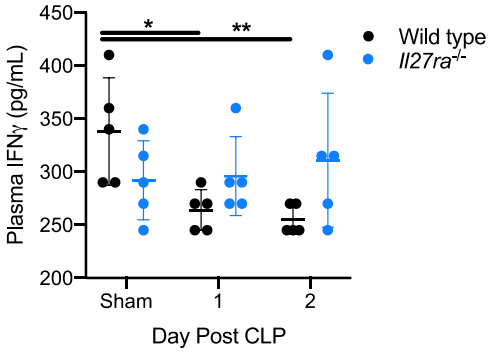

Figure 2.11: Circulating IFNγ is unchanged in *Il27ra*$^{-/-}$ vs wild type mice following CLP (Related to Figure 2.10)

Plasma was purified from the blood of wild type and *Il27ra*$^{-/-}$ mice that underwent sham ("sham") or CLP surgery on days 1 and 2 after surgery.

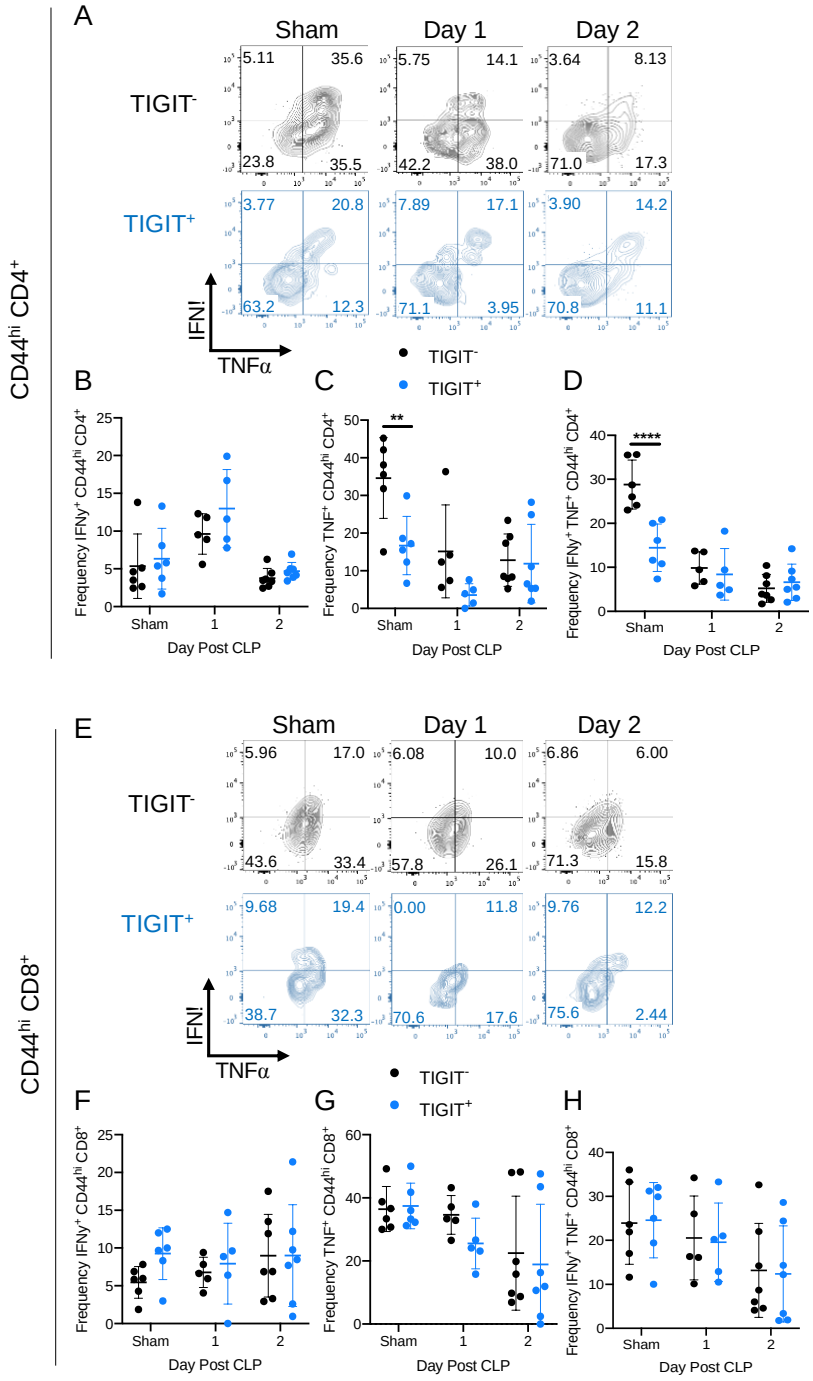

Figure 2.12: TIGIT is not associated with the impairments observed in cytokine production

CLP and sham surgery ("sham") were performed on wild type mice. One and two days after surgery, splenocytes were harvested for stimulation with PMA/Ionomycin or incubated without stimulation. Following incubation, samples were stained for TIGIT, IFN-γ and TNF-α and assessed by flow cytometry. (**A**) Representative flow cytometric plots of IFN-γ (y-axis) and TNF-α (x-axis) production by TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells following

surgery. (**B**) The frequency of IFN-γ producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells following surgery. (**C**) The frequency of TNF-α producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells following surgery. (**D**) The frequency of IFN-γ and TNF-α co-producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD4$^+$ T cells following surgery. (**E**) Representative flow cytometric plots of IFN-γ (y-axis) and TNF-α (x-axis) production by TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells of wild type mice following sham or CLP surgery. (**F**) The frequency of IFN-γ producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells following surgery. (**G**) The frequency of TNF-α producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells following surgery. (**H**) The frequency of IFN-γ and TNF-α co-producing TIGIT$^-$ (black) and TIGIT$^+$ (blue) CD44^{hi} memory CD8$^+$ T cells following surgery. Data are representative of two experiments with n=5-7 mice per group. $^{**}p<0.01$, $^{****}p<0.0001$.

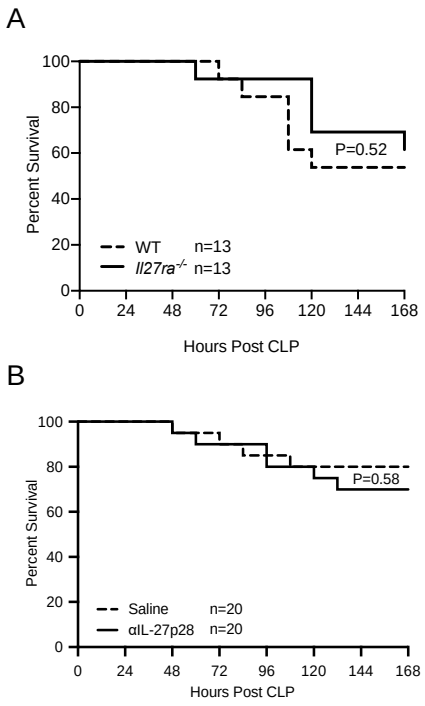

Figure 2.13: IL-27 signaling does not impact the survival of septic mice
(**A**) Wild type (WT, dotted line) and *Il27ra*$^{-/-}$ (KO, solid line) mice underwent CLP and were followed for 7 days for survival. Each group contained 13 animals and was age and gender matched. (**B**) Wild type mice underwent CLP and received either saline (dotted line) or anti-IL-27 neutralizing mAb (solid line). Mice were followed for survival for 7 days following surgery. Each group contained 20 animals and was age and gender matched. All data shown are pooled from 2-3 independent experiments.

CHAPTER 3: CONCLUSION

Recent studies show that the cytokine IL-27 induces co-inhibitory marker expression on T cells. Their induction is most prominent in the setting of chronic viral infections and cancer and include PD-1 and TIGIT. PD-1 and TIGIT also associate with T cell dysfunction during sepsis, so I hypothesized that IL-27 causes their upregulation. As memory T cells are particularly susceptible to this dysfunction, we focused on the link between IL-27, PD-1, and TIGIT in these cells. Using a mouse model of polymicrobial sepsis induced by cecal ligation and puncture (CLP), I was able to understand what these T cells might experience in humans with sepsis.

While I found evidence that IL-27 induces TIGIT expression on memory $CD4^+$ T cells, IL-27 was not associated with TIGIT expression on $CD8^+$ memory T cells during sepsis. In addition, IL-27 was not associated with PD-1 expression on memory $CD4^+$ or $CD8^+$ T cells during sepsis. Mice lacking IL-27 signaling did not have alterations in cytokine production, memory T cell apoptosis, or Treg frequency compared to wild type mice. These phenotypic results contrast with the findings in earlier studies of chronic infection and cancer and suggest that IL-27 functions in a context and/or disease-state dependent manner (summarized in Figure 3.1).

One context that may affect the T cell response to IL-27 is the location of the T cell receiving the signal within the body. A T cell in the circulation sees a different microenvironment (cytokines, chemokines, etc.) that cause differences in effector function and maintenance compared to a tissue resident T cell. Therefore, IL-27 may have a different effect on T cells in the circulation compared to the tissues. The study that described the regulation of co-inhibitory markers (including TIGIT and PD-1) used primarily tissue resident cells. In the setting of cancer, the authors used T cells infiltrating

the tumor, while the chronic infection data came from a previously published dataset that used T cells from the liver, lungs, blood, and brain (Chihara, Madi et al. 2018). This means that the microenvironment of T cells in those settings is fundamentally different from the environment that circulating T cells see.

The idea that the sepsis microenvironment differentially affects tissue resident and circulating T cells is not new. Previous studies found greater alterations in T cells from the circulation compared to tissue resident cells; both the number and effector function of circulating T cells is reduced compared to tissue resident cells (Cavaillon and Annane 2006, Danahy, Anthony et al. 2017). This is not to say that tissue resident cells are unimpaired by sepsis, but that tissue resident cells are more insulated from the effects of systemic dysfunction in the immune system. This insulation might reduce cytokine signaling that would otherwise compete with IL-27 signaling and dilute its impact on the T cell response.

In the present study, I did not assess tissue resident memory T cells or effector memory T cells that migrated the site of infection. By focusing on T cells in the spleen, I primarily assessed central and effector memory T cells. I did not distinguish between these two populations, so it is possible that IL-27 has a differential impact depending on the phenotype of memory T cell. Future studies are necessary to determine if the effects of IL-27 signaling are similar among central and effector memory T cell populations. Further studies should also determine whether sepsis induces similar effects in tissue resident memory T cells as observed during cancer and chronic viral infection.

While I found that memory cells expressing IL-27Rα continue to produce inflammatory cytokines, it is unclear whether they contribute to clearing the infectious insult during sepsis. While there was a baseline reduction in IFNγ production by memory

$CD4^+$ T cells in *Il27ra*$^{-/-}$ mice, sepsis did not further reduce its production and the deficiency was not associated with a reduction in circulating IFNγ at any time point. There was no difference in TNF production between wild type and knockout mice, but in wild type mice we saw an inverse relationship between TIGIT expression and TNF production in memory $CD4^+$ T cells at baseline. Following CLP, TNF production significantly declined within $TIGIT^-$ memory $CD4^+$ T cells but remained unaltered within the $TIGIT^+$ population. It is possible that the increase in frequency of TIGIT expressing cells accounts for this difference, but it is also possible that these cells change their phenotype based on the inflammatory conditions seen in sepsis. Further studies comparing wild type and TIGIT knockout mice are necessary to better understand the relationship between TIGIT and TNF production.

Previous studies indicated that IL-27 exerts much of its immunomodulatory effects through the induction of Tregs. However, in this study IL-27 was not linked to any alterations in the Treg compartment, as mice lacking IL-27Rα had similar frequencies of Treg compared to wild type mice. In addition, the frequency of Treg increased in both wild type and knockout mice, suggesting that other cytokines or environmental signals play a larger role in the relative increase of Treg during sepsis. The most likely explanation for the increase in Treg during sepsis is a decrease in the frequencies of other T cell populations—if IL-27 further increased Treg frequencies there should have been a stark difference in the frequency observed between wild type and knockout mice The failure to observe a difference in Treg frequency is not inconsistent with previous studies on the role of IL-27 in the regulation of co-inhibitory markers. While some studies linked IL-27 to the induction of co-inhibitory markers on Treg, other studies found similar regulation within T effector and helper subsets.

Given the relationship between IL-27 and other cytokines such as IL-17 and IL-33 (discussed in the introduction), the cytokine milieu of the circulation may alter the ability of IL-27 to induce the expression of co-inhibitory markers. Since T cells express hundreds to thousands of different receptors, no signal happens in isolation. While IL-27 inhibits the Th17 and Th2 response under normal conditions, increases in circulating IL-17 and IL-33 may compete with the downstream signaling processes with IL-27 and result in disruption or redundancy of downstream gene expression. This might allow IL-27 to continue to induce TIGIT expression while IL-27 induced PD-1 expression is supplanted by stronger signals from other cytokines. This signaling redundancy would help protect the host from excessive inflammation in the absence of other T cell regulatory pathways. Future research assessing the signaling in memory T cells of both wild type and *Il27ra*$^{-/-}$ mice are necessary to determine how IL-27 impact the downstream gene expression of TIGIT, PD-1, and other co-inhibitory molecules in the context of sepsis.

Previous studies noted a significant improvement in survival when IL-27 signaling was blocked, but we were unable to replicate these results. Although different models of sepsis were used, and with different severities, one major difference was the reagents used to block IL-27 signaling. Early studies used polyclonal antibodies to neutralize circulating IL-27 and soluble IL-27Rα. In contrast, our study used a monoclonal antibody specific to IL-27p28, one of the subunits of IL-27 (the other is EBI3, shared with IL-35). The expression of the subunits are regulated independently of one another, and the IL-27p28 subunit can be released independently in mice (Bosmann, Russkamp et al. 2014, Yan, Mitra et al. 2016, Kilgore, Pennock et al. 2020), but not humans (Pflanz, Timans et al. 2002, Muller, Friedl et al. 2019). The site of binding for the previously used reagents is

not clear, making it possible that they more effectively bound IL-27 and IL-27p28 than the monoclonal antibody targeting IL-27p28 used in this study.

Our study measured circulating IL-27p28 levels, as a surrogate for dimeric IL-27, so it is possible that EBI3 (and dimeric IL-27) did not increase to a similar extent. This could have resulted in the false impression that overall IL-27 was increasing just from seeing IL-27p28 increase. In addition, some of the IL-27p28 produced by the cells may be binding through mechanisms other than the IL-27Rα and diluting the effect caused by IL-27. Since most commercially available IL-27 ELISA kits in mice are specific for the IL-27p28 subunit, circulating IL-27 would appear increased even if the dimeric cytokine was not. In contrast, cancer and chronic infection could be different from sepsis by prioritizing the production of the dimeric cytokine rather than IL-27p28. As humans cannot express IL-27p28 on its own (Pflanz, Timans et al. 2002, Muller, Friedl et al. 2019), it will be important to use mice that do not independently produce IL-27p28 to assess T cell function (Muller, Friedl et al. 2019).

It is also unclear if the gene signature used in the studies linking IL-27 to the expression of co-inhibitory markers during cancer and chronic viral infection reflect IL-27 and/or IL-27p28 signaling. The literature does not specify a difference in signaling outcomes from either cytokine receptor, making it unclear whether the findings in those studies were due to IL-27 or merely the IL-27p28 subunit. If the gene signatures reflected one situation and not the other, this may also help explain the discrepancy in the sepsis disease state compared to the other settings. To determine whether IL-27p28 or IL-27 induces of the co-inhibitory markers, humanized mice that express full IL-27 would eliminate the impact of IL-27p28. Likewise, the site where IL-27p28 and EBI3 join to

form IL-27 could be altered to prevent the release of full IL-27 from the cell, allowing for analysis of IL-27p28 on its own.

Another important consideration for our results is the percentage of IL-27Rα that was bound by circulating IL-27 *in vivo*. T cells can release a soluble version of IL-27Rα that acts as a sink to reduce the concentration of circulating IL-27. While the overall concentration of IL-27p28 appeared increased via ELISA, this does not mean that IL-27 circulates long enough to occupy receptors on T cells. Many other cells express IL-27Rα, so any cells expressing a higher density of IL-27Rα on their surface would compete for IL-27 signaling with T cells. In addition, some of the cells that release IL-27 also express the IL-27Rα (particularly dendritic cells), allowing it to be self-regulatory.

Based on our new understanding of IL-27, there is no clear rationale to pursue the use of IL-27 blockade as a sepsis therapeutic. Although IL-27 concentrations increase in many sepsis patients, it does not appear to be significantly associated with alterations in lymphocyte function or overall mortality during sepsis. This is not completely surprising, since IL-27 concentrations have not consistently associated with sepsis outcomes in humans and rejected for use in diagnostic biomarker testing. However, the studies that found a clear link between IL-27 and co-inhibitory marker expression suggested that IL-27 would be a useful upstream target to prevent T cell exhaustion from ever developing. Based on our findings, sepsis induced alterations in circulating T cells do not reflect what was seen in the previous studies that utilized tissue resident T cells.

Although clinical trials targeting inflammatory cytokines have failed, it does not appear that the answer is to instead target anti-inflammatory cytokines like IL-27. This is exemplified by findings that IL-10 blockade increases sepsis mortality (Kato, Murata et al. 1995, van der Poll, Marchant et al. 1995). While there have been many positive animal

studies showing the beneficial effect of targeting cytokines during sepsis, this has not translated into improvements in clinical treatment; no clinical trials so far have led to an approved therapeutic. The reasons behind this are multifactorial and partially stem from a failure to consider the interaction between individual cytokines and the larger cytokine milieu. In addition, the cytokine milieu varies between septic patients, making it difficult to distinguish any benefit in large studies of heterogenous patients. Therefore, combinations of existing co-inhibitory blockade drugs seem more promising, as they have already shown minor benefits in early clinical trials in sepsis patients and were met with success in cancer patients (Francois, Jeannet et al. 2018, Hotchkiss, Colston et al. 2019).

Although the failure of cytokine-based therapy in septic patients has been disappointing, recent phase I clinical trials (such as IL-7 infusion) have shown the potential benefit of immunomodulation (Francois, Jeannet et al. 2018). However, before cytokines are considered as therapeutic targets for sepsis, further work needs to be done to define the alterations that occur across the cytokine milieu during sepsis and distinguish how individual cytokines interact and modulate the effects of one another. However, there are clearly other factors regulating T cell function that are unknown. The interaction between cytokines and other signaling molecules and memory T cells must be understood in the context of sepsis before targeted therapies can succeed in lowering sepsis mortality. Even then, it is possible that only certain subgroups of sepsis patients benefit from any given therapy, due to differences in patient immune responses, sources of infection, and other factors.

The present model also only considered what occurs in immunologically naïve mice. Humans are far from immunological naivety, and therefore have a significantly enhanced memory T cell compartment that is not reflected in most murine studies. Future

studies should also determine the impact of IL-27 signaling in mice with a memory T cell compartment that better reflects what is seen in humans. Our laboratory developed a model of "memory mice" by sequentially infecting naïve C57BL/6 mice with *Listeria monocytogenes* followed by lymphocytic choriomeningitis virus (LCMV) (Xie, Chen et al. 2019). The sequential infection results in substantially increased frequencies of memory CD8 T cells (from 18% to 55%) and increased memory CD4 T cells (from 20% to 38%) (Xie, Chen et al. 2019).

Following CLP-induced sepsis, the frequency of memory T cells expressing the co-inhibitory markers 2B4 (Xie, Chen et al. 2019) and TIGIT (Zhang et al. in revision) increases, as does the expression of the co-stimulatory marker CD28 (Sun, Chen et al. in revision). The upregulation of 2B4 was tied to the apoptosis of memory CD8 T cells in both mice and humans (Xie, Chen et al. 2019). In addition, 2B4 knockout mice had significantly improved mortality compared to wild type mice following CLP (Xie, Chen et al. 2019). In contrast, treating memory mice with anti-TIGIT significantly reduced sepsis survival (Zhang et al. in revision). Anti-TIGIT also contributed to the apoptosis of memory T cells in this model. Even though CD28 is co-stimulatory and not co-inhibitory, anti-CD28 improved survival in memory mice (Sun, Chen et al. in revision). Anti-CD28 exerted these effects by inducing increased production of IL-10 in $FoxP3^+$ regulatory T cells (Sun, Chen et al. in revision).

Based on these results, it is unclear what effect IL-27 would have in the memory mouse model compared to the present study in naïve mice. It is possible that the unnatural memory T cell compartment observed in previously naïve mice may impact the response of the mice to the cytokine. In addition, memory T cells may play a different level of importance if there are more present. In the current setting of few memory T cells,

they may not have played a key role in survival just because they were such a rare cell population compared to naïve T cells.

In this study, we aimed to address the function of IL-27 in sepsis-induced T cell impairment and exhaustion. In contrast to studies in cancer and chronic viral infections, we found that IL-27 had a limited impact on co-inhibitory expression on memory T cells in the circulation. However, we found that IL-27 induced TIGIT expression on memory $CD4^+$ T cells during sepsis, uncovering the regulatory role that IL-27 plays in the regulation of TIGIT on non-T regulatory cells during sepsis.

Figures

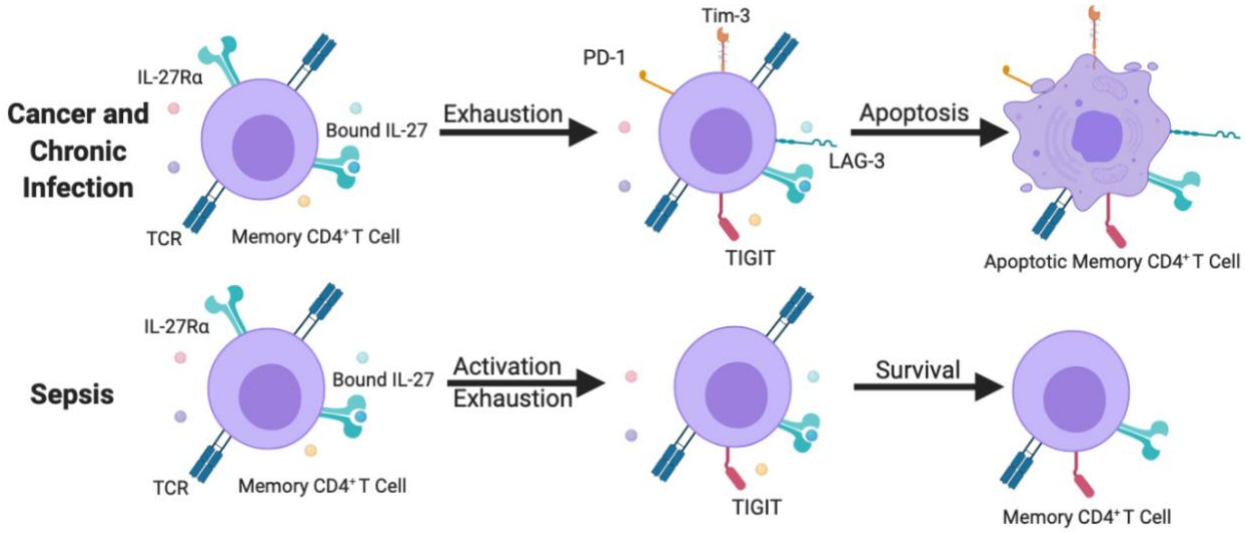

Figure 3.1 Impact of IL-27 signaling during chronic viral infections and cancer versus sepsis
In the setting of cancer and chronic infections, IL-27 signaling causes T cells to upregulate the co-inhibitory markers PD-1, Tim-3, LAG-3, and TIGIT in a TCR independent manner. These markers make T cells exhausted and trigger them to undergo programmed cell death (apoptosis). The apoptosis of the T cells leads to impairments in resolving the infection and clearing tumors. In contrast, exposure to IL-27 during sepsis results in an activated/exhausted phenotype of T cells characterized by the upregulation of TIGIT. During sepsis, these T cells do not undergo apoptosis, and remain alive and functional.

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