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Immunophenotyping and Functional Assessment of Antiviral CD8+ T Cells in a CLP Mouse Model of Immunosuppression

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Abstract

Sepsis causes millions of deaths every year with 70% of them being attributed to the immunosuppressive phase of the syndrome, which occurs shortly after the onset and leads to a severe immunodeficiency. Currently, there is not enough known about antigen specific CD8+ T cell responses during the immunosuppressive phase of sepsis and the studies that have been done, have shown controversial findings. In my thesis research, I have utilized the cecal ligation and puncture (CLP) model of polymicrobial sepsis – which closely mimics the progression of human sepsis – to study antigen specific CD8+ T cell responses by examining their exhaustion phenotype and functionality. Contrary to my initial thoughts, I had discovered that primary and recall CD8+ T cell responses are enhanced during protracted sepsis within the spleen. I demonstrated that antigen specific CD8+ T cells were higher in frequency within the septic spleen; however, they possessed similar absolute numbers compared to the sham control. Additionally, these antigen specific CD8+ T cells had an increased functionality as demonstrated by their increased ability to produce IFN-γ and granzyme B, while maintaining their polyfunctionality. Furthermore, I also discovered that antigen specific CD8+ T cells within the septic spleen were phenotypically more exhausted as these cells had an enhanced co-expression of the different exhaustion markers examined. Herein, I conclude that sepsis-induced immunosuppression leads to an enhanced antigen specific CD8+ T cell response within the spleen, which is an important finding as it currently contrasts the commonly thought notion that CD8+ T cell responses are dampened during protracted sepsis. This research may alter the way we think about sepsis-induced immunosuppression as I have shown that antigen specific CD8+ T cell responses were enhanced, rather than dampened.

Keywords: Sepsis-induced immunosuppression, CLP, CD8+ T cell, Immunophenotyping, CD8+ T function, Spleen

Summary for Lay Audience

Sepsis is a severe syndrome, which causes millions of deaths every year and is currently one of the leading causes of death in the intensive care unit (ICU). Sepsis is characterized as a syndrome that has two distinct phases. The onset of sepsis is characterized by a hyper-inflammatory state, which is relatively short-lived and causes significant mortality; however, the subsequent immunosuppressive phase causes the majority of deaths from this deadly syndrome in developed countries with better critical care facilities. During the second phase of the syndrome, patients are often quite susceptible to infections caused by either newly encountered or previously encountered microbial species. My thesis research has focused on a specific immune cell subset named CD8+ T cells, which are critical for responding to a variety of disease-causing organisms, including new and old microbes. CD8+ T cells are highly specific for microbes that are encountered, and they develop into memory CD8+ T cells, which deal with an infection that has been previously encountered. During sepsis, these cells are reportedly impaired in their ability to deal with microbes. I set out to better understand these cells during sepsis-induced immunosuppression by looking at their frequencies, absolute numbers, cell surface expression of different molecules, which would give us insight into their activation state and function. In my research, I used a clinically relevant mouse model of sepsis-induced immunosuppression and examined specific antiviral CD8+ T cell responses. Contrary to what has been thought previously, I found that memory CD8+ T cells specific for Influenza A Virus (IAV), to my surprise, were higher in frequency, showed signs of increased activation and exhaustion, and were more functionally active during a memory response within the spleen. This was also recapitulated when I investigated primary CD8+ T cell responses to newly encountered IAVs. Currently, it is viewed that CD8+ T cell responses are less activated and less functional within the immunosuppressive phase of sepsis; however, herein I show that they are more activated and functional, which contradicts the current notion.

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my attention to detail and the way I think about different experiments and in designing experiments. I have learned very much from Dr. Haeryfar and all of the wonderful people that he has employed; I consider myself to me extremely lucky to have been able to work alongside him and the members of his lab.

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List of Abbreviations

ACK = Ammonium-Chloride-Potassium

ACVS = Animal Care and Veterinary Services

ALI = Acute lung injury

APACHE II = Acute physiology and chronic health evaluation II

aPTT = Activated partial thromboplastin clotting time

ARDS = Acute respiratory distress syndrome

B2M = β2-microglobulin

B6 = C57BL/6

BALF = Bronchoalveolar lavage fluid

Bcl-2 = B-cell lymphoma 2

BFA = Brefeldin A

BM = Bone marrow

BrdU = Bromodeoxyuridine

BTLA = B and T lymphocyte attenuator

CASP = Colon ascendens stent peritonitis

CCL… = C-C chemokine ligand type …

CCR… = C-C chemokine receptor type …

CD… = Cluster of differentiation…

CD8+ Trm = Tissue-resident memory CD8+ T cells

CDC = Centers for Disease Control and Prevention

cDCs = Conventional dendritic cells

CIITA = Class II transactivator

CLP = Cecal ligation and puncture

CMV = Cytomegalovirus

cRPMI = Complete Roswell Park Memorial Institute

CSFE = Carboxyfluorescein N-succinimidyl ester

CTCF = CCCTC-binding factor

CTLA-4 = Cytotoxic T-lymphocyte-associated protein 4 or CD152

DAMPs = Damage-associated molecular patterns

DCs = Dendritic cells

DIC = disseminated intravascular coagulation

DMSO = dimethyl sulfoxide

DNA = Deoxyribonucleic acid

DR5 = Death receptor 5 or TRAIL receptor 2 (TRAILR2)

EAE = Experimental autoimmune encephalomyelitis

EBV = Epstein-Barr Virus

EDTA = Disodium Ethylenediaminetetraacetic acid

Fas = Fas receptor or CD95

FasL = Fas ligand or CD95L

FBS = Fetal bovine serum

Fc = Fragment crystallizable

FcRγ II/III = Fc receptor γ II/III or CD32/CD16

FDA = Food and Drug Administration

FIP = Fecal-induced peritonitis

FoxP3 = Forkhead box P3

FSC-A = Forward scatter-area

FSC-H = Forward scatter-height

FSC-W = Forward scatter-width

GISRS = Global Influenza Surveillance and Response System

GLUT1 = Glucose transporter 1

gMFI = Geometric mean fluorescence intensity

GP_{33} = glycoprotein$_{33\text{-}41}$ peptide

HA = Haemagglutinin

HBV = Hepatitis B virus

HHV-6 = Human Herpesvirus 6

HLA = Human leukocyte antigen

HLA-DR = Human leukocyte antigen DR allomorph

HSV = Herpes Simplex Virus

I.P. = Intraperitoneal

IAV = Influenza A Virus

ICAM1 = Intercellular adhesion molecule 1

ICS = Intracellular staining

ICU = intensive care unit

IFN-γ = Interferon γ

IFN-α = Interferon α

IFN-γR1 = Interferon-γ receptor 1

IFN-I = Type I interferon

IFNAR = Interferon-α/β receptor

IL-… = Interleukin …

IL-1β = Interleukin 1β

IL-1ra = Interleukin 1 receptor antagonist

IL-2Rα = Interleukin 2 receptor α or CD25

iNOS = Inducible nitric oxide synthase

INR = International normalized ratio

IPAC = Infection Prevention and Control Canada

JC and BK = Polyomaviruses

KLRG1 = Killer cell lectin-like receptor subfamily G member 1

LAG-3 = Lymphocyte activation gene-3 or CD223

LCMV = Lymphocytic choriomeningitis virus

LCMV-Arm = LCMV-Armstrong

LPS = Lipopolysaccharide

M = Matrix protein

M1 = Matrix protein 1

$M1_{128}$ = Matrix protein $1_{128\text{-}135}$ peptide

M2 = Matrix protein 2

MAP = Mean arterial pressure

MCMV = Murine cytomegalovirus

MDCK = Madin-Darby canine kidney cells

MFI = Mean fluorescence intensity

MHC = Major histocompatibility complex

MIP-1β = Macrophage inflammatory protein-1β

mLN = Mesenteric lymph nodes

MOG = Myelin oligodendrocyte glycoprotein

mRNA = Messenger ribonucleic acid

MSS = Murine sepsis score

NA = Neuraminidase

NK = Natural killer cells

NLR = NOD-like receptors

NLRP3 = NLR family pyrin domain containing 3

NO = Nitric oxide

NOD = Nucleotide-binding and oligomerization domain

NP = nucleoprotein

NP_{366} = Nucleoprotein$_{366-374}$ peptide

NS1 = Non-structural protein 1

NS2 = Non-structural protein 2

$NS2_{114}$ = Non-structural protein $2_{114-121}$ peptide

OVA = Ovalbumin

PA = Polymerase acidic protein

PA_{224} = Polymerase acidic protein$_{224-233}$ peptide

PAMPs =Pathogen-associated molecular patterns

PB1 = Polymerase basic protein 1

PB1-F2 = Polymerase basic protein 1-F2

$PB1\text{-}F2_{62}$ = Polymerase basic protein-$F2_{62-70}$ peptide

$PB1_{703}$ = Polymerase basic protein $1_{703-711}$ peptide

PB2 = Polymerase basic protein 2

PBS = Dulbecco's phosphate-beffered saline

PD-1 = Programmed cell death protein 1

PD-L1 = Programmed cell death ligand 1

pDCs = plasmacytoid dendritic cells

PECs = Peritoneal exudate cells

PFU = Plaque forming units

PHA = Phytohaemagglutinin

PMA = Phorbol 12-myristate 13-acetate

Poly-A tail = Polyadenylated tail

PR8 = A/Puerto Rico/8/1934/H1N1

pre-mRNA = Precursor messenger ribonucleic acid

PRRs = Pattern recognition receptors

qSOFA = Quick Sequential Organ Failure Assessment

RdRP = RNA-dependent RNA polymerase complex

RIG-I = Retinoic acid-inducible gene I

RNA = Ribonucleic acid

ROS = Reactive oxygen species

SBP = Systolic blood pressure

SCLO1a1 = Organic anion transporter protein 1

SD = Standard deviation

SLC10a1a = Sodium/bile acid co-transporter

SOFA = Sequential Organ Failure Assessment

SSC-A = Side scatter-area

STAT5 = Signal transducer and activator of transcription 5

T-bet = T-box expressed in T cells (T-box transcription factor 21 (TBX21))

TAP = Transporter associated with antigen processing

$TCID_{50}$ = Median tissue culture infectious dose

TCR = T cell receptor

Tfh = T follicular helper cells

TGF-β = Transforming growth factor β

Th1 = T helper type 1

Th2 = T helper type 2

TIGIT = T cell immunoreceptor with Ig and ITIM domains

TIL = Tumor infiltrating lymphocyte

TIM-3 = T cell immunoglobulin and mucin domain-containing protein 3

TLR = Toll-like receptor

TNF-α = Tumor necrosis factor α

TNFR = Tumor necrosis factor receptor

TRAIL = Tumor necrosis factor-related apoptosis-inducing ligand

TTV = Torque Teno Virus

US = United States

VCAM1 = Vascular cell adhesion molecule 1

VISTA = V-domain Ig suppressor of T cell activation

WBC = White blood cell

WHO = World Health Organization

Chapter 1: Introduction and Literature Review

1.1 Sepsis: Overview

1.1.1 Impact on Human Health

Sepsis is currently defined as a life-threatening organ dysfunction due to a dysregulated host immune response caused by an infection[1]. Currently, this is the third definition of sepsis to date with previous definitions focused on the acute inflammatory processes. Revision of the definition was needed as further evidence has shown that sepsis is not just an inflammatory response but encompasses anti-inflammatory processes as well[2]; additionally, revision was required to better characterize and diagnose the disease. Within the literature, it has been demonstrated that sepsis causes both inflammatory and anti-inflammatory responses at the initial septic event; however, the net immunological response depends on the dominant response at that time[3]. Sepsis is characterized as a biphasic syndrome as there are two phases to sepsis, there is the acute hyper-inflammatory phase and the subsequent chronic immunosuppressed phase; however, it is important to note that these phases may not be experienced in all septic patients as this syndrome is quite heterogeneous[2,4,5] (Figure 1). For example, some patients may present with excessive uncontrollable inflammation leading to early death, while others may experience immunosuppression at the onset of sepsis with little inflammatory responses. Hence, when trying to provide immunotherapy to septic patients, it is important to consider each individuals immunological response as it is quite heterogeneous.

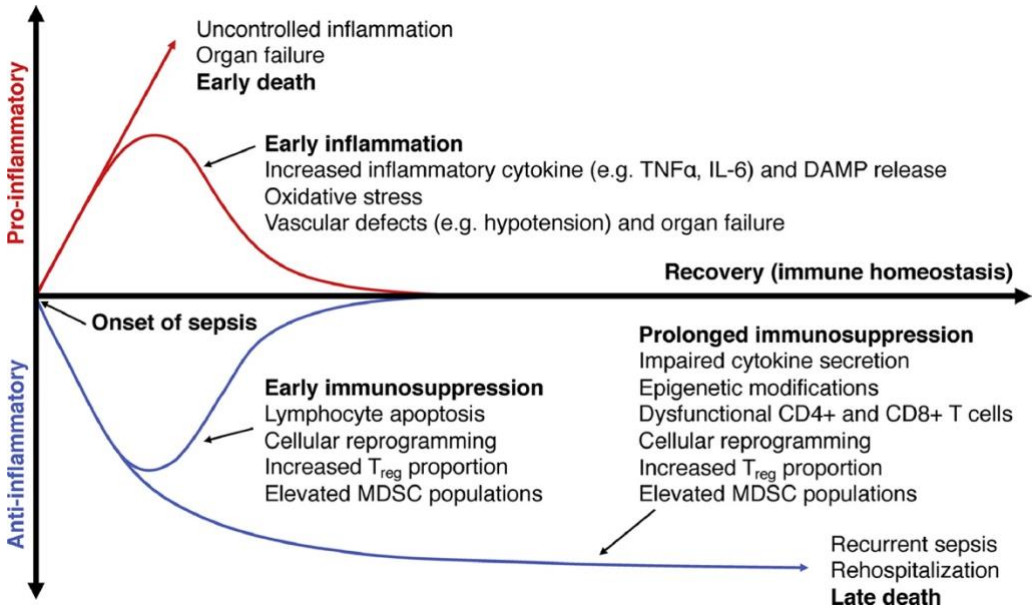

Figure 1. Immune dysregulation in sepsis. Figure adapted from "Exploring the pathophysiology of post-sepsis syndrome to identify therapeutic opportunities", Bouma H.R., Van Der Slikke E.C., An A.Y., Hancock R.E.W.[2], used under license CC BY 4.0 'https://creativecommons.org/licenses/by/4.0/'.

In 2017, there were 49 million cases of sepsis world-wide with an associated mortality rate of ~20%, which accounts for 9.8 million people[1]. These cases and deaths associated with sepsis reflect what is seen during the acute phase of sepsis. Despite the deaths in the hyperinflammatory phase being significant, they are minimal compared to the death rate during the protracted phase. It has been described that sepsis displays a trimodal pattern of death rate distribution with the initial peak representing the onset of sepsis, the second peak occurring weeks after, and the final peak occurring after the patient is discharged from the hospital[6,7]. The magnitude of the peaks increases over the course of sepsis, and the final peak occurs 60 to 90 days after the initial septic event with death rates following the same trend. Moreover, five-year mortality rates for septic patients is abysmal with 60% of patients succumbing to the disease[7], which is considerably higher than the ~20% mortality reported initially. Alarmingly, the precise causes of this apparent increase in mortality over time are not fully understood. However, several groups have suggested that co-morbidities and persistent organ dysfunction work together to create a state of chronic disease, which encompasses immunosuppression, immune dysfunction, and persistent low-grade

inflammation[8,9]. Additionally, patients affected by the chronic phase of sepsis experience a diminished quality of life with systemic physiologic and cognitive impairments[6,10,11].

Despite sepsis being widely known in the medical profession, currently, there are no Food and Drug Administration (FDA) approved curative treatments to alleviate mortality and morbidity[12]. There have been over 100 clinical trials to find a cure for sepsis; however, they have all failed[12]. One potential reason for this downfall was the targeting strategy used in the previous clinical trials. Most experimental drugs tested to date have been used during the initial hyperinflammatory phase to suppress the immune system; however, in doing so, they failed to consider that there are often both inflammatory and anti-inflammatory processes during the initial phase. Drugs targeting inflammatory responses during the first phase would result in (1) exacerbation of the post-septic immunosuppressive environment causing further immunosuppression and (2) preventing early inflammatory responses necessary for dealing with and clearing the primary infection leading to a persistent infection, which can harm the host and increase the initial mortality seen within sepsis.

1.1.2 Etiology and Pathophysiology

Etiology of sepsis

Sepsis can be caused by multiple different pathogens and insults such as bacteria, viruses, fungi, parasites, traumatic insult, and burns. The most common cause of sepsis is due to a bacterial infection with Gram-negative bacteria being the most frequent cause of sepsis; however, there is evidence to suggest that Gram-positive organisms causing sepsis have increased in frequency over time and are now almost as common as Gram-negative infections[13–16]. During the treatment for sepsis, physicians administer broad-spectrum antibiotics, and this is problematic as this can result in increased bacterial resistance over time[17,18]. Furthermore, increased bacterial resistance to broad-spectrum antibiotics has been shown to prolong septic patient's length of hospital stay and duration of mechanical ventilation[16,17,18], which is problematic for septic patients because if bacterial resistance continues to rise over time, there may be no effective strategy to treat the initial infection causing the sepsis.

To elucidate the most common pathogens causing sepsis, there was a study by Vincent et al.[22], which examined the prevalence of different infections causing sepsis at the time of ICU admission. It was reported that Gram-negative bacteria were associated with 62.2% of the infections, while Gram-positives were associated with 46.8% of the infections; among these patients, there were septic events caused by the co-infection with Gram-negative and Gram-positive bacterial species. The predominant Gram-negative bacterial species causing sepsis were *Pseudomonas spp.* (19.9%), *Escherichia coli* (16%), *Klebsiella spp.* (12.7%), *Acinobacter spp.* (8.8%) and *Enterobactor spp.* (8.8%), while the predominant Gram-positive bacterial species causing sepsis were *Staphylococcus aureus* (21.5%), *Enterococcus spp.* (10.9%), *Streptococcus epidermis* (10.8%), *Streptococcus pneumoniae* (4.1%). Furthermore, different fungi can also cause sepsis with *Candida spp.* being attributed to 17% of infections and *Aspergillus* causing 1.4%. Parasites account for 0.7% of sepsis causing infections.

Risk factors affecting the outcome of sepsis

In sepsis, the type of pathogenic organism is an important factor to consider as it determines patient outcome. In a large meta-analysis of 510 studies[23], the authors reported that Gram-negative bacteremia is associated with higher mortality compared to Gram-positive bacteremia, on average. For example, bacteremia caused by the most prevalent Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* and *Pseudomonas spp.*, respectively) had a mortality rate of 25% and 31%, respectively. Despite the significant death associated with Gram-negative and Gram-positive bacterial species, fungal infections tend to be more deadly than bacterial infections. For instance, fungal infections due to *Candida spp.* had a bloodstream infection mortality rate of 43%, which is higher than the previously mentioned bacteremia mortality rate.

One other factor to consider is the site of infection as it has been demonstrated that risk of death due to sepsis is dependent on the site of infection[24]. For instance, in the meta-analysis study[23], pneumonia caused by the most prevalent Gram-positive and Gram-negative bacteria resulted in 32% and 57% mortality, respectively. Respiratory tract infections are the most common site of infection leading to sepsis and is associated with the highest mortality[25]. Furthermore, infections stemming from other sites are important; however, they show different mortality rates. For example, within the same meta-analysis[23], it was shown that the most prevalent Gram-positive and Gram-negative bacterial infections within the urinary tract led to 9% and 0% mortality, respectively. Moreover, within another study[26], patients acquiring sepsis due to urinary tract infections had a 28-day mortality of 20.9%, while patients acquiring sepsis from a pulmonary infection had a 28-day mortality rate of 33.6%.

Another important factor to consider is the source of infection, whether it is community- or hospital-acquired. It has been discovered that sepsis mortality due to hospital-acquired infections causes 45% mortality, and this is due to patients acquiring an infection within the ICU, which ultimately leads to sepsis. Community-acquired infections leading to sepsis, which is acquired outside of the hospital is associated with a mortality rate of 15%[27–29]. Taken together, sepsis is caused by multiple different infectious agents; however, the

type of infectious pathogen, the site of the infection and the route of acquiring the infection are important factors that affect patient outcome during sepsis.

Furthermore, in addition to the aforementioned factors influencing septic patient outcome, there are also a few other factors that are important to consider such as age, co-morbidities, and the timing of presentation[30–32]. In terms of age, within a study by Yao et al.[31], it was demonstrated that septic patients over the age of 60 have a significantly higher mortality rate compared to septic patients under the age of 60, 52.1% vs 34.2%, respectively. Furthermore, within a paper by Wang et al.[30], the authors have shown that age was an independent risk factor for the outcome of septic patients. In terms of co-morbidities, Wang et al.[30] have demonstrated that various pre-existing conditions significantly increased the risk of mortality from sepsis. For example, pre-existing malignancies and cardiovascular disease significantly increased the risk of mortality for septic patients by two-fold; the authors have shown that septic patients with these underlying conditions have a 2.43 and 2.15 odds ratio score, respectively, for the risk of mortality from sepsis. In terms of the timing of presentation, within a study by Liu et al.[32], the authors have discovered that the clinical presentation of sepsis (i.e. signs, symptoms and pace of onset) is extremely heterogeneous among septic patients and this impacts the timing of treatment. For example, pain was associated with later antibiotic intervention, while fever was associated with earlier antibiotic therapy and lower mortality from sepsis. Furthermore, it has been demonstrated that earlier therapeutic intervention is extremely important in dampening the mortality caused by sepsis[30]. For instance, administration of antibiotics within one hour of sepsis onset decreased the risk of mortality by 42%, while adequate fluid resuscitation within three hours from the onset of sepsis decreased the risk of mortality by more than 50%. Hence, considering the affect that the heterogeneity of sepsis clinical presentation has on the timing of treatment, it may be concluded that the heterogeneity of clinical presentation effects the outcome of septic patients.

Pathophysiology of sepsis

Next, I want to briefly address the pathophysiology of sepsis by examining the different dysregulated immune mechanisms that occur during sepsis. Sepsis is caused by pathogenic microbe(s) leading to the systemic activation of various pattern recognition receptors (PRRs) by different pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[33]; the latter are self-molecules that either get released or produced during infection (i.e. apoptotic cells and release of reactive oxygen species (ROS), among many others) and the former being an exogenous source (i.e. the infectious agent). Receptor signalling from PAMPs and DAMPs leads to the widespread activation of innate immune cells, but also adaptive immune cells, which results in the initiation of bacterial killing mechanisms, such as bacterial phagocytosis and the release of massive amounts of both pro- and anti-inflammatory cytokines[33]. For instance, tumor necrosis factor α, interleukin 1β, interleukin 6, interleukin 8 and interleukin 10 (TNF-α, IL-1β, IL-6, IL-8, and IL-10) are released[33]; this is not an exhaustive list as there are many others that get released. This massive surge of cytokines is typically referred to as the 'cytokine storm' and is a major contributor in the pathophysiology of sepsis[34,33].

In response to the 'cytokine storm', endothelial cells will upregulate various adhesion molecules (E-selectin, intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), etc.) leading to the binding of immune cells to the endothelial cell surface[33,35–37]. Furthermore, gap junctions between the endothelial cells of the circulatory system widen, which permits immune cells to extravasate into the different tissues[33,35–37] (i.e. infected and/or inflamed tissues). This massive infiltration of immune cells within the different tissues will lead to excessive tissue damage resulting in organ dysfunction and failure[33,35–38]. For example, the septic 'cytokine storm' is a major risk factor for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), regardless of the anatomical location of infection source[37–40]. Furthermore, within a study by Neumann et al.[37], the authors utilized a colon ascendens stent peritonitis (CASP) model of polymicrobial sepsis in mice and demonstrated that capillaries within the lungs are more permeable compared to sham mice. Moreover, the researchers have also discovered that there was a higher

presence of granulocytes, monocytes, and natural killer (NK) cells within the septic lungs with an associated increase in their capability for oxidative burst and production of serine proteases. In addition, within another study by Qi et al.[38], the authors utilized a CLP mouse model of polymicrobial sepsis and demonstrated by immunohistochemical methods that there was an increased presence of neutrophils capable of producing myeloperoxidase (MPO) with an associated increase in lung structure damage, alveolar reduction and edema within the septic lungs compared to sham mice (Figure 2).

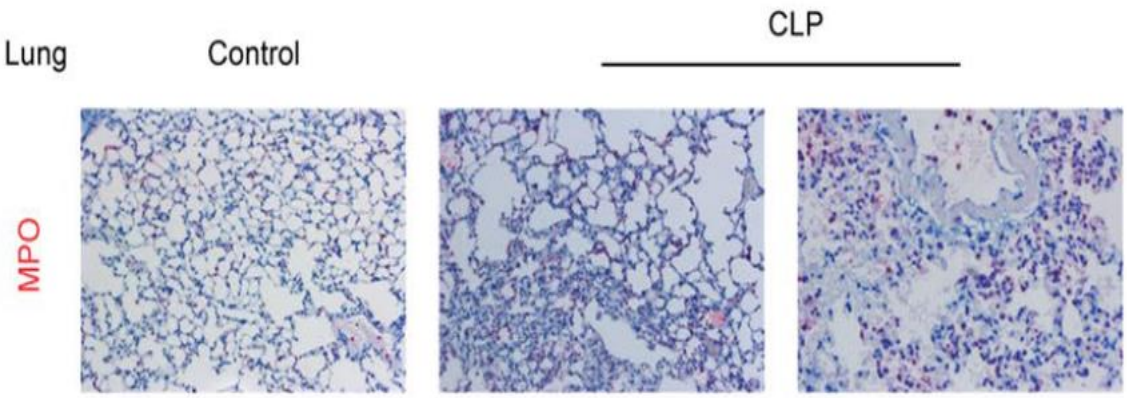

Figure 2. Immunohistochemical analysis of the septic lungs within a CLP mouse model... Changes in lungs at 96 hours post-CLP. MPO is represented by red. Figure adapted from "Identification and characterization of neutrophil heterogeneity in sepsis", Sun B., Qi X., Yu Y., Sun R., Huang J., Liu L., Yang Y., Rui T. [38], used under license CC BY 4.0 'https://creativecommons.org/licenses/by/4.0/'.

Another important aberration seen in sepsis is the dysregulation of the coagulation cascade. During sepsis, the coagulation cascade will be systemically activated, which leads to a condition known as disseminated intravascular coagulation (DIC)[41,42]. DIC leads to the blockade of small blood vessels, which is partially responsible for the organ dysfunction seen during sepsis. Another important aspect of DIC is the widespread bleeding within septic individuals. Septic patients will have excessive bleeding, which can occur in virtually any part of the body because clotting factors are used up during the widespread activation of platelets leading to a lack of clotting factors necessary for maintaining the integrity of the vasculature[41,42].

Another major contributor to the pathophysiology of sepsis is the cardiovascular dysfunction that occurs. During sepsis, nitric oxide (NO) gets secreted and contributes to vasodilation and increased vascular permeability[43–46]. Dilatation of the blood vessels results in less peripheral resistance within the circulatory system, which in turn directly affects the blood pressure by decreasing the blood pressure. Low blood pressure leads to less tissue oxygenation as it would decrease the perfusion of blood into different tissues, which ultimately leads to hypoxia. Furthermore, it has been shown that changes within the macro- and micro-vasculature can directly affect myocardial function[47]. Many causes exist for myocardial dysfunction, for example, coronary microvascular changes seen during sepsis contributes heavily to the dysfunction. Among these changes, as previously alluded to, there is a massive release of cytokines, which has been shown to affect the microvasculature in the heart[48,49]. The production of NO through the activation of inducible nitic oxide synthase (iNOS) contributes in part to these changes. NO and ROS contribute to microvascular changes by triggering apoptotic pathways in cardiomyocytes and by causing mitochondrial dysfunction[47]. Furthermore, the upregulation of NO and adhesion molecules within cardiomyocytes contributes to decreased contractibility by modifying the actin-myosin cytoskeleton[50,51].

1.1.3 Symptoms and Treatment

Symptoms and signs of sepsis

In terms of symptomology, septic and septic shock patients present with many aberrations and clinical signs[52,53]. Diagnosis for sepsis is done by the presence (probable or confirmed) of infection with the systemic manifestations outlined in Table 1. Severe sepsis is diagnosed as sepsis plus sepsis-induced organ dysfunction or hypoperfusion, which is outlined in Table 2. Septic shock is severe sepsis but with persisting hypotension despite adequate fluid resuscitation.

Patients arriving into the clinic will present with many different aberrations such as fever (38.3 °C), hypothermia (36 °C), a faster-than-normal heart rate (>90min^{-1} or more), tachypnea, altered mental state (confusion), hypotension (systolic blood pressure (SBP) <90 mmHg), a blood creatinine increase (>0.5 mg/dL), coagulation abnormalities (activated partial thromboplastin time (aPTT) >60 seconds), thrombocytopenia (platelet count <100000 uL^{-1}) and hyperlactemia (>1 mmol/L). This is not an exhaustive list; however, these are some of the major aberrations seen in sepsis. Furthermore, the Sequential Organ Failure Assessment (SOFA) is highly used for sepsis diagnosis and monitoring; it has been demonstrated that patients with a suspected infection and a score of two or higher on the SOFA scale best predicted hospital mortality[54]. The SOFA score is very intricate, so a quick SOFA (qSOFA) score was developed to make a simpler clinical screening tool, which was demonstrated to perform well in identifying adult patients with suspected infection who were expected to have poor outcomes[53]. The qSOFA measures three clinical parameters: respiratory rate (> 22 breaths/minute), Glasgow Coma Scale (<15; assesses a patient's ability to perform eye movements, speech, and motor ability) and SBP (<100 mmHg). If a patient receives a score of two or higher with qSOFA, then clinicals should assess for further organ damage, provide more therapy, and send the patient to the ICU.

In terms of severe sepsis, some of the symptoms that are typically observed are decreased urine output (0.5 mL $kg^{-1}h^{-1}$ for more than 2h despite fluid resuscitation), acute lung injury (Pao_2/Fio_2 (Ratio of arterial oxygen partial pressure to fractional inspired oxygen) <250 in absence of pneumonia or <200 in presence of pneumonia), high blood creatinine (>2.0mg/dL) and high blood bilirubin (>2.0mg/dL).

Currently, there is a new consensus on septic shock and it is now defined as a 'subset of sepsis in which circulatory, cellular and metabolic abnormalities are associated with a greater risk of mortality than sepsis alone'[55].There are two conditions that need to be met before clinicians characterize the patient as having septic shock: need vasopressor therapy to maintain a mean arterial pressure of >65 mmHg and serum lactate > 2mM, which is persistent even after adequate fluid resuscitation.

Table 1. Diagnostic criteria for sepsis. Adapted from Surviving Sepsis Campaign[52]. SBP = systolic blood pressure, MAP = mean arterial pressure, SD = standard deviation, PaO_2/FiO_2 = ratio of arterial oxygen partial pressure to fractional inspired oxygen (Horowitz index or Carrico index) to determine severity of lung injury, INR = international normalized ratio calculated as INR = (tested prothrombin time/normal prothrombin time)*international sensitivity index, aPTT = activated partial thromboplastin clotting time

Infection, documented or suspected, and some of the following:	
General variables	
	Fever (>38.3°C)
	Hypothermia (core temperature <36°C)
	Heart rate >90 min^{-1}
	Tachypnea
	Altered mental status
	Significant edema or positive fluid balance (>20 mL/kg over 24 h)
	Hyperglycemia (plasma glucose >140 mg/dL or 7.7 mmol/L) in absence of diabetes
Inflammatory variables	
	Leukocytosis (White blood cell (WBC) count >12,000 μL^{-1})
	Leukopenia (WBC count <4,000 μL^{-1})
	Normal WBC count with greater than 10% immature forms
	Plasma C-reactive more than two SD above the normal value
	Plasma procalcitonin more than two SD above the normal value
Hemodynamic variables	
	Arterial hypotension (SBP <90 mmHg, MAP <70mmHg, or an SBP decrease >40 mmHg in adults or less than two SD below normal for age)
Organ dysfunction variables	
	Arterial hypoxemia (PaO_2/FiO_2 <300)
	Acute oliguria (urine output < 0.5 mL kg^{-1} h^{-1} for at least 2 hours despite adequate fluid resuscitation)
	Creatinine increase >0.5 mg/dL or 44.2 μmol/L
	Coagulation abnormalities (INR >1.5 or aPTT > 60 s)
	Ileus (absent bowel sounds)
	Thrombocytopenia (platelet count <100,000 μL^{-1})
	Hyperbilirubinemia (plasma total bilirubin >4 mg/dL or 70 μmol/L)
Tissue perfusion variables	
	Hyperlactatemia (>1 mmol/L)
	Decreased capillary refill or mottling

Table 2. Diagnostic criteria for severe sepsis. Adapted from Surviving Sepsis Campaign[52]. PaO_2/FiO_2 = ratio of arterial oxygen partial pressure to fractional inspired oxygen (Horowitz index or Carrico index) to determine severity of lung injury, INR = international normalized ratio calculated as INR = (tested prothrombin time/normal prothrombin time)*international sensitivity index.

Severe sepsis definition = sepsis-induced tissue hypoperfusion or organ dysfunction	
Criteria to determine severe sepsis	
	Sepsis-induced hypotension
	Lactate above upper limits laboratory normal
	urine output < 0.5 mL kg^{-1} h^{-1} for at least 2 hours despite adequate fluid resuscitation
	Acute lung injury with PaO_2/FiO_2 <250 in the absence of pneumonia as infection source
	Acute lung injury with PaO_2/FiO_2 <200 in the presence of pneumonia as infection source
	Creatinine >2.0 mg/dL or 176.8 μmol/L
	Bilirubin >2.0 mg/dL or 34.2 μmol/L
	Platelet count <100,000 μL^{-1}
	Coagulopathy (INR >1.5)

Treatment for sepsis

Next, I will discuss the treatment and clinical management for sepsis. Currently, there is no FDA approved drugs to cure sepsis, and there has been over >100 clinical trials failing to show efficacy in curing the condition[12]. However, despite there being a lack of FDA-approved drugs, within the past decades, sepsis management has become better due to advancing modern medicine but also the revision of the definition for sepsis[56]. In my discussion about management and treatment, I will discuss the initial management of sepsis and briefly touch upon the management for more severe forms of sepsis[52,53].

The first step when a patient comes to the hospital is to perform fluid resuscitation with saline, this is considered standard care for all patients with sepsis. The second step is to screen for potential infectious agents by culturing their blood to determine the pathogen causing the sepsis. Cultures are done prior to antimicrobial intervention – unless there is a delay of 45 minutes or more – and at least two sets of blood cultures are performed to assess for both aerobic and anaerobic pathogens. Studies have shown that there is a benefit to patient outcome when antimicrobials are administered quickly, which would likely target the causative agent of the sepsis[57].These antimicrobials should be given within one hour from the patients time of admission. Furthermore, imaging studies should be performed to confirm a potential source and location of the infection because depending on the location and type of infection, different drugs will be used. The third step is source control. After identification of the specific site of infection and the type of infection present, clinicians make an effort to perform source control methods such as drainage of an abscess, debridement of necrotic tissue and removal of a potentially infected device. These are just some of the methods that are typically used for source control. The fourth step is to prevent infection by using selective oral decontaminates, for instance, oral chlorhexidine gluconate can be used as a form of oropharyngeal decontamination to reduce risk of ventilator-associated pneumonia in septic patients. All of these steps are typically taken for all septic patients.

The next steps discussed will be for more severe forms of sepsis. The fifth step is for hemodynamic support and adjunctive therapy. Fluids containing different proteins are intravenously administered, typically it is saline supplemented with albumin. These different types of fluids are needed to aid in restoring the patients' blood pressure. The sixth step is to administer vasopressors to patients with a mean arterial pressure of <65 mmHg, in which norepinephrine is the first-choice vasopressor. The seventh step, if patients do not get adequate restoration of hemodynamic stability, then intravenous hydrocortisone is administered in a continuous flow and when patients do not need vasopressors and hydrocortisone, then hydrocortisone will be discontinued in a tapered manner. Taken together, the management of sepsis requires very quick intervention and is very extensive.

1.2 Sepsis-Induced Immunosuppression

1.2.1 Impact on Human Health and Sources of Mortality

Impact of protracted sepsis on human health

The initial phase of sepsis causes high mortality with approximately a ~20% mortality rate, and septic shock causing up to ~40% mortality[1]. Although these deaths are significant, the death toll due to the immunosuppressive phase is more plentiful, accounting for 70% of the deaths associated with sepsis[58]. Furthermore, death rates over time are abysmal with 60% of patients succumbing to the syndrome within five years[7]. Currently, it is not well understood why the death rates are so high among patients during the chronic phase of sepsis; however, several groups have come to the consensus that co-morbidities and persistent organ dysfunction work together to create a state of chronic immunosuppression, immune dysfunction, and persistent low-grade inflammation[8,9]. Additionally, individuals who are affected by the chronic phase of sepsis possess a diminished quality of life with systemic physiologic and cognitive impairments[6,10,11].

Sources of mortality in protracted sepsis

Deaths during the immunosuppressive phase are typically attributed to either (1) the failure to clear the primary infection causing the sepsis[59,60], (2) the persistent organ dysfunction that occurs during the post-septic environment[59–61] and (3) secondary infections acquired during their hospital stay or outside the hospital after discharge[60,61]. There is increasing evidence to suggest that patients with sepsis have a hard time fighting the primary infection as evidenced by a post-mortem study which demonstrated that ~80% of patients still presented with unresolved septic infections, despite antibiotic treatment[60]. Furthermore, it was also discovered that ~90% of sepsis/septic shock patients that were treated within the ICU for longer than seven days had an unresolved septic focus at the time of post-mortem analysis, which strongly suggests that the primary infection was unresolved during protracted sepsis. In terms of organ dysfunction, it has been demonstrated by many groups

that this is a significant cause of mortality during protracted sepsis[59–61]. For instance, within a study by Goldenberg et al.[59], the authors examined the causes of death in septic patients at later time points and discovered that ~58% of the deaths were attributed to organ dysfunction without the presence of microbial species. Hence, the persistent organ dysfunction seen within the post-septic environment is a significant cause of mortality during protracted sepsis.

The main focus of this introduction will address the secondary infections acquired during protracted sepsis; however, it is important to note the contributions to mortality during sepsis-induced immunosuppression from persistent organ dysfunction and the uncleared primary infection. Within a study by Otto et al.[61], it has been discovered that infections during sepsis-induced immunosuppression increase over time within the post-septic environment and lead to increased requirements for mechanical ventilation and renal replacement therapy. The authors demonstrated that between day one-to-five post-diagnosis of sepsis, there were 19.6% of septic patients displaying a positive blood culture; however, between day 16-to-150 post-diagnosis of sepsis, there were 27.9% of septic patients displaying a positive blood culture. Furthermore, between day 16-to-150 post-diagnosis of sepsis, septic patients with positive blood cultures displayed higher requirements for mechanical ventilation (79.2% vs 44.8%) and renal replacement therapy (85.7% vs 48.2%) compared to septic patients without positive blood cultures. Hence, over the course of sepsis, septic patients will experience higher bacterial burden with an associated increase in morbidity. Furthermore, it is commonly seen in septic patients that microorganisms that are opportunistic cause significant mortality, such as *Acinetobacter spp.*, *Pseudomonas spp.* and *Enterococcus spp.*[62–64]. Corroborating the findings that septic patients are more susceptible to secondary infections, I can examine hospital re-admission rates. Multiple studies have shown that ~25% of patients who survive sepsis and get discharged from the hospital will be re-admitted within 30 days[65–68]. The leading cause of re-admission is typically due to an infection[69–71].

In addition to secondary infections acquired during protracted sepsis, septic patients often experience the reactivation of latent viruses. For instance, it has been shown that viruses like cytomegaloviruses (CMVs)[72] and herpes simplex viruses (HSVs)[73] get reactivated during sepsis-induced immunosuppression. Recently, within a study by Walton et al.[74], the authors examined the reactivation of multiple endogenous viruses during sepsis-induced immunosuppression; the researchers examined viruses such as herpes viruses (CMV, Epstein-Barr virus (EBV), HSV-1, human herpesvirus 6 (HHV-6)), polyomaviruses (JC and BK; both of the names are initials from patients infected with these viruses), and anellovirus (Torque Teno Virus (TTV)). The authors discovered that 42.7% of septic patients had viremia with two or more of the viruses examined, which demonstrates that the host immune system is extremely impaired in protracted sepsis; however, these estimates may be an underestimate as not all patients were tested for each virus. Despite the power of this study, the researchers assumed that these infections were due to viral reactivation and not an acquired infection as the majority of adults have been infected with these viruses at some point within their lives, which is a limitation of this study. For instance, the majority of adults have been reported to have been infected with HHV-6[75], ~90% of adults have been infected with EBV[76], and the seroprevalences for HSV-1[77], HSV-2[77], JC virus[78,79] and BK virus [78,80] are 58%, 17%, 70-80%, and 60-70%, respectively. Moreover, within this study, the authors also examined the length of ICU duration, severity of illness and mortality. It was discovered that patients that presented with reactivation of all of the viruses except for JC and BK had a longer stay within the ICU with HSV and CMV doubling the patient's length of stay. Viremia with CMV, EBV, HSV and HHV-6 in septic patients was associated with a higher SOFA score, which is indicative of a more profound organ dysfunction and typically led to a worse outcome. In terms of mortality, it was demonstrated that septic patients positive for CMV had an increased 90-day mortality rate compared to CMV negative patients; at 90 days, CMV positive septic patients had a survival rate of ~40%, while CMV negative septic patients had ~65% survival.

1.2.2 Dysregulation of the Innate Immune System

In this section, I will focus on the dysregulation seen within the innate immune system during sepsis by describing the impairments seen within the different innate immune cells. I will be discussing the different impacts sepsis has on neutrophils, monocytes and macrophages, dendritic cells (DCs), and natural killer (NK) cells (Figure 4).

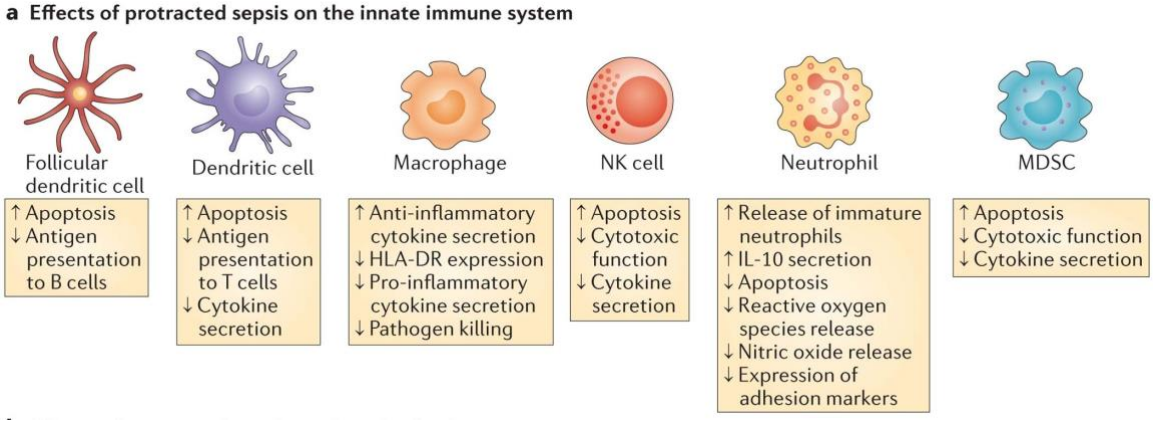

Figure 3. Cellular dysfunction from protracted sepsis within innate immune cells. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Reviews Immunology, Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy, Hotchkiss R.S, Monneret G., Payen D. [81], Copyright 2013.

Neutrophils

Neutrophils are produced within the bone marrow (BM) and are released within the circulation in mass quantities; they are the most abundant WBC within the circulation, but they are also present in small numbers within different tissues such as the spleen, lungs and liver[82,83]. These cells are critical for early immune responses as they tend to be the initial responders during infection[84,85]. Neutrophils are short-lived granulocytes that are constitutively pro-apoptotic and typically die off within 24 hours from being released from the BM[84,86].

During the early stages of sepsis, neutrophil numbers increase within the circulation due to delayed apoptosis[87], which has been demonstrated in human septic patients during the first

24 hours of sepsis diagnosis[88]. There is evidence to suggest that neutrophils are needed during the initial septic insult; however, they later become pathogenic. In a study by Hoesel et al.[89], it was reported within a CLP mouse model that depleting neutrophils prior to the septic insult led to increased mortality; however, depleting neutrophils 12 hours post-CLP resulted in enhanced bacterial clearance and improved survival. These findings suggest that neutrophils become pathogenic later in sepsis, which could be due to their dysfunctional state within the septic host as multiple groups have shown that neutrophils are persistently dysfunctional late in sepsis[90–93]. For instance, it has been shown that the BM releases immature neutrophils, which are deficient in their capability for oxidative burst, complement activation, cell migration, and bacterial clearance[90–93]. Additionally, it has been demonstrated that these cells are capable of suppressing adaptive immune responses by the expression of programmed cell death ligand 1 (PD-L1), which is one of the ligands for programmed cell death protein 1 (PD-1). In a study by Wang et al.[94], the authors examined neutrophil PD-L1 expression at 12, 18 and 24 hours post-sepsis onset within a CLP mouse model and human septic patients. It was discovered that PD-L1 was maximally expressed at 24 hours. Furthermore, it was shown by another group[95] that expression of PD-1 on B and T cells increased 12 hours after sepsis onset. From this information, neutrophils can provide a negative signalling to the adaptive immune cells, which would dampen their responses. Neutrophils further exacerbate the organ damage during sepsis as they can migrate into different tissues to cause harm[8,87]. For example, neutrophil infiltration into the lungs is a hallmark of sepsis-induced acute lung injury[40]. Moreover, in a CLP model of sepsis, immunohistochemical and immunofluorescence experiments demonstrated massive influx of neutrophils within the lungs four days post-CLP compared to sham and this led to significant lung damage, alveolar reduction and interstitial edema[38]. Moreover, they can infiltrate into multiple organs, even the brain. In a study by Saito et al.[96], they demonstrated in a CLP mouse model that neutrophils in septic mice had an increased presence within the cerebral cortex. From all this information, I may state that neutrophils cause severe organ damage to distal organs. Neutrophils may be a potential biomarker to assess patient outcome as neutrophil counts have been shown to correlate with septic patient outcome. Patients with septic shock develop lymphopenia with increased neutrophil counts resulting in a decreased lymphocyte-to-neutrophil ratio, which

is sustained at day 7 after septic insult[97] and has been shown to be implicated in poor prognosis for septic patients[98,99].

Monocytes and macrophages

The most well-characterized defect within this cell population during sepsis-induced immunosuppression is endotoxin tolerance. Endotoxin tolerance is characterized as the diminished capacity of production and release of pro-inflammatory cytokines in response to pathogen components such as lipopolysaccharide (LPS) and other toll-like receptor (TLR) stimuli[100,101]. During immunosuppression, monocytes and macrophages are significantly hindered in their ability to produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12; however, their anti-inflammatory capacity is not affected as shown by their ability to persistently produce interleukin 1 receptor antagonist (IL-1ra) and IL-10[102–105], which might suggest that these cells shift from a pro-inflammatory to an anti-inflammatory phenotype increasing the risk of secondary infections and increased mortality[106]. During the early phase of sepsis, monocytes and macrophages are major producers of pro-inflammatory cytokines and chemokines, which initiates the inflammatory responses and could contribute to increased mortality in sepsis. During the progression of sepsis, there is a massive cell loss of monocytes and macrophages that could result in immunosuppression and a higher risk to succumbing to secondary infections[61,107–109].

In terms of monocytes, the most well-characterized dysfunction is the downregulation of human leukocyte antigen DR allomorph (HLA-DR) seen in septic patients[110,111]. Currently, reduced HLA-DR expression on monocytes in the clinic is the most reliable biomarker to determine immune status in critically ill patients[109,112–114]. Furthermore, reduced expression or failure of HLA-DR expression restoration within septic patients is indicative of immunosuppression, susceptibility to secondary infections and sepsis mortality[114–117]. Reduced HLA-DR expression on monocytes is a marker for monocyte anergy as it correlates with reduced expression of TNF-α and IL-1β in response to microbial challenges and has been also shown to affect antigen presentation through HLA class II proteins[118,119].

To provide a mechanism for this phenomenon, a research group decided to look at the transcriptional regulation of different major histocompatibility complex (MHC) class II molecules within postoperative abdominal septic patients and discovered that compared to control patients, there was a selective and persistent increase in CCCTC-binding factor (CTCF) enrichment at binding sites near HLA genes[110]. This finding resulted in an associated decrease in the transcription of genes: *HLA-DRA*, *HLA-DRB1*, *HLA-DPA1* and *HLA-DPB1* as well as the Class II transactivator (CIITA). Furthermore, the authors also performed correlational analysis and found that CIITA transcription levels were strongly correlated with survival time of septic patients. Moreover, the authors demonstrated that enhanced CTCF binding within MHC-II regions was persistent during the first week of sepsis onset; however, they found a correlation between long-term survival and recovery of transcriptional expression of CIITA.

In terms of macrophages, numerical reduction within this population may severely impact the host's ability to respond to the primary and secondary infections in sepsis as they are highly important for maintaining and activating the host inflammatory response[120,121]. Commonly, there are two types of macrophages: M1, which is associated with pro-inflammatory processes; M2, which is involved in suppression of immune responses by anti-inflammatory processes and tissue repair. Upon phagocytosis and excessive inflammatory responses, macrophages tend to switch from an M1 to an M2 phenotype and have increased production and secretion of anti-inflammatory cytokines such as IL-10 and IL-1ra [122,123]. It has been shown in septic patients that there is an increase in lactate levels, which is currently used as one of the markers to diagnose sepsis. Recently, it has been shown that lactate has the ability to polarize macrophages towards an M2 phenotype[44,124]. There is evidence to suggest that macrophages can cause organ damage by infiltrating into different organs. For example, it was recently shown by Zhou et al.[125] that there was an increase in the presence of macrophages within the myocardium during late sepsis with the ability of these cells to cause inflammation within this tissue as measured by their TNF-α and Interferon γ (IFN-γ) production capabilities. Furthermore, it has also been shown that in septic mice, there was a higher presence of macrophages within the thymus, which ultimately led to the apoptosis of double-positive thymocytes leading to thymic atrophy[126].

Dendritic cells

DCs are produced within the BM and are released as immature cells that are capable of phagocytosis and processes associated with antigen uptake. DCs are the most potent antigen-presenting cells and are necessary for microbial sensing, regulation of the immune response and inflammation[127,128] by being the bridge between the innate and adaptive immune responses[129,130]. Conventionally, there are two types of dendritic cells: conventional DCs (cDCs), which tend to secrete IL-12; plamacytoid DCs (pDCs), which are involved in secreting Interferon alpha (IFN-α).

During sepsis, there is a massive amount of PAMPs and inflammatory mediators, which causes the systemic activation of DCs, subsequently leading to their maturation[131]. Widespread maturation of immature DCs leads to the depletion of immature DCs, which are necessary for responding to newly encountered pathogens and naïve T cell priming[131–133]. Similar to what has been seen within the monocyte and macrophage populations, both dendritic cell populations go through a dramatic reduction within the circulation and spleen due to sepsis-induced apoptosis[130,133–135]. The loss of DCs has been shown to be associated with worse septic patient outcome and increased susceptibility to secondary infections[134,135]. The DCs remaining after widespread apoptosis show significant functional alterations. For instance, within many studies[131,136,137], it was demonstrated that upon stimulation, DCs have a reduced ability to produce pro-inflammatory cytokines. Furthermore, DCs isolated from septic mice had an impaired ability to produce and release IL-12 and TNF-α upon stimulation with LPS or CpG, which indicates that sepsis reduces their functionality. Further groups have shown that lung-resident dendritic cells from septic mice have a decreased methylation of histone H3 Lys4 (H3K4; transcription-permissive) and increased methylation of H3K27 (transcription-repressive) within the promoter of the *Il12* gene, which correlated with decreased IL-12 production in response to TLR agonist[138]. However, despite their inability to produce pro-inflammatory cytokines, they tend to produce higher levels of the anti-inflammatory cytokine IL-10[130,131,138,139], which contributes to the immunosuppression seen in sepsis. They also possess a reduced capacity to act as an antigen presenting cell as evidenced by their reduced surface expression of

cluster of differentiation 40 (CD40), CD86 and HLA-DR, which would lead to either T cell anergy or T regulatory (Treg) cell expansion[131,140,141]. DCs within the septic host become immunosuppressive and fail to activate immune cell responses and results in an immunosuppressive condition with associated organ dysfunction[139,142–146]. It has been suggested that the depletion of DCs can be used as an early predictive biomarker for patient outcome for sepsis[134,147].

NK cells

During sepsis, NK cells are depleted within the circulatory system as evidenced by their lower absolute numbers within the blood of septic patients[148–150]. Furthermore, this decrease in number of NK cells persists for several weeks after the onset of sepsis[151] and is associated with increased mortality in septic patients[149,152–156]. It has been shown that the NK cells that survive the initial apoptotic event are severely affected in terms of their functionality as shown by their reduced cytotoxic function and their decreased ability to produce IFN-γ[149,155,157,158]. This loss of functionality seen within NK cells leads to increased risk of secondary infections after sepsis onset[158–163]. Furthermore, during sepsis-induced immunosuppression, NK cells may upregulate PD-1, PD-L1 and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), which negatively affects their function as it has been shown that interrogation of septic mice with antibodies against these exhaustion molecules have been shown to restore NK cell function[164–166]. However, the cytotoxic ability of NK cells during sepsis may be controversial as some groups have reported that NK cells under septic conditions have a higher expression of intracellular granzyme A and B[167–169]. In a study by Laorden et al.[169], it was discovered that within the acute phase of septic peritonitis (between 0-20 hours post sepsis induction), NK cells were the major producers of granzyme A and B. Moreover, it was demonstrated within the peritoneal exudate cells (PECs) that there is a continuous decline in frequency of NK cells expressing both granzyme A and B; however, they show a continuous increase on a per-cell basis. Within the blood, there was a different pattern of expression of granzyme A and B in NK cells; NK cells had an increased frequency of cells expressing granzyme A and B with a peak expression 14 hours post sepsis induction, while granzyme A over the

study period decreased and granzyme B increased on a per-cell basis. Furthermore, this group had also examined the effect of granzyme A and B on bacterial growth. It was discovered that within the blood and PECs, mice either lacking granzyme A or B or both had an increased bacterial burden compared to wild-type mice at 20 hours post sepsis induction, while within the lungs and liver, mice lacking either granzyme A or B or both had increased bacterial burden at 14 hours post sepsis induction. Furthermore, in a study by Arias et al.[168], they examined the contribution of NK cells to produce granzyme A during sepsis and their survival rates. It was demonstrated that mice devoid of granzyme A had enhanced survival after *Brucella microti* infection causing sepsis with an associated reduction in DIC and levels of proinflammatory cytokines. However, despite these experiments, the NK cell contribution was not elucidated as granzyme A was knocked out in every cell in the mouse. To address this, they adoptively transferred NK cells either possessing granzyme A or lacking it into either WT mice or mice lacking granzyme A. it was reported that transferring NK cells competent in granzyme A production into mice lacking granzyme A restored the susceptibility to sepsis induced mortality, which suggests that NK cells are the major source of granzyme A during sepsis induced by *Brucella microti* and are pathogenic. Furthermore, in a study by Tituana et al.[167], as what was demonstrated within the previous studies, the authors determined that the major producer of granzyme A after sepsis induction by CLP were NK cells. They showed that mice devoid of granzyme A within NK cells were protected from CLP induced mortality and had reduced systemic proinflammatory cytokines. The researchers had also demonstrated that pharmacological inhibition of granzyme A after CLP increased survival and reduced inflammation.

1.2.3 Dysregulation of the Adaptive Immune System

In this section, I will discuss the aberrations within the adaptive immune cells due to sepsis with a heavy focus on T cell impairments (Figure 5).

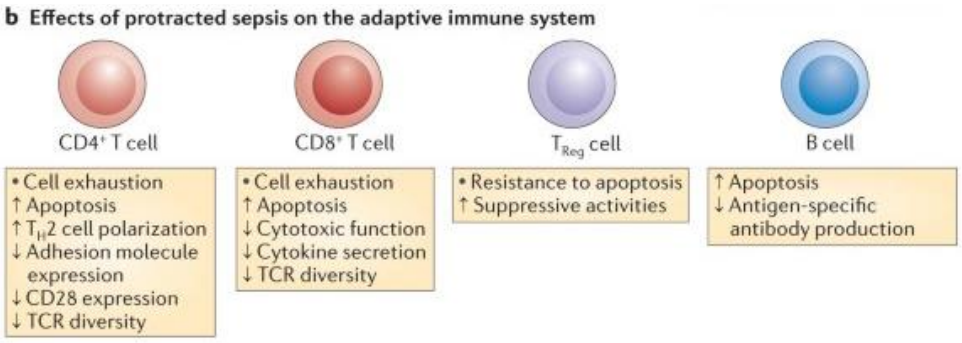

Figure 4. Cellular dysfunction from protracted sepsis within adaptive immune cells. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Reviews Immunology, Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy, Hotchkiss R.S, Monneret G., Payen D. [81], Copyright 2013.

B cells

Within the literature, it has been shown that B cells during sepsis decrease in absolute numbers, which was maintained for 28 days after the initial septic event[150,170,171]. One research group went beyond examining B cells as a whole, and decided to examine the effects of sepsis on different B cell subsets in septic shock patients[170]. Prior to discussing their results, I will discuss the markers they had used to define the different subsets. They utilized the markers CD23, CD69 and CD5 in conjunction with the common CD19 B cell marker: CD23 is mainly expressed by regulatory B cells; CD69 is expressed by early activated B cells; CD5 is expressed on B1a B cells. It was discovered that CD19+CD23+ B cells were significantly reduced in septic shock patients at ICU admission. Furthermore, the authors noticed that compared to non-survivors, survivors had an increase in this B cell subset at ICU admission and 7-day follow up, however, the levels normalized 28 days after the initial septic event. Moreover, the researchers decided to determine whether the levels of CD19+CD23+ B cells at the different time points could be used to predict patient

mortality and discovered that, at the time of admission, the level of this B cell subset accurately predicted patient outcome with a 90.9% sensitivity and 80% specificity. CD19+CD69+ B cells displayed a different trend within septic shock patients as they increased in frequency at ICU admission and at 28-day follow-up. This finding may suggest that during early sepsis, there is an increased activation of B cells that are maintained at later sepsis time points, which could contribute to the excessive inflammation, which has also been demonstrated by another research team[172]. CD19+CD5+ B cells were significantly reduced at ICU admission, however, the levels normalized at 28-day follow-up. From this information, I can conclude that during sepsis, there is a massive shift in B cell subsets that could be contributing to the septic disease. In a study by Kelly-Scumpia et al.[172], it was discovered that B cells enhanced early innate immune responses during bacterial sepsis. In their study, they utilized the CLP model of polymicrobial sepsis and found that mice deficient in B cells, but not T cells, displayed decreased levels of inflammatory cytokine and chemokine production with an associated decrease in survival early after sepsis induction. Furthermore, the authors went on to characterize the activation of B cells during early sepsis by measuring the expression of CD69 and discovered higher frequencies of CD69+ B cells, which was due in part by the type I interferon (IFN-I) signalling as mice devoid of interferon-α/β receptor (IFNAR) had a significant reduction in CD69+ B cells with decreased cytokine production. Moreover, the researchers also demonstrated that T-cell independent B cell functions and antibody production provided protection within the septic environment. These findings demonstrate that early B cell functions are important in protecting against sepsis, presumably because they aid in dealing with the initial infection. Despite these findings displaying increased B cell function during sepsis, there is some evidence to suggest that the humoral response is defective at later stages of sepsis and is associated with increased susceptibility to secondary infections[173–177]. In a study by Sjaastad et al.[173], it was discovered that there was a reduced antibody production in response to IAV in early (2 days post-CLP) and late sepsis (30 days post-CLP). The authors further went on to discover the mechanisms by which this impaired response occurs. It was demonstrated that compared to sham mice, the septic mice had a deficiency in antigen specific B cell differentiation and class-switching due to a failure of antigen specific CD4+ T cells to expand and differentiate into T follicular helper cells

(Tfh), which aid in antigen specific B cell differentiation. Taken together, during early septic events, B cells contribute to the inflammatory response, which contributes to clearing the primary infection, however, in early and late sepsis, they are deficient in mounting an appropriate response to newly encountered antigens as they are unable to differentiate and class switch to produce good quality antibodies.

T cells

T cell apoptosis and composition alterations during sepsis

T cells are severely affected by sepsis-induced apoptosis as they numerically plummet within different tissues of the body during immunosuppression, some of which are the spleen and gut, amongst others[8]. Moreover, it has been reported within a CLP mouse model that both the use of caspase inhibitors and the overexpression of B-cell lymphoma 2 (Bcl-2) within lymphocytes improves sepsis survival and prevents massive lymphocyte depletion[178]. In terms of T cells, it has been demonstrated within a CLP mouse model of sepsis that overexpression of Bcl-2 within T cells led to these mice having an enhanced survival and were protected from sepsis-induced apoptosis as their numbers were comparable to sham, which suggests that the primary mechanism of sepsis-induced T cell loss is apoptosis[179]. Despite the T cell numerical loss during acute sepsis, there have been reports of recovery through thymus-independent, antigen-independent and -dependent homeostatic mechanisms in the CD4+ T cell and CD8+ T cell pool[180–185,185–188], which I will further discuss later. Although there is a recovery of T cells after the initial septic event, it will lead to significant alterations within their compositions. For instance, sepsis has been shown to reduce the production of new naïve T cells by limiting thymic output[189,190], which is partially due to the accelerated apoptosis of immature single and double positive thymocytes leading to less naïve T cells being released from the thymus[190]. This diminished output of new naïve T cells from the thymus may severely hinder the septic patient's ability to mount an appropriate response to newly encountered pathogens as there would be a 'gap' within the naïve T cell compartment. Interestingly, it has been reported within septic shock patients that susceptibility to secondary infections and mortality were

strongly inversely correlated with T cell receptor (TCR) diversity and ability to increase TCR diversity during the course of sepsis[191].

In terms of alterations to the naïve CD4+ T cell pool, there have been multiple studies suggesting a significant change in their composition[182,183,192]. For example, in a study by Perez et al.[182], the authors utilized a CLP mouse model and examined the absolute numbers of six different endogenous naïve antigen specific CD4+ T cells (different precursor pool size, immunodominance and clonotype composition) within the spleen at two and 30 days post-CLP. It was reported that there was a reduction in numbers within all of the different antigen specific CD4+ T cells at two days post-CLP compared to sham; however, at 30 days post-CLP, there was either an increased, decreased or similar absolute number of the different antigen specific CD4+ T cells compared to sham. Furthermore, within a different study by Herrmenau et al.[183], the authors had induced a primary response 30 days post-sepsis induction and then examined the expansion of naive antigen specific CD4+ T cells within the spleen. It was discovered that antigen specific CD4+ T cells at the peak primary CD4+ T cell response within the septic condition had a reduced frequency and absolute number of these cells compared to sham. This information suggests that at 30 days post-sepsis induction, there was a reduced frequency and absolute number of precursor antigen specific CD4+ T cells. Moreover, within a study by Perez et al.[192], it was demonstrated that two days post-CLP, there was a reduction in frequency and absolute number of naive antigen specific CD4+ T cells; however, at 30 days post-CLP, there was a similar frequency and absolute number of naïve antigen specific CD4+ T cells compared to sham. From this information, it could be suggested that different naïve antigen specific CD4+ T cells have a differential capacity to recover, ultimately leading to an altered composition within the naive CD4+ T cell compartment.

In terms of naïve CD8+ T cells, sepsis has been shown to severely alter their composition[180,193]. For instance, in a study by Condotta et al.[180], it was discovered that at 30 days post-CLP, there were septic mice that possessed two distinct populations of CD44+CD11a+CD8+ T cells, either low or high, which indicates either a low or high level of homeostatic proliferation, respectively. The authors decided to look at these populations

in conjunction with inducing a primary response for naïve antigen specific CD8+ T cells; it was demonstrated that mice possessing the high CD44+CD11a+CD8+ T cell phenotype had reduced numbers and responses from antigen specific CD8+ T cells at the peak primary CD8+ T cell response compared to low group, in which the low group had a similar response compared to sham. This information indicates that higher homeostatic proliferation leads to a change in the composition of the naïve CD8+ T cell compartment resulting in the 'gaps' within this population , which has been corroborated within another group[193]. Furthermore, Condotta et al.[180] had also assessed the absolute numbers of naïve antigen specific CD8+ T cells at different time-points post-CLP within the spleen; it was discovered that at two days post-CLP, there was a significant reduction of naïve antigen specific CD8+ T cells, which was maintained at day 30 post-CLP. Moreover, within a different study by Lin et al.[193], the authors had irradiated mice and infected them with lymphocytic choriomeningitis virus (LCMV), then subsequently isolated splenocytes and examined the number of seven different LCMV-specific memory CD8+ T cells six month after infection. It was discovered that mice that had undergone antigenic proliferation during lymphopenia compared to wild-type mice, had a significant accumulation of LCMV-specific memory CD8+ T cells which accounted for roughly 34% of the memory CD8+ T cell pool, while wild-type mice had approximately 8%. From this information, it may be stated that during a secondary infection after lymphopenia, naïve antigen specific CD8+ T cells will have an exuberant expansion leading to an overrepresentation within the memory CD8+ T cell compartment, which is detrimental as it would lead to a lower frequency of memory CD8+ T cells specific for other pathogens.

Memory T cells also go through a drastic change within their composition. Within a study by Jensen et al.[194], it was reported that after CLP, central memory CD8+ T cells will progressively increase over time leading to their overrepresentation within the memory CD8+ T cell compartment. Furthermore, the authors also demonstrated that this increase leads to a differential tissue localization of memory CD8+ T cells, altered genetic profile and reduced capacity to control re-infection. In terms of memory CD4+ T cells, in a study by Sjaastad et al.[195], it was demonstrated that memory CD4+ T cells preferential deplete compared to naïve CD4+ T cells within the septic condition; however, they numerically

recovered 30 days post-CLP. Furthermore, the authors also discovered that antigen specific memory CD4+ T cells were significantly reduced two days post-CLP; however, these cells numerically recovered 30 days post-CLP. Despite this recovery, there was a difference in the kinetics of antigen specific memory CD4+ T cell subsets to recover. It was demonstrated that at seven days post-CLP, there was a numerical recovery of forkhead box P3 (FoxP3) antigen specific memory CD4+ T cells, while T-box expressed in T cell (T-bet; T-box transcription factor 21 (TBX21)) antigen specific memory CD4+ T cells only recovered 28 days post-CLP. This information is significant as it demonstrates that sepsis has the ability to alter the composition of the memory CD4+ T cell pool. FoxP3 is the canonical transcription factor that is involved in Treg differentiation, while Tbet is the transcription factor associated with T helper type 1 (Th1)-mediated responses. Hence, during sepsis, there will a shift in memory CD4+ T cells to produce Tregs, which are heavily involved in immunosuppression; this finding has been corroborated by multiple groups[196,197].

Homeostatic proliferation of different T cell subsets during sepsis

Homeostatic proliferation occurs during a lymphopenic environment, which encompasses sepsis[184]. In terms of naïve T cells, homeostatic proliferation is defined as the expansion due to their ligation with self-MHC complexes. During lymphopenia, naïve T cells have been shown to expand in response to self- or commensal-antigens and require MHC interactions and the presence of IL-7[185–187,198–204]. In the context of sepsis, naïve CD4+ T cells have been shown to go through antigenic proliferation by recognizing microbes from the gut microbiota due to leakage from the septic insult[184,192]. Furthermore, in a study by Perez et al.[182], the authors concluded that naïve CD4+ T cells numerically recover by homeostatic proliferation in an antigen independent manner as there was a higher frequency of CD11a+CD49d+CD4+ T cells 30 days post-CLP compared to sham. However, this conclusion may be faulty as the markers CD11a and CD49d have been shown to be exclusively upregulated on antigen specific CD4+ T cells during antigenic proliferation[205,206]. Hence, they may have detected CD4+ T cells that were expanded due to microbial exposure and/or cross reactivity. Naïve CD4+ T cells have the ability to

undergo homeostatic proliferation, however, in the context of sepsis, they may not. In a study by Unsinger et al.[184], it was demonstrated within a CLP mouse model that CD8+ T cells, but not CD4+ T cells, were capable of undergoing homeostatic proliferation. To accomplish this, the authors had adoptively transferred either naïve OT-I CD8+ T cells and OT-II CD4+ T cells or naïve wild-type CD8+ T cells and CD4+ T cells seven days post-CLP, which were labeled with carboxyfluorescein N-succinimidyl ester (CSFE), then subsequently looked at their proliferation 21 days post-CLP. It was discovered that naïve CD8+ T cells underwent multiple rounds of proliferation, while naive CD4+ T cells did not. However, it was demonstrated that 21 days post-CLP, the CD4+ T cell compartment did numerically recover, which made them further question the mechanism of recovery. It was concluded that CD4+ T cells recovered through the endogenous memory CD4+ T cell population rather than from naïve CD4+ T cells. To provide a mechanism as to why naïve CD8+ T cells perform homeostatic proliferation, while naïve CD4+ T cells do not, it has been demonstrated in multiple studies that naïve CD8+ T cells are more readily able to expand and fill the 'hole' within the T cell compartment during lymphopenia[184,207–209], while hampering naïve CD4+ T cell homeostatic proliferation[184]. Despite the power of this study, naïve CD4+ T cell homeostatic proliferation during sepsis is still questionable. Within the study by Unsinger et al.[184], the authors had adoptively transferred a large amount of naïve T cells into the septic mice seven days post-CLP, which is problematic because (1) there is typically about 20-200 precursor naive CD4+ T cells[210] and 80-1200 naïve CD8+ T cells, rather than 1 million, which is the quantity that they had adoptively transferred and (2) the authors had adoptively transferred these cells seven days post-CLP, which may be problematic as they would lack the T cell intrinsic problems incurred on them during sepsis. Furthermore, within a study by Perez et al.[192], it has been demonstrated that naïve antigen specific CD4+ T cells recognizing segmented filamentous bacterium in septic mice lacking segmented filamentous bacterium had recovered their numbers 30 days post-CLP and had a higher frequency expressing CD44, which indicates that naive CD4+ T cells during sepsis have the ability to numerically recover through homeostatic proliferation. These controversial findings could perhaps be explained by the clonotype selection that these cells experience. Within a study by Foulds et al.[211], it was demonstrated that high levels of monoclonal CD4+ T cells proliferate poorly during antigen stimulation,

while monoclonal CD8+ T cells proliferated extensively and quickly; it was also demonstrated that both polyclonal CD4+ and CD8+ T cells proliferated extensively. This information suggests that high levels of monoclonal CD4+ T cells do not proliferate well as there is competition, which has been illustrated by multiple other studies[186,207,212,213]. Naïve CD4 T cells are more stringent in their requirements to undergo homeostatic proliferation compared to naïve CD8+ T cells as they require the ligation with MHC class II molecules, which are typically found within lymphoid organs, and require specific self-peptides to expand[186,208,213,214]. Hence, naïve CD4+ T cells after sepsis, will be more restricted in their ability to expand through homeostatic mechanisms, however, they still possess the capability to expand through antigenic stimulation, which has been demonstrated by a few groups[184,192]. Naïve CD8+ T cells during sepsis have the ability to undergo homeostatic and antigenic proliferation, with the latter being due to commensal microbes and/or due to the exposure of cognate antigen[198], albeit homeostatic proliferation is the main mechanism of expansion for naïve CD8+ T cells[184].

Homeostatic proliferation of naïve T cells encompasses an alteration in their phenotype. Several groups have reported that naïve T cells upon homeostatic proliferation have an increased expression of CD44, although they did not express common markers of activation such as CD69, CD25, CD49d nor downregulate CD62L[185–187,215–218]. However, this newly adopted phenotype was only transient as they reverted back to a naïve state[218]. Furthermore, this newly acquired phenotype by naïve T cells is associated with a greater functionality as demonstrated by their increased cytotoxic effector ability and their increased capability to produce cytokines compared to phenotypically naïve T cells, although they are not as potent as true memory T cells[185,218].

Compared to naïve T cells, memory T cells neither require self-antigens nor MHC interactions to undergo homeostatic proliferation[219–224]. Instead, memory CD8+ T cells heavily depend on cytokines IL-7 and IL-15, while memory CD4+ T cells do not require these cytokines to perform homeostatic proliferation[221,224–229]. There is evidence to suggest that memory CD4+ T cells decline steadily compared to memory CD8+ T cells, which persist in the host[230]. However, in the context of sepsis, it has been shown by Skirecki et

al.[231], memory CD4+ T cells preferably proliferate within the bone marrow after the septic event, which was heavily dependent on IL-7. The authors had discovered that tissue resident effector memory antigen specific CD4+ T cells were spared from sepsis-induced apoptosis within the bone marrow; additionally, the bone marrow was the main site of expansion of these cells, which allowed them to exit and enter the circulation and filling in the missing CD4+ T cells. Furthermore, the researchers had also demonstrated that CD4+ T cells have an impairment in migrating to the bone marrow after the septic event. This information provides strong evidence that memory CD4+ T cells are one of the major contributors to reconstitution of the CD4+ T cell pool after sepsis-induced lymphopenia, which occurs within the bone marrow of septic individuals. Despite numerical recovery of memory T cells during a lymphopenic environment, it has been reported that non-MHC mechanisms of homeostatic proliferation render these memory T cells less functional and less able to protect against previously encountered pathogens[223,232].

Intrinsic and extrinsic mechanisms of T cell dysregulation during sepsis

In addition to a numerical loss, other intrinsic and extrinsic mechanisms of T cell dysregulation exist in sepsis. Firstly, T cells have been shown to go through a metabolic reprogramming from oxidative phosphorylation to glycolysis, which negatively impacts their function[233,234]. Recently, it was reported that the intracellular level of ROS partially dictates the signalling responsible for T cell immunometabolism reprogramming, with increased ROS leading to the switch from oxidative phosphorylation to glycolysis[234]. Increased ROS is seen in septic patients[235,236] and is partly caused by the decreased activity of autophagy seen during the course of sepsis[237]. Autophagy has been shown to be protective in a mouse model of sepsis by preventing apoptosis, preserving mitochondrial functions and limiting ROS production[237]. Furthermore, it has been demonstrated that suppressing T cell autophagy negatively affects viability and function of T cells[238], which contributes to immunosuppression[239]. Secondly, within septic patients, there is a reduction in serum IL-7, which has profound effects on the T cell compartment[240,241]. The IL-7-IL-7R axis is heavily involved in T cell development, differentiation, and survival of naïve T cells, as well as the generation and maintenance of memory T cells[242,243]. Examining

peripheral blood lymphocytes isolated from septic patients, it was shown that there is a significant reduction in the expression of IL-7R by T cells[244–246]. IL-7/IL-7R signalling occurs via signal transducer and activator of transcription 5 (STAT5) to enhance the protein kinase B signalling pathway in T cells, which is required to maintain their normal metabolism, in part by upregulating glucose transporter 1 (GLUT1)[242]. GLUT1 is essential for oxidative phosphorylation in T cells[247] and in preventing apoptotic cell death[244,248–250]. Treatment with IL-7 demonstrated efficacy in managing T cell dysfunction in sepsis by increasing GLUT1 expression, absolute T cell numbers, activation of T cells and T cell functionality[242,244,247,249–252]. Taken together, it is reasonable to suggest that the accumulation of ROS in T cells leading to the metabolic reprogramming is partially explained by the decreased IL-7R signalling and the defective autophagy response, which has been well characterized in sepsis and leads to their impairment.

On top of T cell intrinsic impairments, it has been shown that multiple populations of cells influence T cell responses during sepsis. DCs have been shown to be severely impacted by the widespread apoptosis, which directly contributes to impaired CD8+ T cell responses[133]. Furthermore, DCs in the context of sepsis-induced immunosuppression have been shown to induce T cell anergy, T helper type 2 (Th2)-mediated T cell immunity, Treg differentiation, and despite DC maturation occurring during sepsis, it failed to initiate a Th1 immune response[131,133,139,141,253,254]. There are reports of an increased frequency of FoxP3+ Tregs, however, this is due to the preferential loss of other CD4+ T cell subsets rather the absolute increase in number[255–257]. Moreover, Tregs contribute to the immunosuppression seen in sepsis as they have been linked to increased solid tumor growth during sepsis, release of immunosuppressive cytokines, decreased proliferation of lymphocytes, lymphocyte anergy, and inhibition of immune responses[257–264]. Additionally, it has been reported that the neutralization of immunosuppressive cytokines, IL-10 and transforming growth factor β (TGF-β), leads to decreased percentage of FoxP3+ Tregs and improved septic mice survival[265].

Naïve T cell immune responses during sepsis

Next, I will further discuss the functionality within the naïve T cell compartment during sepsis-induced immunosuppression, with a focus on antigen specific T cells[180,182,183,192,266–269].

In terms of naïve CD4 T cells, it has been shown by Perez et al.[182] that pathogen challenge two and thirty days post-CLP, at the peak primary CD4+ T cell response, there was a reduced capability of antigen specific CD4+ T cells to produce IFN-γ and TNF-α. Furthermore, the authors demonstrated that the quality of response is affected as there was a reduced frequency of double-expressors of IFN-γ and TNF-α, which would suggest that these cells after sepsis, early and late, have a reduced capability to produce a response upon recognition with their cognate antigen. Moreover, they had also examined functionality of bulk and antigen specific CD4+ T cells at a late septic time-point by inducing a primary infection with *Candida albicans* 30 days post-CLP, then at the peak primary CD4+ T cell response stimulated splenocytes *ex vivo* with phorbol 12-myristic 13-acetate (PMA) and ionomycin. It was reported that bulk CD4+ T cells expressed similar frequencies and absolute numbers of cells capable of producing IL-17A, while *C. albicans*-specific CD4+ T cells had a reduced capacity to produce IL-17A. Furthermore, within a different study by Jensen et al.[269], the authors had examined the effect of sepsis on experimental autoimmune encephalomyelitis (EAE), in terms of naïve autoantigen-specific pathogenic CD4+ T cell responses. The researchers had performed either sham or CLP surgery and then proceeded to induce EAE five days post-surgery, then subsequently assessed myelin oligodendrocyte glycoprotein (MOG)-specific CD4+ T cell functionality seven days post EAE induction. It was discovered within the central nervous system that there was a reduced absolute number of MOG-specific CD4+ T cells capable of producing IFN-γ, TNF-α and IL-17A, which would indicate that they have a reduced functionality after sepsis; however, there was a similar frequency of these cells capable of producing the different cytokines. Moreover, within another study by Herrmenau et al.[183], the authors had induced a primary response 30 days post-sepsis induction, then examined the cytokine production 7 days post-infection. It was demonstrated that at the peak primary CD4+ T cell response, there was no

difference in the productive capabilities of the different cytokines examined, which may suggest that at later stages of sepsis, antigen specific CD4+ T cells do not have a significant impairment in their functionality. Furthermore, within another study by Perez et al.[192], the authors had done a similar experimental design as the previous group and discovered that there was a similar frequency and absolute number of antigen specific CD4+ T cells capable of producing IFN-γ. From all this information, it could be suggested that naïve antigen specific CD4+ T cells may have a dampened functionality early in sepsis, however, at a later time point, they may possess a similar functionality.

In terms of naïve CD8+ T cells, many groups have studied their functionality after the septic event[180,266–268]. For instance, within a study by Condotta et al.[267], the authors examined naïve antigen specific CD8+ T cell responses at multiple time points after sepsis induction (two, nine, and 29 days post-CLP), at the peak primary CD8+ T cell response. Two- and nine-days post-CLP displayed a significant impairment in their ability to produce IFN-γ and TNF-α, which was associated with a higher plaque forming unit (PFU) within the kidneys. Furthermore, it was also discovered that at 29 days post-CLP, there was a similar capability of these cells to produce IFN-γ and TNF-α, while controlling the viral burden. This information suggests that early after the septic event, there is a reduced functionality of antigen specific CD8+ T cells to produce a response; however, at later time points, they appear to be functionally competent. Furthermore, in another study by Condotta et al.[180], the researchers examined the peak naïve antigen specific CD8+ T cell response two and 30 days post-CLP. The authors discovered that at both time points within the spleen, there was a reduced expression of IFN-γ, while at the later time point, there was a reduced expression of IFN-γ, TNF-α, IL-2, granzyme B and CD107a. Moreover, within a study by Markwart et al.[266], it was discovered that inducing a primary response 10 days post-CLP and examining the peak primary antigen specific CD8+ T cell response, there was an increased activity of these cells as they possessed higher per-cell expression of IFN-γ compared to sham. From all the information examined, it is clear that inducing a primary response two days post-CLP led to reduced responses from antigen specific CD8+ T cells, however, there are controversial findings for later time points during sepsis. To add to the confusion, early naïve CD8+ T cell responses after sepsis may even controversial. Within

a study by Dahany et al.[268], the authors subcutaneously injected mice with B16-OVA (ovalbumin) melanoma cells and waited for tumor growth, then subsequently intravenously injected OT-I (specific for OVA) into mice 1 day prior to sepsis induction by CLP and examined their activation and proliferation. It was discovered that septic mice had an enhanced response to the tumor as they detected significantly more proliferation through CFSE assays and activation as measured by CD69 expression compared to mice. This information suggests that sepsis has the capability to enhance naïve CD8+ T cell responses to a pre-existing malignancy. Despite the power of this study, the authors had examined the responses during the inflammatory phase of sepsis, which is known for the widespread activation of immune cells, whereas the previous studies and my study have focused on the response during a secondary infection, which may not make them comparable.

Memory T cell immune responses during sepsis

Thereafter, I will further discuss the impact sepsis-induced immunosuppression has on the functionality of memory T cells with a heavy focus on memory CD8+ T cells as this is one of the main focuses of this thesis.

One of the first pieces of evidence to suggest that memory T cell responses are impaired in sepsis was the reported loss of delayed-hypersensitivity reactions in septic patients[270]. Since then, there have been many more studies looking at memory T cells during sepsis. It was discovered that in severe septic patients, there is a reactivation of latent viruses, such as CMV[72,271] and HSVs[73], in which memory T cell responses are heavily involved in controlling these infections. Furthermore, pre-existing memory CD4+ and CD8+ T cells are subjected to sepsis-induced apoptosis during sepsis[195,272]; however, it has been recently shown that sepsis results in the preferential depletion of memory CD8+ T cells compared to naïve CD8+ T cells[273,274]. Moreover, it was found that the CD244 (2B4) coinhibitory receptor was upregulated preferentially on memory T cells, which led to higher levels of capase-3/7 activation indicative of apoptosis[274]. From these data, it can be suggested that memory T cells are more susceptible to sepsis-induced apoptosis compared to other T cell compartments. In both memory CD4+ and CD8+ T cells, not only are the pre-existing cells

depleted but they show impaired antigen-specific expansion and functionality in the septic environment[194,195,271,275–281].

In terms of memory CD4+ T cells, there was a differential proliferative capacity upon recall response during different stages of sepsis with two days post-CLP showing a decreased ability of antigen specific memory CD4+ T cells to expand upon cognate antigen stimulation, however, 30 days post-CLP did not show such a defect[195]. Moreover, in terms of functionality, antigen specific memory CD4+ T cells within the septic condition displayed reduced functional capacity measured by the frequency and absolute number of single- or multi-cytokine producing cells defined as IFN-γ^+, IFN-γ^+TNF-α^+, and IFN-γ^+TNF-α^+IL-2^+ at both time-points compared to sham-control. Furthermore, in another study by Carson et al.[280], the authors examined bulk memory CD4+ T cell numbers and function and discovered that there was a reduced proliferative capacity and reduced cytokine productive capability of both Th1 and Th2 CD4+ T cell subsets, which was associated with an increased repressive histone methylation at the promotors of *Ifng* and *Gata3*, which are responsible for Th1 and Th2 responses, respectively.

In the context of memory CD8+ T cells, one group examined the absolute numbers of pre-existing memory CD8+ T cells, their capacity for secondary expansion, bystander activation and functionality after sepsis induction[275]. The authors demonstrated that pre-existing antigen specific memory CD8+ T cells two days post-CLP were significantly reduced within the peripheral blood, spleen, and lungs with an associated increase in apoptotic capacity as measured by higher activation of caspase-3/7. Furthermore, the researchers also assessed functionality of these antigen specific memory CD8+ T cells by stimulating them *ex vivo* with their cognate peptides and discovered that two days post-CLP, they had a reduced frequency of CD8+ T cells capable of producing IFN-γ, with an associated reduction in antigen-sensitivity as demonstrated by the increased effective concentration of peptide required for obtaining 50% of the maximum IFN-γ production. In terms of secondary-expansion, the authors had infected mice four days post-CLP with LCMV-Armstrong (LCMV-Arm; acute infection model) and then examined their absolute numbers within the peripheral blood six days post-infection. It was reported that these cells

were severely decreased in their absolute numbers compared to sham, which would indicate that recall response reactions during the immunosuppressive phase of sepsis have a reduced capacity to undergo secondary expansion. Furthermore, the investigators had also examined the innate memory functionality of antigen specific memory CD8+ T cells and endogenous memory CD8+ T cells during sepsis. Virulent-*Listeria monocytogenes* (LM) was administered (does not contain LCMV glycoprotein$_{33\text{-}41}$ peptide (GP_{33}), which is the antigen the memory CD8+ T cells are specific to) five days post-CLP, and then spleens were collected 20 hours post-infection, which then they measured their functionality by examining their frequencies and absolute numbers of cells capable of producing IFN-γ. It was demonstrated that within the CLP condition, there was a significant reduction in both absolute numbers and frequencies of antigen specific memory CD8+ T cells and endogenous memory CD8+ T cells capable of producing IFN-γ, while antigen specific memory CD8+ T cells had a reduced frequency of cells expressing CD25, CD69, and granzyme B compared to sham. This information suggests that bystander activation and functionality of both antigen specific and endogenous memory CD8+ T cells are compromised after sepsis. Within another study by Jensen et al.[194], pre-existing memory CD8+ T cells were examined in their ability to recover from sepsis-induced cell loss, their phenotypic changes and their functionality. The authors discovered that within human septic patients, there was an increased proliferative capacity of different memory CD8+ T cell subsets, with central memory CD8+ T cells having the highest capacity to proliferate and these experiments used the expression of intracellular Ki-67. Corroborating their findings within the human septic blood specimens, they found similar results in the spleen within their CLP mouse model. After CLP, they monitored Ki-67 expression of their antigen specific memory CD8+ T cells (P14, which are stimulated by LCMV-Arm) over a period of 16 days and discovered that for both central and effector memory antigen specific CD8+ T cells, there was an increased expression of Ki-67 within the CLP condition, with a major difference at day nine post-CLP, which indicates that these pre-existing antigen specific memory CD8+ T cells have an increased proliferative capacity compared to sham. Moreover, the investigators wanted to further characterize this proliferative enhancement by administering bromodeoxyuridine (BrdU) at day nine (highest proliferative capacity) and examining the amount of BrdU incorporation seven days later; BrdU gets incorporated

into deoxyribonucleic acid (DNA) and a higher presence of BrdU indicates higher proliferation. Consistent with their previous findings, it was discovered that both central and effector memory P14 CD8+ T cells and endogenous central and effector memory CD8+ T cells had an increased incorporation of BrdU within the septic condition, which gives strong evidence that both antigen specific and non-antigen specific memory CD8+ T cell populations have an increased proliferative capacity. Moreover, they demonstrated through these mice experiments that compared to effector memory CD8+ T cells, central memory CD8+ T cells had a higher proliferative capacity throughout. In addition, the authors had also assessed the phenotypic changes that occur 30 days post-CLP. They discovered that within the septic condition, there was a shift of pre-existing memory CD8+ T cells from an effector memory to a central memory CD8+ T cell phenotype due to the higher proliferative capacity of central memory CD8+ T cells, which is consistent with previous reports indicating that central memory CD8+ T cells have a higher capacity to undergo homeostatic proliferation[282–284]. From this information, it may be suggested that during sepsis, upon homeostatic proliferation of memory CD8+ T cell populations, central memory CD8+ T cells will proliferate more within the septic environment leading to their domination within the memory CD8+ T cell pool. Furthermore, they had also assessed for late septic functionality (>30 days post-CLP) of pre-existing antigen specific memory CD8+ T cells within the spleen. It was reported that there is an increased functionality of pre-existing antigen specific memory CD8+ T cells as measured by the expression of IFN-γ and IL-2; compared to sham, there were similar levels of cells expressing IFN-γ, however, they possessed a higher frequency of cells expressing IL-2 and co-expressing IFN-γ and IL-2, which would indicate that these cells are more polyfunctional and active compared to sham. In another group that examined human cytomegalovirus reactivation in severe septic patients within three-days of ICU admission[271], they examined the function of memory CD8+ T cell responses and discovered that in terms of singly-expressing cytokines (IFN-γ, TNF-α, CD107a, macrophage inflammatory protein-1β (MIP-1β)), there was no difference between septic patients that either had CMV reactivation or not, however, septic patients that had CMV reactivation had significant reduction in polyfunctional CD8+ T cells. This information suggests that polyfunctionality of memory CD8+ T cells is needed to control latent viruses after the onset of sepsis and could potentially mean that the loss of

polyfunctionality may lead to reactivation of latent viruses, however, they did not establish a causal relationship as it was correlation. Furthermore, this study did not address the differences between healthy controls and severe septic patients with or without hCMV reactivation. Furthermore, within a study by Xie et al.[285], the authors had assessed the frequency and absolute numbers of pre-existing antigen specific memory CD8+ T cells three days post-CLP within the spleen; moreover, they had also assessed the phenotype and functionality of these cells at that time-point. It was discovered that the absolute number and frequency of antigen specific memory CD8+ T cells were significantly reduced compared to sham. In terms of phenotypic differences, it was demonstrated that both antigen specific and bulk memory CD8+ T cells had an upregulation of 2B4; furthermore, within the non-antigen specific memory CD8+ T cell compartment, there was no difference in 2B4 expression compared to sham. This information suggests that 2B4 could potentially be upregulated specifically on antigen specific memory CD8+ T cell subsets. Moreover, the authors had also performed similar experiments in 2B4 knockout mice and discovered that, at three days post-CLP, septic mice lacking 2B4 expression had similar frequencies and absolute numbers of antigen specific memory CD8+ T cells, which would indicate that 2B4 may be a major player in sepsis-induced lymphopenia, which has been shown by others[274,286]. In regard to functionality, the researchers had shown that splenic antigen specific memory CD8+ T cells lacking 2B4 had a significant enhancement in their ability to produce IFN-γ compared to septic wild-type mice, however, the authors did not compare to the responses to a sham-control. Within the previous studies examined, most of the groups have focused on memory CD8+ T cells within the blood or spleen, which are systemic organs. However, it is also important to address tissue-resident memory CD8+ T cells (CD8+ Trm). Recently, it has been shown that CD8+ Trm cells may not be numerically and functionally affected by sepsis[272,287]. It was demonstrated by Dahany et al.[272], that CD8+ Trm do not suffer from a numerical decline nor functional impairments during sepsis, while circulating memory CD8+ T cells did. However, sepsis does impair bystander recruitment of circulating CD8+ T cells and antigen specific memory CD8+ T cells at the site of CD8+ Trm activation. It was concluded that sepsis reduces the expression of interferon-γ receptor 1 (IFN-γR1) on the vascular endothelium, which decreases sensing of CD8+ Trm produced IFN-γ leading to decreased expression of various chemokines

and/or adhesion molecules required for T cell recruitment. Furthermore, in another study by Dahany et al.[287], the authors adoptively transferred naive P14 CD8+ T cells into mice, then infected them with LCMV-Arm, after 24 days, the authors injected mice subcutaneously with B16-melanoma cells, and then performed CLP or sham surgery 19 days post-tumor injection, two days post-CLP (or -sham), they analyzed P14 memory CD8+ T cells within the tumor and blood. It was discovered that within the blood, there was a massive reduction of antigen specific memory CD8+ T cells, while antigen specific memory CD8+ T cells within the tumor were numerically similar compared to sham. From this information, I could state that memory CD8+ T cells within the circulatory system are numerically and functionally affected, however, CD8+ Trm cells may not experience this decline in number and function. Despite the previous findings, in a more recent study by Moioffer et al.[276], it has been demonstrated that this resistance of sepsis-induced dysfunction and numerical loss in CD8+ Trm may not be fully true. The authors reported that the severity of sepsis determines the degree of impairment for CD8+ Trm cells. The researchers discovered that compared to circulatory memory CD8+ T cells, which were numerically and functionally diminished by both moderate and severe sepsis, CD8+ Trm cells were only affected numerically and functionally by severe sepsis.

1.3 Influenza A Viruses

1.3.1 IAV properties

General characteristics of Influenza Virus

Influenza viruses are respiratory pathogens that cause mild to severe respiratory infections in humans typically referred to as influenza, and commonly known as “the flu”. They are a part of the *Orthomyxoviridae* family of viruses and there are four types of influenza viruses, influenza A, B, C and D. There are three types of influenza virus that are capable of infecting humans (A, B and C), and their classifications are primarily based on antigenic differences in their nucleoprotein (NP) and matrix (M) proteins[288,289]. While the Influenza D virus infects animals like cattle and pigs[290,291], there is evidence of potential human infection as there have been reports of Influenza D specific antibodies within human serum samples. Influenza A viruses are the most common cause of seasonal flu epidemics and pandemics in humans[292,293] due to their ability to genetically mutate and reassort[294–298]. IAV’s natural reservoir is in avian species, and it has been shown that IAV has had multiple incidents of zoonotic transmission into human populations that have given rise to stable seasonal and pandemic IAV strains[299].

The genome of IAV consists of eight segmented, negative-sense ribonucleic acid (RNA) that codes for 11 protein products: haemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), NP, non-structural protein 1 (NS1), non-structural protein 2 (NS2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1-F2 (PB1-F2). Each RNA segment forms a complex with NP, which combines with the RNA-dependent RNA polymerase complex (RdRP; composed of PB1, PB2 and PA), which in here we refer to as vRNPs. The viral envelope, which derives from the host upon budding, contains the proteins NA (11 subtypes), HA (18 subtypes) and M2. NA is essential for the spread of the newly generated virions from host cells as it cleaves the interaction between sialic acid residues on the surface of host cells and HA, which leads to viral release. HA is essential

for viral attachment and entry as it recognizes and binds to sialic acid receptors on the surface of host cells in the respiratory tract. IAV strains are partly named by the subtypes of HA and NA that they possess: type/(host if originated from non-human source)/location of isolation/isolation number/year of strain isolated/surface antigen subtypes. For example, the virus that was utilized in my thesis study was A/Puerto Rico/8/1934/H1N1 (PR8). HA and NA are used in the classification of IAV subtypes as these proteins go through antigenic drift and shift. Antigenic drift is the accumulation of random mutations within the RNA genome due to the nature of the RdRP and antigenic shift is the new combination of full RNA sequences from two or more flu strains as they can re-assort their eight different segments during co-infection. These mechanisms of genetic differences have the capability to generate new viruses that are distinct from pre-existing subtypes and are capable of avoiding pre-existing adaptive immune responses to cause infection[300–303]. M2 is essential for the release of the IAV genome into the cytoplasm; it is a proton-selective ion channel that is activated by the drop in pH seen within the endosome after receptor-mediated endocytosis. It acidifies the viral core by transporting protons into the core and this results in the fusion of the viral and endosomal membranes and release of vRNPs into the cytosol of the host cell[304,305]. Just beneath the viral envelope, the M1 protein forms the matrix layer of the IAV virus, it has also been shown to interact with the vRNPs by aiding in their export of the host nucleus in late IAV infection[306]. The proteins PB1, PB2 and PA constitute RdRP, which is responsible for the transcription and replication of the viral genome. In terms of transcription, the IAV virus utilizes a "cap-snatching" mechanism. PB2 recognizes the 5' cap of host precursor messenger ribonucleic acid (pre-mRNA), and PA cleaves host pre-mRNA to generate 5'-capped RNA fragments that PB1 utilizes to synthesis viral messenger ribonucleic acid (mRNA)[307–311]. Compared to transcription, replication of the IAV genome does not require this mechanism. The RdRP creates a positive-sense RNA strand that is used as a template for the RNA genome and the initiation of this process occurs through a priming loop[311,312]. NS1 is essential for the inhibition of host antiviral responses, regulating viral RNA and protein synthesis[313–319]. NS1 has been shown to suppress the expression of host mRNAs, preventing the expression of IFN-induced antiviral genes. Furthermore, it has also been shown to inhibit caspase-1 activation and production of IL-1β, and hinders retinoic acid-inducible gene I (RIG-I) antiviral

signalling pathways. Viruses that lack NS1 are very attenuated. NS2 is necessary for the nuclear export of newly generated vRNPs out of the nucleus after the amplification of the RNA genome and delivery to the cell membrane for progeny budding[320,321]. PB1 contains an additional open reading frame that codes for PB1-F2, which is currently not well understood, however, it has been shown to contribute to the inflammatory response and flu-induced pathogenesis by activating the NLR family pyrin domain containing 3 (NLRP3) inflammasome[322].

IAV replication cycle

Next, I will discuss the viral replication cycle of IAV (Figure 3). In humans, the virus will be taken up, although, to achieve successful infection, IAV must pass through the respiratory systems mucus layer, which is the first-line defense. Within the mucus layer of the respiratory tract, there are sialylated decoy receptors which prevent viral entry; however, IAV is able to bypass this first-line of defense due to the ability of NA to cleave the bond between HA and sialylated decoy receptors[323]. Next, IAV will bind to sialic acid receptors within the respiratory tract by the binding of HA, and this will lead to virus uptake into the host cells by receptor mediated endocytosis. Upon acidification of the endosome, M2 will transport hydrogen ions into the viral core to acidify the core leading to the fusion of the viral and endosome membranes, which results in the release of vRNPs into the cytosol. The vRNPs are then transported into the nucleus via nuclear pore complexes where they will be subjected to viral transcription and replication. In terms of transcription, the RdRP will steal the 5' cap from host pre-mRNA and use these short 5'-capped RNAs as a primer for transcription of viral RNA. The end of the viral transcripts also possess a polyadenylated tail (poly-A tail), which is created by the "stuttering" action of the viral polymerase[324]. These viral mRNAs will then get transported out of the nucleus and get translated by host ribosomes. Certain proteins from the viral mRNA will get transported back into the nucleus to form vRNPs complexes with newly generated RNA genomes or perform actions within the nucleus, others will perform actions within the cytosol, and others are destined to be integrated into the host membrane. In terms of replication, the RdRP complex will generate some positive-sense RNA strands that will be used to generate

new negative-sense RNA genomes, and this action is independent of cap-snatching mechanisms, however, they utilize a priming loop mechanism which is built in the RNA genome. These newly generated genomes will associate with viral proteins creating vRNPs and are escorted out of the nucleus by NS2 and get localized to the host membrane rich in lipid rafts containing HA, NA and M2 and then bud out of the cell and get cleaved by NA to be released.

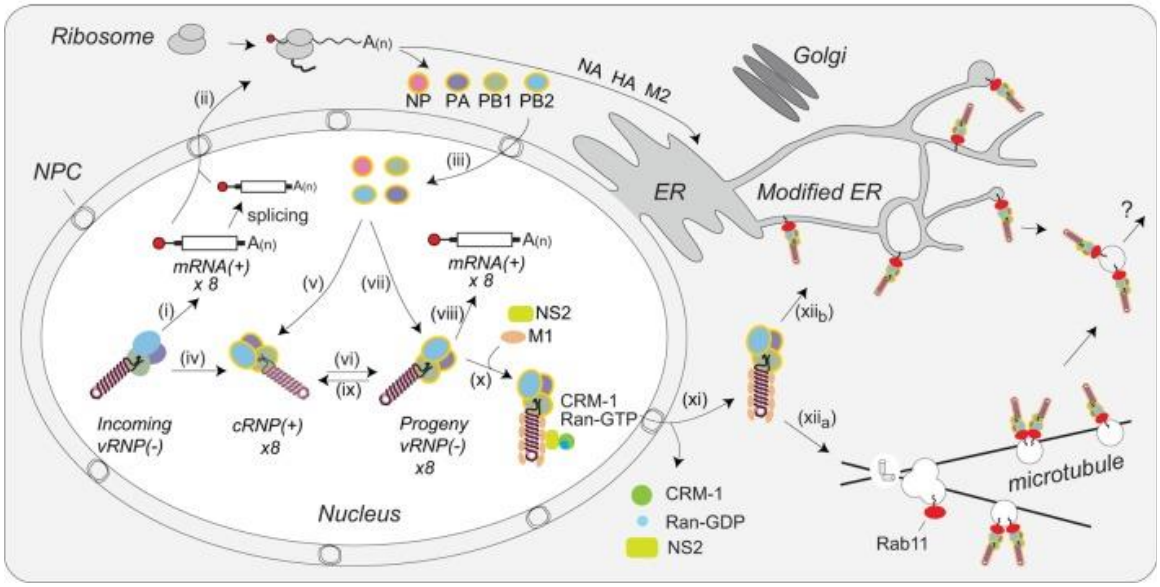

Figure 5. Replication cycle of IAV. Figure adapted from "Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement", Daniels R., Dou D., Revol R., Ostby H., Wang H.[325], used under license CC BY 4.0 'https://creativecommons.org/licenses/by/4.0/'.

1.3.2 Impact on Human Health

Every year, worldwide seasonal flu is estimated to cause 1 billion infections and typically around 290,000 to 650,000 deaths according to Infection Prevention and Control Canada (IPAC)[326]. Seasonal influenza infects people of all ages; however, it is more likely to cause severe disease requiring hospitalization in patients with complications and in high-risk populations, such as children (6-23 months), elderly people (65+), pregnant women and patients with pre-existing co-morbidities[326,327]. In Canada, Influenza and pneumonia are ranked among the top 10 causes of death in Canada[326]. Furthermore, approximately 12,200 people are admitted to the hospital for influenza infection and roughly 3,500 patients die due to influenza[326]. Moreover, on top of people losing their lives due to influenza infection, there is a significant economic burden associated with influenza. While there are many estimates of how IAV affects economic burden, one study that considered hospital costs and productivity loss found within the United States (US) a 11.2-billion-dollar economic burden[328]. Direct medical costs were 3.2 billion, while indirect costs were 8.0 billion dollars. Despite effective vaccination methods, there is still a huge economic burden and a possibility of new emerging strains as evidenced in the 2009 pandemic when the "swine flu" took place. The "swine flu" originated from Mexico around March 19th, 2009 and spread worldwide despite the protective immunity from vaccination[329]. Statistics from the Centers for Disease Control and Prevention (CDC) estimated that within the US from April 12th 2009 to April 10th 2010, there was an estimated 60.8 million cases with 274,304 hospitalizations and 12,469 deaths due to this strain[330]. Furthermore, within Canada, it was reported that the H1N1 pandemic strain was confirmed in all territories and provinces with an associated mortality rate of 1.3 per 100,000 people, which accounts for 1,300 deaths. Moreover, comparing the IAV cases and deaths from the previous year, there was an approximate 73% increase in cases and a 456% increase in death associate with this pandemic strain[329].

There is a dire need for universal vaccines that are efficient in dealing with IAV infections to dampen the cost of IAV on society and the health burden associated with IAV. Currently, the most common way that flu vaccines are made is by using an egg-based manufacturing process. This method has been used for over 70 years and has been efficient in dealing with IAV infections, however, there is some serious downfalls. The process begins with the CDC or another laboratory (partner in the World Health Organization (WHO) and Global Influenza Surveillance and Response System (GISRS)) providing the manufacturers with the most common strains of influenza that circulated within the previous flu season and then they administer those viral strains into fertilized chicken eggs. The infected chicken eggs will then be incubated for several days to allow for the virus to replicate and then the fluid containing the virus is harvested (allantoic fluid). For the "flu shot", the vaccine viruses will be inactivated (killed) and then viral antigens will be purified, packaged, and distributed. Despite this method being efficient in controlling IAV infections, it can still be subjected to newly emerging strains, which was seen in the 2009 "swine flu" outbreak. These flu-based vaccination generation methods are costly and are labor intensive and are required to be made every year due to the nature of the virus. Current methods for vaccine development are specific for NA and HA, however, due to the ability of the virus to mutate by accumulation of random mutations (antigenic drift) due to the error-prone nature of the viral polymerase and the new combinations of sequences from two or more influenza strains (antigenic shift), this method is faulty as new viral strains can arise which can lead to influenza pandemics[300–303]. There is a need for a universal vaccine strategy that would lead to protective immunity to IAV for years to come.

1.3.3 Transmission, Symptoms and Pathophysiology

Influenza virus is transmitted in humans through direct contact and droplets within the air[331]. Influenza epidemics usually occur during the cold seasons, when humidity and temperature is low as these conditions have been suggested to prolong viral shedding and transmission[332]. On top of these conditions, dense populations has also been shown to enhance the transmission of this virus[333,334]. In terms of the reservoirs that IAV have, the primary reservoir is an avian source, however, IAV is also found within pigs[335]. In terms of avian influenza, the major subtypes of IAV that are threats to human health are H5N1 and H7N9[336], which is widely found in domestic farms and has been shown to cause severe disease in humans[336,337]. For example, in terms of H5N1, it first originated from a poultry outbreak at the end of 2003 in Thailand, which resulted in 840 cases of human infections in 15 different countries, which had a mortality rate of more than 50%[335]. In terms of H7N9, this too was a confirmed transmission from poultry to human and was associated with more than 560 cases, which lead to severe pneumonia with multiorgan failure and mortality rates of ~40%[335,338]. Both of these avian IAVs are now stable within the human population. Furthermore, the 2009 "swine flu" pandemic was confirmed as a triple reassortant virus that was transmitted from birds to pigs then to humans[339]. Pigs as a reservoir is a major problem and has been considered a "mixing vessel" for IAV viruses as pigs possess both avian and human influenza receptors[340,341], which allows them to mix and match different IAV strains from different origins[340,341].

The IAV incubation period is 24 to 48 hours before symptoms arise and viral shedding peaks[317]. Influenza virus can be asymptomatic or mild, however, in rare cases, IAV can induce complicated disease with severe pneumonia, which may lead to multiorgan failure or exacerbation of previously established diseases. Onset of symptoms happens after the incubation period and they include headache, muscle weakness and pain, felling unwell, chills, cough, and fever which can persist for two to eight days. Viral shedding and contagiousness may persist after the resolution of symptoms for four to five days[342]. Furthermore, on top of these symptoms, pandemic strains can cause gastrointestinal illness such as vomiting and diarrhea. It has been shown in severe IAV infections, the virus can

reach the alveolar compartment. Alveolar infections can cause severe damage to this tissue. In terms of histology, alveolar infections present with intra-alveolar hemorrhagic edema accumulation, massive infiltration of white blood cells, fibrin deposition, and bronchial and alveolar cell apoptosis[343] which can lead to progressive respiratory failure ultimately leading to acute respiratory distress syndrome (ARDS) and fatal outcome[344]. Another common cause of IAV-associated death and morbidity is pneumonia due to secondary bacterial infections. There is evidence to suggest that there is a two-way relationship between IAV and certain bacteria. In terms of IAV, IAV makes the host more susceptible to secondary bacterial infections by exhausting the immune system and increasing lung damage. Lung damage increases the risk of bacterial infections because it has been shown to enhance bacterial adherence and invasion into the lungs[345]. Furthermore, certain bacteria have been reported to enhance IAV infection[346].

1.3.4 CD8+ T cell responses to IAV

For the focus of this thesis, I will not discuss the immune responses from other immune cells as I am focused on the CD8+ T cell response.

The build-up to the CD8+ T cell response to IAV

The CD8+ T cell response is an important component of the adaptive immune system to combat IAV infection. First, we will discuss the events leading up to the CD8+ T cell response and then discuss their functionality[347–350]. Upon infection, DCs will uptake IAV by either being infected by or by picking IAV antigens within the respiratory tract and migrate to secondary lymphoid organs. This process of migration and taking up antigen will lead to DC maturation, which results in their enhanced ability to present antigens to T cells. Within the DCs, if they are infected by IAV then they will have the presence of IAV proteins within the cytosol and they can be processed and presented on MHC I molecules. When antigens of IAV are taken up by DCs, they are within endosomal compartments which are typically destined for MHC II presentation, however, DCs have an enhanced ability for cross-presentation, which is when there is the leakage of antigens from the endosome into the cytosol[351], and hence they would be able to present antigens on MHC I. During processing of antigenic peptides, the proteasome within the cytosol of DCs will degrade viral antigens to generate short peptides, 7-11 amino acids in length, which then the Transporter Associated with Antigen Processing (TAP) protein will translocate these peptides from the cytosol into the endoplasmic reticulum. Once the peptides reach the endoplasmic reticulum, they will be loaded onto MHC class I molecules and transported to the cells surface for presentation to CD8+ T cells. On top of this process, DCs will also upregulate molecules associated with co-stimulation, for example, they will upregulate CD40, CD80 and CD86 upon maturation and migration, which are necessary for the activation of naïve CD8+ T cells as failure to upregulate these markers while presenting peptides leads to anergy in T cells. Once the DCs are within the secondary lymphoid tissues, presenting the IAV peptide and have co-stimulatory markers present, a naïve T cell, which has a TCR that is specific for the peptide presented on the DC will engage with

the DC and receive co-stimulatory signals. Once the naïve CD8+ T cell forms the immunological synapse with the DC, signals will be transferred to the T cell that will lead to its differentiation into an effector CD8+ T cell with an associated clonal expansion. During their differentiation into effector CD8+ T cells, they will upregulate the C-C chemokine receptor type 5 (CCR5) leading to their accumulation within the lungs through the action of C-C chemokine ligand type 5 (CCL5), where they will continue to proliferate and combat the IAV[352,353]. Furthermore, compared to naïve CD8+ T cells, effector CD8+ T cells do not require prolonged antigen exposure to be activated, they are able to produce cytokines and their cytolytic effector killing behaviours through a short interaction with their cognate peptide presented on MHC I[354], which would be seen within the lungs of host infected with IAV.

CD8+ T cell response to IAV

CD8+ T cells are important for host protection and viral clearance within an individual experiencing IAV infection. Effector CD8+ T cells from the secondary lymphoid tissues will egress to travel to the lung, where they will provide protective immunity through many mechanisms. When they encounter their cognate peptide in the context of MHC class I presentation on virally infected cells, they will become activated and will respond. In terms of their peak responses during primary infection with IAV, they reach a peak response at 8 days post-infection within the draining mesenteric lymph nodes (mLN) and at 10 days post-infection within the bronchoalveolar lavage fluid (BALF)[355]. In terms of their responses that they perform; firstly, activated CD8+ T cells may directly kill the virally infected cells by inducing cytotoxicity. CD8+ T cells will form an immunological synapse with the virally infected cell that is restricted to the cell that is being targeted and will release perforin and granzymes. Perforin permeabilizes the membranes of the virally infected cell by forming multimeric complexes with itself within the membrane to form a pore, which allows the entry of granzymes and these act by inducing apoptosis within the targeted cells[356,357]. Secondly, activated effector CD8+ T cells can also cause cytotoxicity in the host virally infected cell by the TNF receptor (TNFR) family-dependent pathways[356,358,359]. Activated effector CD8+ T cells express the Fas ligand (FasL; CD95L) that can bind to the

Fas receptor (Fas; CD95) on the target cell, and this interaction induces apoptosis within the target cell. Thirdly, effector CD8+ T cells also express TNF-related apoptosis-inducing ligand (TRAIL), which is capable of inducing apoptosis within the target cell by engaging with the death receptor 5 (DR5) on the surface of virally infected cells. Fourthly, activated effector CD8+ T cells produce different pro-inflammatory cytokines with most of these cells expressing IFN-γ, a smaller subset expressing TNF-α, and an even smaller subset of cells expressing IL-2[360]. Effector CD8+ T cells that are polyfunctional as measured by their co-expression of IFN-γ, TNF-α, and IL-2 provide the most effective protection against IAV[360–365]. These cytokines have many different functionalities, and we will briefly discuss their effects in the immune response. In terms of IFN-γ, this is a pleiotropic cytokine that has a multitude of effects on different immune cells and non-immune cells[366,367]. For instance, IFN-γ has been shown to enhance CD8+ T cell motility, cytotoxicity, and target killing. Furthermore, IFN-γ leads to the upregulation of both MHC class I and II pathways leading to enhanced presentation of viral peptides to induce a more robust response; it affects macrophages by enhancing their microbial activities such as increased phagocytosis and upregulation of their anti-microbial products, and this is found in other innate immune cells; it enhances trafficking of immune cells into the site of infection by the upregulation of adhesion molecules and chemokines leading to enhanced response from other immune cells. In terms of TNF-α, it is a pleiotropic cytokine that is typically pro-inflammatory[368]. TNF-α has been shown to enhance cytotoxic ability within CD8+ T cell and NK cells, enhance intracellular viral killing in different immune cells, increased trafficking into the site of infection by the upregulation of different adhesion and chemokine molecules, and enhances T cell activation and proliferation. In terms of IL-2, it has been shown that this cytokine drives the expansion of activated T cell subsets[369]. Recently, it has been shown that upon viral infection, CD8+ T cells express heterogenous levels of interleukin 2 receptor α (IL-2Rα or CD25): CD8 T cells expressing low levels of CD25 tend to upregulate CD127 and CD62L, which would preferentially give rise to a memory CD8+ T cell population; CD8+ T cells expressing high CD25 will proliferate more when they encounter IL-2 and display an effector CD8+ T cell phenotype, and contract more during the contraction phase[370]. Furthermore, during CD8+ T cell exhaustion, IL-2 is one of the first cytokines that gets downregulated when they are exhausted[371]. Effector CD8+ T cells

within the lungs are also major producers of IL-10, which is responsible for the dampening of inflammatory response. Within previous studies by Sun et al.[372], they demonstrated that effector CD8+ T cells were a major producer of IL-10 within the lungs of mice infected with a sublethal IAV challenge and blockade of IL-10 resulted in increased pulmonary inflammation and lethal injury.

CD8+ T cell responses during IAV infection are important for controlling viral load and reducing mortality. In a study by Bender et al.[373], they demonstrated that mice devoid of β2-microglobulin (B2M), which lack CD8+ T cells, had a delayed viral clearance with an associated increase in mortality following IAV infection. Furthermore, it has been shown by another group that adoptively transferring effector and memory IAV-specific CD8+ T cells into naïve mice and then challenging them with lethal IAV infection lead to decreased mortality and viral load[374]. From these experiments, it can be concluded that CD8+ T cell-mediated immunity is a critical part of the response to IAV infection, which would help clear the virus and aid in supporting survival.

CD8+ T cell-mediated immunity to IAV is typically seen as protective[375–380]. However, it has also been shown that they are capable of causing immunopathology within different tissues during infection[381–385]. One of the first studies that demonstrated evidence of this phenomena was from 1981[385]. They reported that athymic mice, which lack functional T cells, had a longer survival and a slower onset of lung pathology compared to wild-type mice, however, in the long run, they had increased mortality, persistent lung injury and failed to clear the virus when administered lethal dose of IAV. Furthermore, in subsequent studies, they utilized a non-viral infection model, which had constitutive expression of HA on alveolar epithelial cells and then adoptively transferred CD8+ T cells specific for HA into these mice. They reported that these mice had severe lung injury as the recipient mice had a progressive decline in weight, interstitial pneumonitis, increased inflammatory cytokines within the lungs, compromised lung structure and function[384,386]. From this information, it is evident that during IAV infection, CD8+ T cells could contribute to lung pathology. More recent studies using this model have demonstrated that the cytokine secretion from CD8+ T cells enhanced lung immunopathology by enhancing inflammatory

cytokine production from other immune and non-immune cell types, and non-specific apoptosis of alveolar epithelial cells, which contributes heavily to lung injury and inflammation[387]. This is more evidence to suggest that during IAV infection, despite CD8+ T cells being protective, they are also pathogenic. However, despite these findings, this system is very artificial as they constitutively express HA on their alveolar epithelial cells, which does not recapitulate the *in vivo* IAV infection, as this virus gets cleared. Despite these downfalls, it is still good evidence to suggest that CD8+ T cell during IAV infection contributes to immunopathology, however, this system may only be relevant for patients that suffer from chronic IAV infection as they would have the presence of their antigens for an extended period of time without the ability to clear it. In more recent studies, research groups had wanted to elucidate the mechanisms by which CD8+ T cells contribute to lung pathology during IAV infection. Through their experiments, it was discovered that TNF-α secretion by IAV-specific CD8+ T cells was necessary to induce lung injury during IAV infection[382,383]. Corroborating the previous findings, in a study by Srikiatkhachorn et al.[381], the authors discovered that interference of TNF-α signalling within lung epithelial cells dampened CD8+ T cell-mediated lung injury during IAV infection. From these studies, it is evident that CD8+ T cells responding to IAV infection and are producers of TNF-α contribute heavily to the lung pathology seen during IAV infection. Furthermore, TNF-α released from IAV-specific CD8+ T cells within the lungs particular signals through lung epithelial cells which lead to lung pathology. During IAV infection, the infiltration of IAV-specific CD8+ T cells is essential for viral clearance and protection, however, there must be a balance in response as they can contribute to lung immunopathology.

Importance of studying immunodominance

One important fact to consider for IAV-responding CD8+ T cell clones is their immunodominance status. Immunodominance is the concept of having an immune response against a handful of antigenic peptides out of the many produced. During the generation of antigenic peptides, there will be thousands of peptides generated during IAV infection, however, only a certain few will make the cut leading to a detectable, and sometimes robust, CD8+ T cell response. There are many factors that influence the generation of immunodominant epitopes. For instance, the availability of the antigenic peptide is one determinant, the binding affinity of the peptides generated to MHC class I molecules, the frequency of T cell clones to a peptide, immunodomination which is the concept of suppression or elimination of subdominant T cells by dominant T cells[388]. Herein, I present a few determinants of immunodominance, however, there are many other factors that contribute.

In terms of studying CD8+ T cell responses, it is extremely important to consider immunodominance. During an infection, there is a massive CD8+ T cell response to different antigenic epitopes; however, certain CD8+ T cell clones will be more preferentially utilized and possess higher frequencies within the host. For example, within C57BL/6 (B6) mice, during a recall response to HKx31 after the initial priming with PR8, up to 80% of the IAV-specific CD8+ T cells are nucleoprotein$_{366\text{-}374}$ peptide (NP_{366})-specific CD8+ T cells[389–392]. This is a large proportion of the IAV-specific CD8+ T cells responding to one antigenic peptide and failing to examine this dominant response would hinder the ability of the researcher to make conclusions on IAV-specific CD8+ T cell responses. This idea can be applied to many different infections as nearly all infections would generate CD8+ T cells that are either dominant or subdominant. When performing experiments that examine cognate CD8+ T cell responses, it is also important to examine subdominant CD8+ T cells. Subdominant CD8+ T cells contributes to heterosubtypic immunity, which is the concept of having multiple CD8+ T cells specific for the antigen of interest. Heterosubtypic immunity contributes heavily to the protection of a host because it is better to have multiple responders rather than just one or two; in an analogy, it is bad to

have all of eggs in one basket compared to having eggs in multiple baskets. Within my study, I utilized the concept of immunodominance to perform the experiments. In B6 mice, there are six immunogenic epitopes that have been discovered[355,389,393,394]: NP_{366}, polymerase acidic protein$_{224\text{-}233}$ peptide (PA_{224}), polymerase basic protein $1_{703\text{-}711}$ peptide ($PB1_{703}$), non-structural protein $2_{114\text{-}121}$ peptide ($NS2_{114}$), polymerase basic protein-$F2_{62\text{-}70}$ peptide (PB1-$F2_{62}$), and matrix protein $1_{128\text{-}135}$ ($M1_{128}$). During the primary response, the majority of the CD8+ T cell response is from T cell specific for: NP_{366} and PA_{224}, while the others are subdominant. In terms of the recall response, NP_{366}-specific CD8+ T cells are dominant and constitute approximately 80% of the IAV-specific CD8+ T cells[389–392]. Despite our experiments not examining the subdominant IAV-specific CD8+ T cell responses, we did examine the dominant responses. This would give us a good indication on how IAV-specific CD8+ T cells during sepsis are responding, however, in future studies, we may broaden our scope to examine subdominant CD8+ T cell responses to further characterize heterosubtypic immunity.

1.4 Project Rationale

1.4.1 Rationale for Studying CD8+ T Cell Responses During Protracted Sepsis

Within the introduction of this thesis (section 1.2.3), I examined the current knowledge on sepsis induced immunosuppression and the affect it has on naïve and memory CD8+ T cell responses. Hence, within this section, I will just provide an overview on this research, rather than discussing in-depth the findings.

Rationale for examining antigen-specific naïve CD8+ T cell responses during sepsis-induced immunosuppression

In terms of naïve CD8+ T cells, it has been previously shown that these cells experience a numerical loss and faulty recovery as evidenced by the 'gaps' within their TCR repertoire[180,193] and this has clinical implications as TCR diversity inversely correlates with susceptibility to secondary infections and mortality within septic patients[191]. Multiple groups have shown that naive CD8+ T cell functionality in response to cognate antigens are severely diminished within the septic environment as evidenced by the reduced expression of various cytokines and cytolytic effector molecules, with both early and late responses being diminished[180,267]. However, despite these findings, there have been reports of either unaffected or enhanced responses by naïve antigen specific CD8+ T cells during a primary response[266,267]. All the studies that have examined early responses (two days post-CLP) found a dampened response from naïve antigen specific CD8+ T cells; however, this phenotype might just be due to the numerical cell loss that occurs during sepsis, as it has been previously shown that lymphopenia is most pronounced two days after sepsis[395,396]. Furthermore, the efficacy of naïve CD8+ T cell responses is determined by the number of naïve antigen specific CD8+ T cells at the time of infection[397,398], so it makes sense that groups would notice a severe impairment within their responses. In terms of later responses after sepsis, there is more controversial findings. For instance, within a study by Condotta[267], it was discovered that inducing a primary response nine days post-CLP led to a diminished response from naïve antigen specific CD8+ T cells; however, inducing a

primary response 30 days post-CLP, there was no reduction in responses compared to sham. Within another study by Condotta et al.[180], the authors discovered that inducing a primary response 30 days post-CLP led to a reduced response from naive antigen specific CD8+ T cells. Moreover, within a study by Markwart et al.[266], it was demonstrated that inducing a primary response 10 days post-CLP led to a significantly higher functionality of naive antigen specific CD8+ T cells. From all this information, it is clear that the effect sepsis has on naïve antigen specific CD8+ T cell responses are still controversial, especially at later time points. Currently, it is not well understood how sepsis affects naïve antigen specific CD8+ T cell responses during a primary infection in the context of sepsis-induced immunosuppression, which is why my study is important as I will address this gap within the literature. Compared to other groups, I have chosen a day-four timepoint after sepsis induction to induce a primary response, which I then examined naïve antigen specific CD8+ T cell responses at the peak primary CD8+ T cell response; this has not previously been done before. This time point was chosen based on the kinetics of immunosuppression in CLP survivors. Many groups have discovered that at this timepoint, there is a partial recovery of the naïve CD8+ T cell pool[273,395] and this may severely affect the results as having more antigen specific naïve CD8+ T cells will enhance the responses. The point of this study is to examine the primary response at four days post-CLP to get a better understanding of the naïve CD8+ T cell responses during sepsis, previously no one has looked at this time point, which would provide incremental knowledge on sepsis progression and their responses. Furthermore, in my research, I can make comparisons between the naïve and memory CD8+ T cell responses as we have done both experiments, which has not previously been done.

Rationale for examining memory CD8+ T cell responses during sepsis-induced immunosuppression

In terms of the memory CD8+ T cell responses, there is evidence to suggest that sepsis-induced immunosuppression has the capacity to dampen recall responses[275], however, there is a lot missing information from this pool of knowledge. First, I will briefly touch upon the research that has been done, then I will discuss what has not been done. Currently, there are many studies that have examined pre-existing memory CD8+ T cell numbers and function after sepsis[194,271,272,275,399]. Despite these studies being powerful, they had not addressed the recall responses at the peak memory CD8+ T cell response *in vivo.* Although, there is only one study, as far as I know, that addresses recall responses within the context of sepsis-induced immunosuppression. Within a study by Duong et al.[275], the authors had examined antigen specific memory CD8+ T cell secondary expansion. The researchers induced a recall response four days post-CLP, then examined the absolute numbers and frequency of antigen specific memory CD8+ T cells six days post-infection within the blood. They had discovered that within the septic condition, there was a reduced frequency and absolute number of antigen specific memory CD8+ T cells, which would indicate that memory CD8+ T cells within the septic condition may have a decreased ability to undergo antigenic proliferation upon re-encounter of cognate antigen in the context of sepsis induced immunosuppression. Currently, there are no studies that have examined the antigen specific memory CD8+ T cell functionality during a recall response in the context of sepsis-induced immunosuppression; furthermore, the one study that has been done had only studied the secondary expansion within the blood, which discounts the expansion within different tissues such as the spleen, lungs, and liver, amongst others. In terms of functionality, all of the aforementioned studies have addressed the functionality of pre-existing antigen specific memory CD8+ T cells by stimulating them *ex vivo* with their cognates peptides, however, the researchers had not induced a recall response, which would affect their results as they are not measuring the functionality at the peak memory CD8+ T cell response. These methods are good for examining early responses to cognate antigen; however, it is extremely important to examine their responses at the peak T cell response during a recall response to get a full understanding of how these cells are affected during

sepsis and their ability to respond to previously encountered antigens. To date, there are no studies examining the antigen specific memory CD8+ T cell functionality at the peak T cell response during a recall response in the context of sepsis-induced immunosuppression. From all the aforementioned reasons, our study is extremely important in filling in the 'gaps' on how memory CD8+ T cells respond during sepsis-induced immunosuppression. Our study is novel as no one has previously addressed phenotypic differences, functionality, and numerical differences of antigen specific memory CD8+ T cells during an *in vivo* recall response at the peak memory T cell response within different tissues.

Rationale for utilizing the CLP mouse model of polymicrobial sepsis

In performing my study, I have chosen the CLP mouse model of polymicrobial sepsis as it has many advantages over other models of sepsis[400–402]. Firstly, the major advantage that it possesses is that it accurately recreates human sepsis progression. Compared to human sepsis, the CLP model causes similar hemodynamic and metabolic alterations within the mice. Furthermore, within this model, fluid resuscitation is required as failing to administer intravenous fluid will prevent the mouse from experiencing the different stages of hemodynamic states; this is advantageous as it more closely resembles the therapeutic treatment in septic patients. In addition to mimicking the hemodynamic and metabolic alterations, within this model, both inflammatory and anti-inflammatory processes are present; mice go through the initial hyperinflammatory phase, then the subsequent immunosuppressive phase, which closely mimics the sepsis progression within humans. Secondly, this model has a similar development of polymicrobial peritonitis with an associated tissue ischemia, which is due to the traumatic insult from the surgery. Due to the nature of the surgery, there is the presence of a septic focus, which is extremely important for peritonitis as it allows the leakage of microbial pathogens into the peritoneum. This septic focus is advantageous over methods that provide a bolus of pathogenic material because (1) it provides a continuous source of microbial species, which is clinically more relevant and (2) it provides diverse microbial species as it is not limited to the composition of the bolus. Furthermore, the tissue ischemia is also an advantageous aspect to CLP as it also contributes to the immune dysfunction observed in sepsis. Third,

sepsis severity may be altered by changing the length of ligation, the number of punctures within the cecum and the size of the needle used. Fourth, there is the presentation of organ dysfunction. For example, it has been demonstrated within the CLP mouse model that there is the presence of ALI, among other organ dysfunctions. This makes the model more clinically relevant as it mimics the sequelae from sepsis. Fifth, there is an opportunity to make the CLP mouse model more clinically relevant by either administering antibiotics or performing source control methods, which are commonly seen during the treatment for sepsis. Hence, considering all these reasonings, the CLP mouse model of polymicrobial sepsis is a great model that closely mimics the progression of human sepsis.

1.4.2 Importance of Studying CD8+ T Cell Responses in the Context of Sepsis-Induced Immunosuppression

Studying CD8+ T cell responses during sepsis-induced immunosuppression is important for our understanding of sepsis because they are major effectors in the immune response to different pathogens. It has been previously shown that knocking out CD8+ T cells enhances susceptibility to pathogens and increases mortality, with multiple groups showing that CD8+ T cells are required to protect against infections, such as IAV, among other deadly diseases[373–380]. In terms of sepsis, it has been previously demonstrated that patients with sepsis experience higher levels of CD8+ T cell exhaustion after the hyperinflammatory phase, which was correlated with poor septic outcome[403–405]. From this information, it could be suggested that CD8+ T cell dysfunction contributes to mortality after the resolution of hyperinflammation during sepsis.

CD8+ T cells may be protective under certain circumstances, but it has been shown that they are also able to contribute to severe pathology. When these cells are hyperactive within tissues, they enhance tissue pathology, as was seen by the studies examining the CD8+ T cell responses during IAV infection. CD8+ T cells are a double-edged sword as they are protective but can also induce pathology[381–386]. In terms of sepsis, it has been shown that memory CD8+ T cells contribute heavily to mortality and tissue pathology during the hyperinflammatory phase as they have enhanced inflammatory mediators and innate immune responses leading to increased mortality and tissue pathology[395,396]. Human populations contain many populations of memory T cells, and this could partly explain why patients are extremely susceptible to sepsis-associated death. Furthermore, the elderly population tends to have a higher mortality from sepsis compared to younger individuals, which is evident within clinical data as the majority of individuals that succumb to sepsis are older patients. This is an important point as elderly individuals tend to be considered a 'memory population' because they are more highly constituted with memory T cells compared to younger individuals. These pieces of information add to the importance of why studying CD8+ T cells in sepsis is important as they could potentially be mediating a significant portion of the aberrant responses seen in sepsis. T cells may be pathogenic and

protective during the course of sepsis; however, it is currently not well understood what occurs to these cells and their responses. From all of the aforementioned reasons and observations, there is a critical need to understand these double-edged swords within the context of sepsis as it would enhance our knowledge on sepsis as a whole, and lead to potential elucidation of therapeutic targets.

1.4.3 Hypothesis and Objectives

Hypothesis: During protracted sepsis, naïve and memory antigen specific CD8+ T cells will demonstrate a reduced frequency and absolute number within the different tissues at the peak CD8+ T cell response; additionally, these cells will possess an enhanced exhausted phenotype with an associated reduced activation phenotype. Furthermore, at the peak CD8+ T cell responses, antigen specific CD8+ T cells will display a reduced functionality in response to their cognate antigens.

Aim 1: Immunophenotyping and functional assessment of IAV-specific CD8+ T cells at the peak memory CD8+ T cell response within different anatomical locations during protracted sepsis.

Aim 2: Immunophenotyping and functional assessment of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response within different anatomical locations during protracted sepsis.

Chapter 2: Methods

2.1 Animal Experiments

2.1.1 Mice Used in Experiments

B6 mice were ordered from Charles River Laboratories and housed in a specific pathogen-free barrier facility in Medical Science Building, room 607/608, Western University. Male B6 mice were ordered at 6 weeks of age and depending upon the experimental aim, their age was different. For aim 1, mice underwent surgery between the age of 18-24 weeks, whereas for aim 2, they underwent surgery from the age of 8-12 weeks. In each independent experiment, age-matched mice were used. Mice were euthanized by cervical dislocation at their humane or experimental endpoint, which will be further discussed in section 2.1.2 (Cecal Ligation and Puncture Procedure). Experimental procedures were approved by the Animal Care Committee at Western University under protocol number 2018-122; Appendix 3)

2.1.2 Cecal Ligation and Puncture Procedure

All mice, regardless of whether they were used in aim 1 or 2 received the same CLP surgery. Surgeries were booked at least 1 week prior to surgery date through the 'Animal Care and Veterinary Services' (ACVS) website. On the website, the link 'Procedure room/anesthetic machine/Or booking' was used to book the surgical suite. All surgeries were performed in Medical Science Building room 5000a. The anesthetic machine, heat lamps, blankets and water-blanket were all provided by ACVS prior to surgery. The list of ingredients and procedure for the surgery are provided (Appendix 1).

Note: For all the surgeries, I have done 2 pokes with a 27.5G needle and a 0.5-cm ligation from the apex of the cecum. Furthermore, within the first 24 hours post-surgery, mice were monitored every two hours to assess for morbidity and mortality. After 24 hours, mice were monitored for morbidity and mortality every 12 hours throughout the duration of the experiment (11 days post-surgery). Mice were monitored using the Murine Sepsis Score

(MSS)[406], which I have provided the monitoring criteria (Appendix 2). Mice were administered two-doses of buprenorphine subcutaneously at the time of surgery and 24 hours after surgery; each dose was at a concentration of 0.05 mg/kg. In terms of humane endpoint, mice displaying a score of ~15-17 or more on the MSS were sacrificed due to severe morbidity.

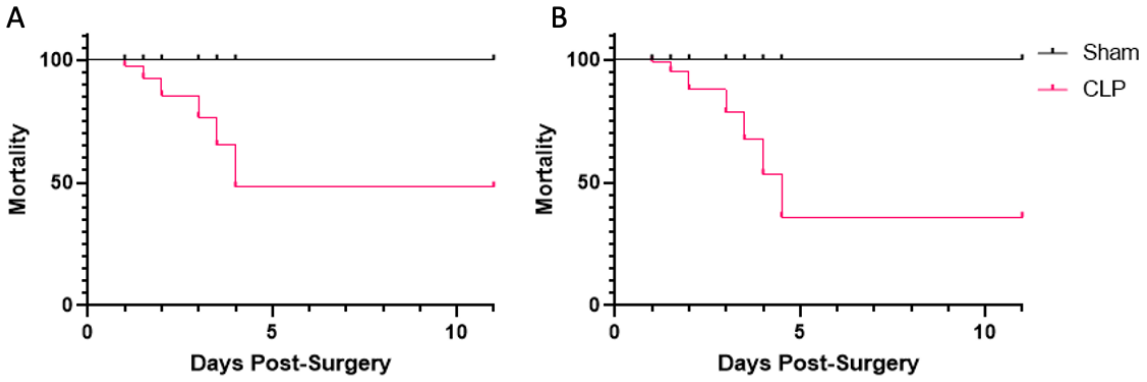

Figure 6. Survival curve for the primary and recall response. This figure represents the mortality of all mice that had undergone surgery based on their experimental aim (recall and primary response). Survival was measured throughout the experimental timeline (11 days after surgery). (A) Survival curve for the mice that had undergone the experimental aim 2 (primary response). This graph overall represents 39 individual mice; 29 mice that had undergone CLP surgery, while 10 mice underwent sham surgery (CLP: n=29; Sham: n=10). This data is pooled from 4 different independent experiments with approximately 7-8 mice that had undergone CLP per experiment and 2-3 mice that had underwent sham surgery per experiment (CLP: 7-8 mice/experiment; Sham: 2-3 mice/experiment). (B) Survival curve for the mice that had undergone the experimental aim 1 (recall response). This graph overall represents 47 individual mice; 32 mice that had undergone CLP surgery, while 15 mice underwent sham surgery (CLP: n=32; Sham: n=15). This data is pooled from 6 different independent experiments with approximately 4-6 mice that had undergone CLP per experiment and 2-3 mice that had underwent sham surgery per experiment (CLP: 4-6 mice/experiment; Sham: 2-3 mice/experiment). (A and B) total number of mice used: 61 mice for CLP and 25 mice for sham (CLP: n = 61; sham: n = 25).

2.2 Cell Culture and Stimulation Reagents

2.2.1 Standard Equipment and Procedures

Cells intended for stimulation assays and culturing were incubated in a HERACELL 150 incubator (Thermo Fisher) set at 37° C with 6% CO_2. All centrifugation was done in either Allegra 6R or Allegra X15R centrifuge (Beckman Coulter). The majority of the centrifugation steps were done at 456 x *g* (~1500RPM) for five minutes in 4° C, which I will further refer to as 'standard spin'. All washes used these settings on the centrifuge.

In terms of counting cells, after processing different tissues, the cells were re-suspended in various amounts of Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich) depending on the pellet size of the leukocytes. For the majority of the cells counted, 20 μL of the processed cells (re-suspended in PBS) was taken out and pipetted into a 96-well U bottom plate and was diluted with 180 μL of PBS (dilution factor = 10). After, 20 μL from the previously diluted well is taken and placed into a new well, and 20 μL of Trypan blue was added to this well (dilution factor = 2). Next, 10 μL of this Trypan blue and cell mixture was placed inside the well of a haemocytometer and counted under the microscope at 40X.

2.2.2 Cell Culture

Cells were cultured in complete Roswell Park Memorial Institute (cRPMI) medium, which consists of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; heat-inactivated and 0.3 μm-filtered, Wisent Bioproducts; two 15-mL tubes containing frozen FBS were allowed to reach room temperature and dumped into a 500 mL bottle of RPMI 1640), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM glutaMAX, 10 mM HEPES, minimum essential medium non-essential amino acids, and 1 mM sodium pyruvate (Gibco).

2.2.3 Stimulating Reagents

To detect antigen specific CD8+ T cells, we stimulated bulk leukocytes with immunogenic peptides (Table 3). These peptides were added to cells at a final working concentration of 500 nM, in a final volume of 200 μL for 5 hours. For more details on the stimulation and intracellular staining (ICS), please refer to section 2.5.3. Purified peptides (>95%) were provided to our lab by Dr. Jack Bennink and Dr. Jonathan Yewdell (National Institutes of Health, Bethesda, MD) and stored at -30° C in dimethyl sulfoxide (DMSO) at a peptide stock concentration of 1 mM. The peptides were purified by high-performance liquid chromatography and analyzed by mass spectrometry at the National Institutes of Health.

Table 3. Immunogenic peptides used in study. This table represents the peptides utilized during the *ex vivo* stimulation assays. All three were used for *ex vivo* stimulation, GP_{33-41} was used a control peptide as it is MHC D^b restricted, just as the other two peptides. $NP_{366-374}$- and $PA_{224-233}$-specific CD8+ T cells were detected using tetramer reagents loaded with MHC D^b molecules loaded with either peptide.

Peptide Name	Virus	The Protein Origin of Antigenic Peptide	MHC-Restriction	Peptide Sequence
$NP_{366-374}$	IAV (PR8)	Nucleoprotein	D^b	ASNENMETM
$PA_{224-233}$	IAV (PR8)	Acidic Polymerase	D^b	SSLENFRAYV
GP_{33-41}	LCMV	Glycoprotein	D^b	KAVYNFATC

2.3 Mouse Cell Isolation

2.3.1 Splenic Cell Isolation

After cervical dislocation, spleens were removed from the abdominal cavity of mice and placed in 15 mL tubes on ice. After the isolation of all tissues, spleens were homogenized in cold PBS using a glass homogenizer with the 'TIGHT' glass pestle. Homogenate was then passed through a 70 μm filter to acquire a single cell suspension and to get rid of loose tissue and debris. Cells were then pelleted using the standard spin on the centrifuge and then subjected to Ammonium-Chloride-Potassium (ACK) lysis buffer to remove red blood cells. Splenocytes were typically incubated in 3 mL of ACK lysis buffer for 3 minutes; however, depending on the size of the white pellet and the amount of red blood cell contamination, the amount and time of ACK lysis buffer incubation may differ. Splenocytes were washed, and re-suspended in cold PBS for cell counting (described in section 2.2.1)

ACK recipe:

1. Put 800 mL of distilled water into a suitable container (glass)
2. Add 8.02 g of ammonium chloride
3. Add 1 g of potassium bicarbonate
4. Add 0.0372 g of disodium Ethylenediaminetetraacetic acid (EDTA)
5. Adjust the pH to 7.2-7.4 (using hydrochloric acid and sodium hydroxide)
6. Add distilled water until the volume is 1 L

2.3.2 Peritoneal Cavity Mononuclear Cell Isolation

After cervical dislocation, mice were placed on their backs. The abdomen was sprayed with 70% ethanol and massaged to dislodge any PECs that are adherent to the peritoneum and organs. A small horizontal cut was made in the abdominal skin, which was used to tear open the skin to expose the peritoneal membrane. A dulled out 18.5G needle (dulled by rubbing the tip of the needle back and forth on a forceps gridded handle) in a 10 mL syringe

filled with 10 mL of sterile cold PBS was gently pierced through the peritoneal membrane. The syringe contents were emptied into the peritoneal cavity, allowing the cavity to expand and then the fluid was aspirated back into the syringe. Once the needle was removed from the peritoneal cavity, it was cut open to collect any remaining PBS. A note of the amount of PBS with PECs that was aspirated was taken to determine cells/mL. The PECs were pelleted by standard spin and depending on if there was presence of red blood cells, this pellet would be subjected to ACK lysis buffer. Either after pelleting or ACK lysis buffer washing, the PECs were re-suspended in cold PBS for cell count.

2.3.3 Lung Non-parenchymal Mononuclear Cell Isolation

Lungs were cut out from the euthanized mouse, without the heart, and placed into a small 1.5 mL tube with cold PBS. The lungs were cut into fine pieces with scissors and placed into a 15 mL tube with 4 mL of collagenase type IV solution (Sigma-Aldrich, 0.5 mg/mL in RPMI 1640). The tubes were subsequently placed in the incubator for one hour, while continuously being rotated at a fast pace. After incubation, the lung homogenate was pushed through a 70 μm filter by a 10 mL syringe plunger. This solution was then washed, and exposed to ACK lysis buffer, and then washed again and re-suspended in cold PBS for cell counting.

2.3.4 Liver Non-Parenchymal Mononuclear Cell Isolation

After cervical dislocation, the liver was excised and placed into a petri dish. A razor blade was used to chop up the liver into smaller chunks to make it easier to homogenize. Once the liver was chopped up into smaller bits, it was placed in a glass homogenizer and homogenized with a ‘loose’ pestle. The homogenized liver was washed and then re-suspended in 12.5 mL/liver of a 33.75% Percoll solution in PBS (GE Healthcare). The mixture was centrifuged at 700 x g for 12 minutes at 20° C without the brake. The pellet consists of non-parenchymal cells, which contains the mononuclear cells from the liver. The supernatant and parenchymal cells were aspirated out and the remaining pellet was

washed. After washing, the cells were subjected to ACK lysis buffer and washed again and then re-suspended in cold PBS for cell counting.

2.4 IAV Inoculation

10-day-old embryonated chicken eggs were used to propagate all IAV strains, then the allantoic fluid was filter-sterilized and stored at -80° C until use. Viral infectious titers were determined through $TCID_{50}$ (median tissue culture infectious dose; dose at which 50% of the cells are infected) assay using Madin-Darby canine kidney (MDCK) cells. For this thesis, only two mouse-adapted strains were used: PR8 (Puerto Rico/8/1934/H1N1) and the X31 reassortant virus (core: Puerto Rico/8/1934/H1N1; coat: A/Hong Kong/1/1968/H3N2). The utility of using the X31 reassortant virus will be discussed further in the section 2.4.2.

Two models of IAV inoculation were used: primary and recall response. These two IAV inoculation methods will be further discussed in the next two sections (2.4.1 and 2.4.2)

2.4.1 Recall anti-IAV Response in Mice Undergoing CLP

In this model, I wanted to look at the memory antigen specific CD8+ T cell response in the context of a recall response during sepsis-induced immunosuppression. The question that this would answer: how are memory IAV-specific (antigen specific) CD8+ T cells responding to IAV during sepsis-induced immunosuppression at the peak memory CD8+ T cell response. This aim would establish the phenotype and functionality of memory IAV-specific CD8+ T cells in the recall response during sepsis-induced immunosuppression.

Two months prior to CLP surgery (or sham), mice were injected I.P. with PR8 mixed with PBS (225 μL of PR8 and 275 μL of PBS; >25,000 $TCID_{50}$). The purpose of waiting two months, prior to the surgery, is to establish immunological memory for this virus in these mice. The immune response is typically about one month with the last week being involved in the contraction phase. To ensure that I was working with a true memory cell population

that is stable, I decided to wait an extra month on top of the month it takes to establish immunological memory. Two months after I.P. injection, mice underwent CLP surgery (or sham). Four days post-surgery, during the immunosuppressive phase of sepsis, the survivors were injected I.P. with X31 reassortant virus mixed with PBS (225 μL of X31 and 275 μL PBS; >3,000 $TCID_{50}$). Seven days post-X31 I.P. injection, at the peak memory CD8+ T cell response, mice were euthanized, and surface staining and ICS were performed. The experimental design is outlined in Figure 5. Of important note, during the initial priming with PR8, mice generate antibodies to the PR8 coat (H1N1). X31 reassortant virus was used to induce a recall response in these mice because they possess a different coat (H3N2), which would avoid the neutralizing antibodies against PR8 during the priming. X31 contains a PR8 core, which contains internal proteins to illicit an immune response, which it originally already developed during the PR8 priming.

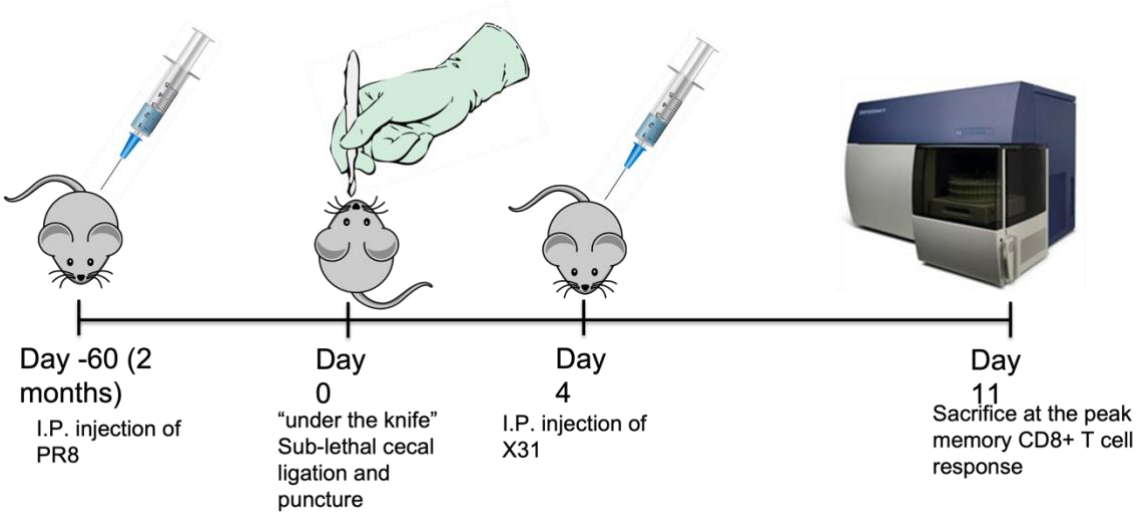

Figure 7. Schematic representation of the recall response experiments.

2.4.2 Primary Anti-IAV Response in Mice Undergoing CLP

In this model, I wanted to look at the antigen specific CD8+ T cell response in the context of a primary response during sepsis-induced immunosuppression. The question that this would answer: how naïve IAV-specific (antigen specific) CD8+ T cells respond to IAV during sepsis-induced immunosuppression. Hence, this aim is to establish the phenotype and functionality of IAV-specific CD8+ T cells in the primary response during sepsis-induced immunosuppression at the peak primary CD8+ T cell response.

CLP surgery (or sham) was performed on these mice, thereafter, four days after the induction of sepsis, the survivors were administered 500 μL intraperitoneally (I.P.) of PR8 mixed with PBS (225 μL of PR8 and 275 μL of PBS; >25,000 $TCID_{50}$). Seven days post I.P. injection of PR8, at the peak CD8+ T cell response, the mice were euthanized, and surface staining and ICS were performed. The experimental design is outlined in Figure 6. Of important note, IAV administered I.P. is unable to produce an active infection because the peritoneal cavity lacks an enzyme involved in the release of new virions. Despite lacking the enzyme, IAV can still replicate its RNA genome and produce its proteins within certain cell types, such as dendritic cells. The dendritic cells will present peptides to mount an immune response against the virus, which makes this model a good IAV vaccination model.

I decided to use PR8 – rather than X31 – because I wanted the priming step in generating the primary CD8+ T cell response to be the same between the recall and primary experiments. During the recall response, PR8 is used to prime the naïve IAV-specific CD8+ T cells, while X31 is used to induce a recall response. X31 contains the same internal proteins; however, it differs to PR8 in the coat. I wanted to ensure that the priming step was the same to avoid any potential confounding factors that X31 priming would consist of.

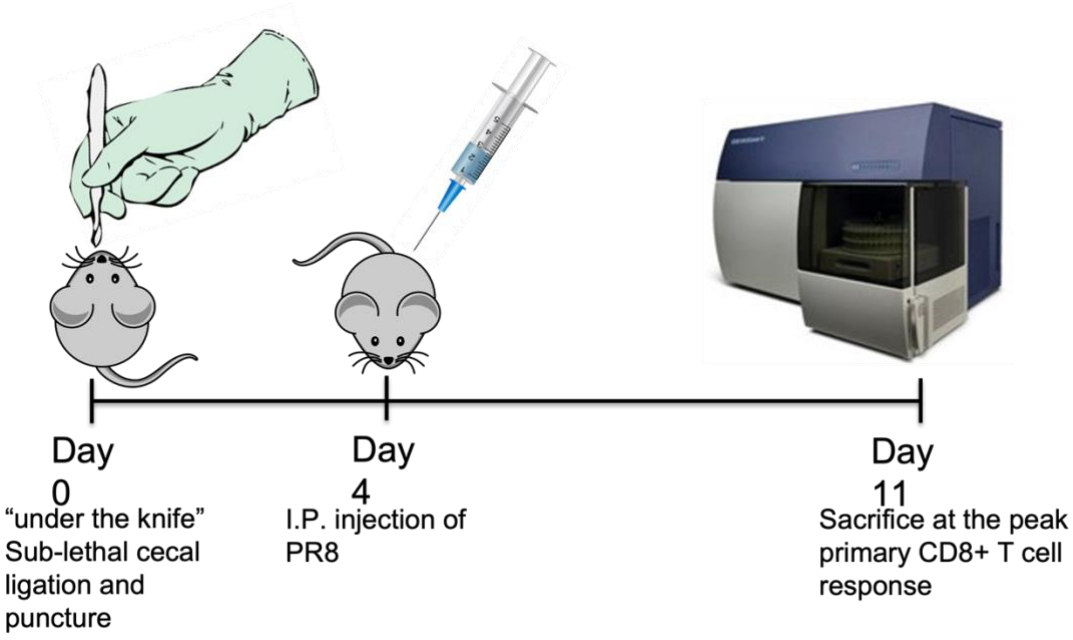

Figure 8. Schematic representation of the primary response experiments.

2.5 Cytofluorimetric Analyses

2.5.1 Equipment and Software

Flow cytometry experiments were performed using a FACSCantoII (Becton Dickinson), which was in the Hearyfar lab (in-house flow cytometer). The software that was used for data collection was FACSDiva software version 6 (Becton Dickinson). Compensation was performed by using OneComp eBeads (Thermo Fisher), with one tube made for each antibody or tetramer color present in the panels. One drop from the OneComp eBeads was put into a 5 mL flow tube and 1 μL of the appropriate antibody (antibody used in the panel) or an antibody with the same color (tetramer) was mixed with the OneComp beads. This mixture was incubated for 15 minutes to allow for the binding of the antibody to the beads. In the majority of the experiments, 1 million cells from each sample were stained, however, depending on cell yield from the tissue, different amounts of cells were stained particularly from the liver, lung and PECs.

The FACSDiva software calculated and applied the compensation to each of the sample tubes prior to the acquisition of data. Following collection of data, FCS files were exported from FACSDiva and transported onto my personal computer for analysis using FlowJo version 10.

Total cell numbers for a specific population were determined by multiplying each gating frequency with the total number of cells found during the cell counting.

2.5.2 Surface Staining

After tissue isolation and processing, all cells were re-suspended in cRPMI at a concentration of 10 million cells per mL (depending on the cell yield of the tissue; needed minimum 0.6 mL for ICS and surface stain). 100 μL of the cell suspension was taken and placed into a 5 mL flow cytometry tube (1 million cells) and was washed twice with PBS + 2% FBS (referred to as staining buffer). The cells were washed twice to get rid of any cRPMI that was within the tube. In terms of the centrifugation, for all washes, they underwent standard spin (456 x g (~1500RPM) for five minutes in 4° C). After the second wash step, the supernatant from the 2.4G2 hybridoma was used as fragment crystallizable (Fc) block in all staining protocols. Of important note, the 2.4G2 hybridoma produces monoclonal immunoglobulin G (IgG) antibodies that bind to the Fc receptor γ II/III (FcRγ II/III; CD32/CD16), which prevents the non-specific antibody binding due to these receptors. Each tube was re-suspended in 20 μL of Fc block and placed in a 4° C fridge for 5-10 minutes in the dark for incubation. Thereafter, antibodies mixed with staining buffer were put in each appropriate tube to bring the total volume to 100 μL. 80 μL was administered to each tube, and antibodies were added at a dilution factor of 200 for the final volume (100 μL /200 = 0.5 μL/tube) and tetramers were added at a dilution factor of 400 for the final volume (100 μL/400 = 0.25 μL/tube). Once added, they were incubated in a 4° C fridge for 20 minutes in the dark, after which, cells were washed twice and re-suspended in 100-200 μL of staining buffer and read on the flow cytometer.

Of important note, tetramers are a reagent that allows the detection of antigen specific T cells by utilizing the MHC complex[407]. Tetramers are composed of four MHC molecules that are biotinylated, which bonds with streptavidin conjugated to a fluorophore. The MHC molecules are loaded with the peptide of interest. For example, in this study, I have used the tetramer that detects NP_{366}-specific CD8+ T cells; each MHC in this tetramer contains the peptide $NP_{366-374}$, which allows CD8+ T cells that are specific to this peptide to bind.

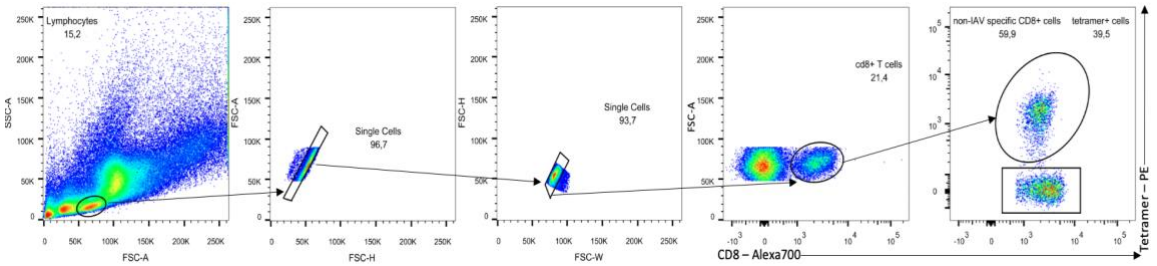

Figure 9. Gating strategy to detect IAV-specific CD8+ T cells by tetramer reagent. First, I had gated on the lymphocyte population within the side scatter-area (SSC-A) vs. forward scatter-area (FSC-A) flow plot. Subsequently, to exclude doublets and dead cells/debris, I had applied two different gates: FSC-A vs. forward scatter-height (FSC-H); FSC-H vs. forward scatter-width (FSC-W). After doublet exclusion, I gated on CD8+ T cells. The final flow plot represents the frequency of tetramer positive CD8+ T cells and the non-tetramer positive CD8+ T cells (non-IAV-specific CD8+ T cells).

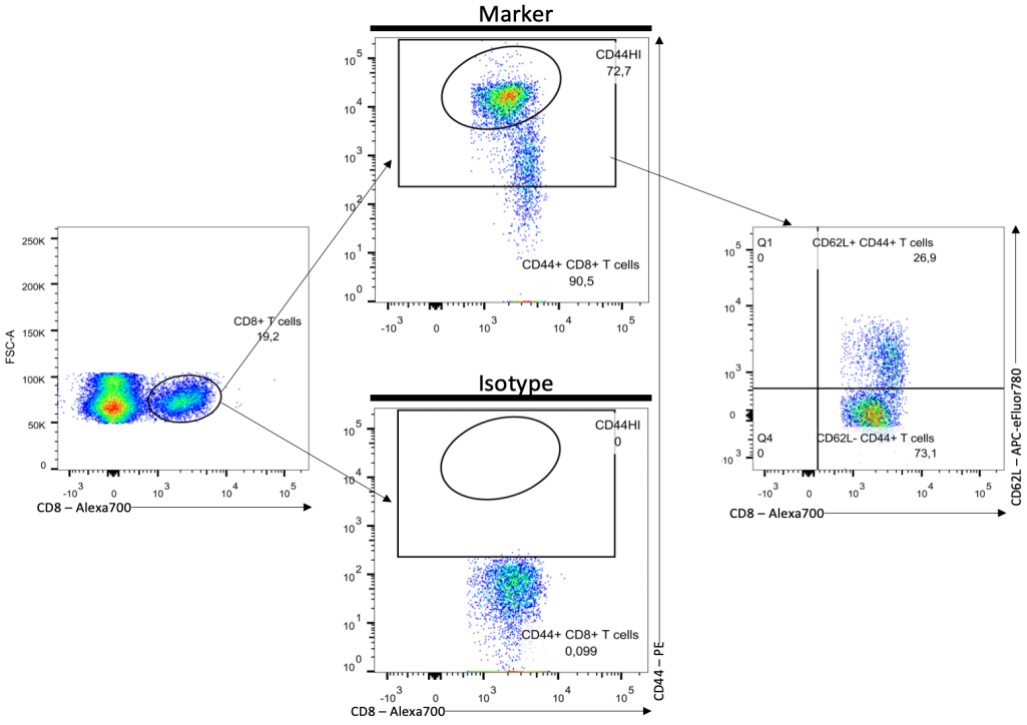

Figure 10. Gating strategy to detect the different memory CD8+ T cells subsets. First, the gating strategy to gate on CD8+ T cells is the same as Figure 9. I gate on CD8+ T cells, then subsequently gate on CD44 expressing CD8+ T cells, which was determined based on an isotype control. The final flow plot represents cells that are either expressing or not expressing CD62L. Central memory CD8+ T cells are CD44+CD62L+, while effector memory CD8+ T cells are CD44+CD62L-.

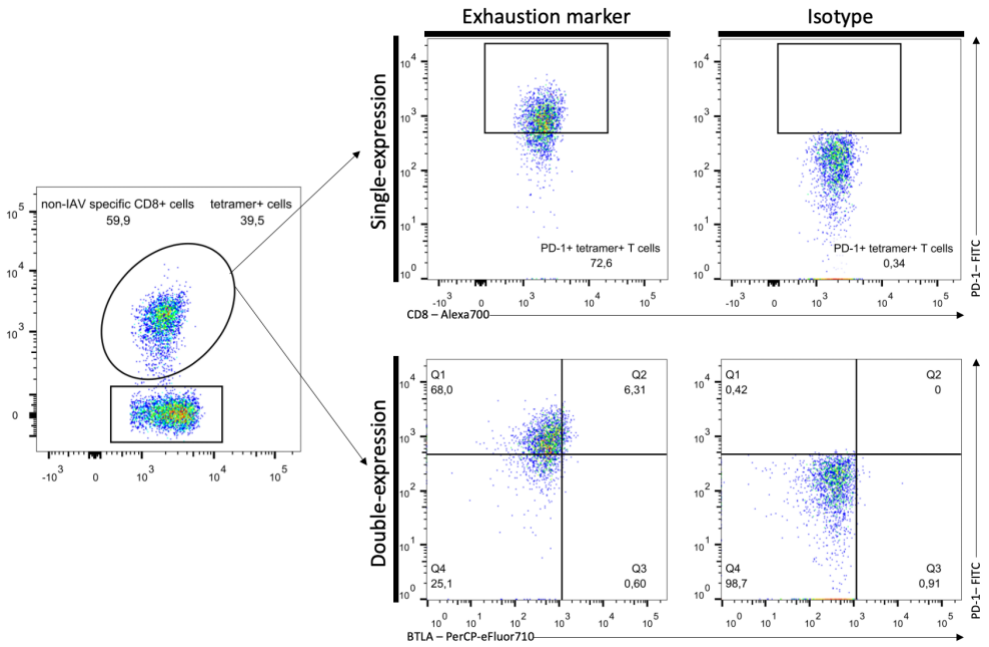

Figure 11. Gating strategy to examine the single- and double-expression of the various markers utilized within this study. Important note: this is an example of the gating strategy used throughout this thesis to examine either the single- or double-expression of the various markers. The gates for both the single- and double-expression were determined based on an isotype control. This strategy was employed for the IAV-specific CD8+ T cells, non-IAV-specific CD8+ T cells and central/effector memory CD8+ T cells.

2.5.3 Conditions and Intracellular Staining

For *ex vivo* stimulation of mouse cells, cells were placed in cRPMI after cell counting at a concentration of 10 million cells per tube (depending on cell yield and the number of cells needed for an experiment). Thereafter, 1 million cells were seeded in a 96-well U-bottom plate (100 μL), and a 100 μL mixture of PBS and the stimulating peptide was placed in each appropriate well, which led to a total volume of 200 μL. Stimulating peptides had a working concentration of 500 nM, which is a dilution of 2000 from the stock solution of peptides (1 mM). Of important note, the conditions were not ideal as they received half of the nutrients they need for the stimulation; however, the stimulation was only 5 hours long and the color of the media faded after this time but not to the point of complete nutrient depletion. After 2 hours of incubation, Brefeldin A (BFA, Sigma-Aldrich), which inhibits protein transport from the endoplasmic reticulum to the Golgi complex, was added at a concentration of 0.01 mg/mL in each well. The plates were then returned to the incubator for another 3 hours.

After 5 hours, the plate was removed from the incubator to perform staining. The cells were spun down at 1026 x g (~2100RPM) in 4°C for 5 minutes to cool down the cells. For all the next spins and washes, the cells were spun down at 1026 x g (~2100RPM) in 4°C for 3 minutes. After the initial spin, cells were re-suspended in 20 μL of Fc block for 5 minutes in the dark at 4°C. Following Fc block incubation, surface staining was performed with a mixture of either antibody or isotype with staining buffer and 50 μL of this mixture was placed into the appropriate wells (antibodies were diluted by 200). The plate was incubated in a 4°C fridge for 20 minutes in the dark. Thereafter, plates were washed twice with PBS (get rid of protein – absolutely no protein for fixation) and cells were fixed in 1% paraformaldehyde (Sigma-Aldrich) at room temperature for 15 minutes. The cells were then washed twice with PBS, and the intracellular antibody cocktail, which contains 0.1% saponin (Sigma-Aldrich) and antibodies at a dilution factor of 200 was added to each well at staining volume of 50 μL. Saponin is used to permeabilize cells, which allows the passage of antibodies into the cell. The plates were wrapped in parafilm and left to incubate

in the 4° C fridge in the dark overnight. Cells were washed twice with staining buffer and transferred to flow cytometry tubes for reading.

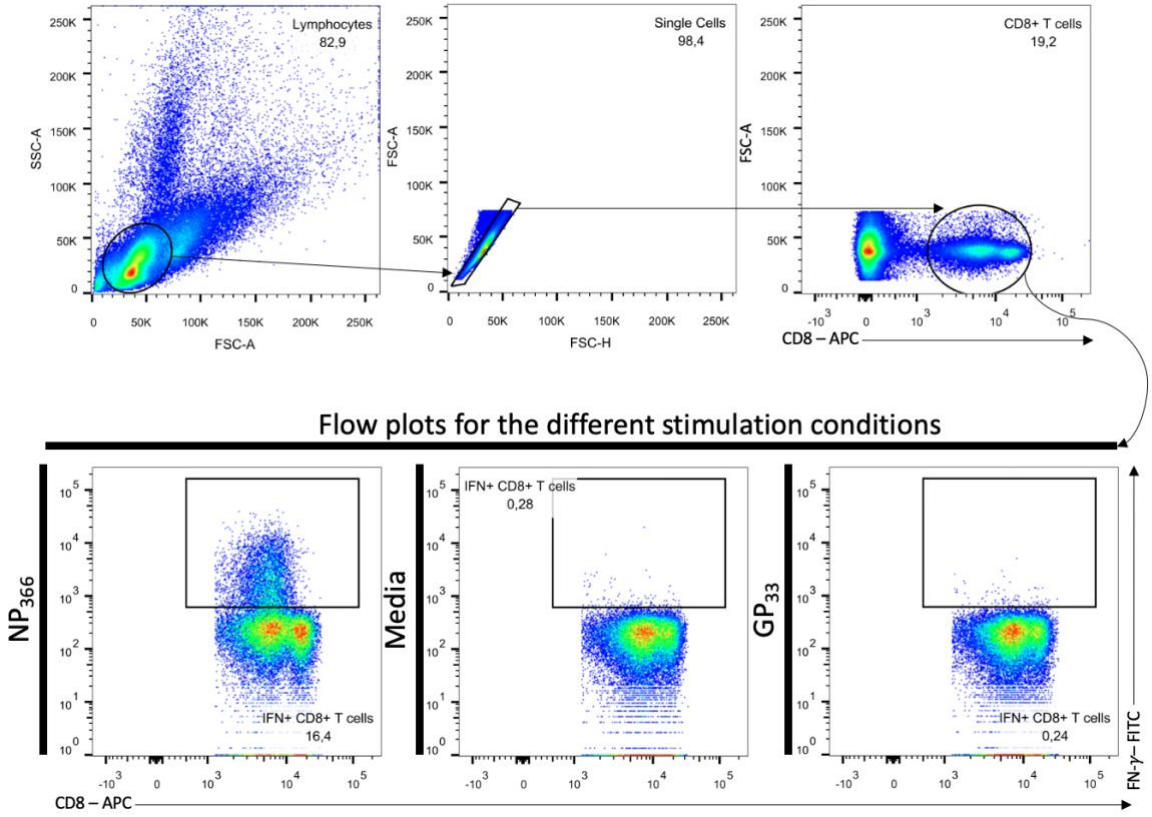

Figure 12. Gating strategy to detect IFN-γ producing IAV-specific CD8+ T cells. First, I gated on the lymphocyte population within the SSC-A vs FSC-A flow plot, then subsequently excluded doublets by gating on the FSC-A vs. FSC-H flow plot. I gated on the CD8+ T cells by gating on the FSC-A vs CD8-APC flow plot. The final flow plots represent the frequency of CD8+ T cells expressing IFN-γ under different stimulation conditions. There were four stimulation conditions: NP_{366}, PA_{224}, media and GP_{33}. The first two stimulation conditions were intended to stimulate the IAV-specific CD8+ T cells of interest, the third condition was intended to set the gate for the IFN-γ-producing CD8+ T cells, while the fourth condition was utilized to ensure that the stimulation was specific for IAV-specific CD8+ T cells as GP_{33} is a peptide to stimulate LMCV-specific CD8+ T cells and this peptide is restricted to the same MHC haplotype as the IAV peptides within this study. Note: the lymphocyte population is not at its regular location (view tetramer gating) due to the fixation step within the ICS protocol.

Table 4. Fluorophore-labeled anti-mouse antibodies

Target	Clone	Manufacturer	Fluorophore	Catalog number	Purpose/Detect
CD8a	53-6.7	eBioscience	APC	17-0081-82	CD8+ T cells
IFN-γ	XMG1.2	eBioscience	FITC	11-7311-82	Functionality and detect IAV-specific CD8+ T cells
CD8a	53-6.7	eBioscience	Alexa700	56-0081-82	CD8+ T cells
CD44	IM7	eBioscience	APC-eFluor 780	47-0441-82	Activation and memory subsets
CD44	IM7	eBioscience	PE	12-0441-82	Activation and memory subsets
CD62L	MEL-14	eBioscience	APC-eFluor 780	47-0621-82	Memory subsets
CD62L	MEL-14	eBioscience	PE-eFluor 610	61-0621-82	Memory subsets
B and T lymphocyte attenuator (BTLA)	6F7	eBioscience	PerCP-eFluor 710	46-5950-82	Exhaustion/activation
PD-1	J43	eBioscience	FITC	11-9985-82	Exhaustion/activation
Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)	UC10-4B9	eBioscience	APC	17-1522-82	Exhaustion/activation
TIM-3	RMT3-23	eBioscience	PE-Cy7	25-5870-82	Exhaustion/activation
2B4	M2B4 (B6) 458.1	Biolegend	APC	133518	Exhaustion/activation
Lymphocyte activation gene-3 (LAG-3)	eBioC9B7W	eBioscience	PE-Cy7	25-2231-82	Exhaustion/activation
V-domain Ig suppressor of T cell	MIH64	eBioscience	PerCP-eFluor 710	46-1083-82	Exhaustion/activation

activation (VISTA)					
T cell immunoreceptor with Ig and ITIM domains (TIGIT)	GIGD7	eBioscience	FITC	11-9501-82	Exhaustion/activation
CD25	PC61.5	eBioscience	PE-eFluor 610	61-0251-82	Early activation
CD69	H1.2F3	eBioscience	PerCP-Cy5.5	45-0691-82	Early activation
CD95	15A7	eBioscience	Alexa488	53-0951-82	Cell death
CD127	A7R34	eBioscience	PE-Cy7	25-1271-82	Memory generation/activation/ memory maintenance
TNF-α	MP6-XT22	eBioscience	PE-eFluor 610	61-7321-82	Functionality
Granzyme B	NGZB	eBioscience	PE	12-8898-82	Functionality
IL-2	JES6-5H4	eBioscience	PE-Cy7	25-7021-82	Functionality
Killer cell lectin-like receptor subfamily G member 1 (KLRG1)	2F1	eBioscience	PerCP-eFluor 710	46-5893-82	Exhaustion/activation/ functionality/memory generation/survival

2.6 Statistical Analyses

Flow cytometry results were analyzed using FlowJo version 10.0.7 software (Tree Star, Ashland, OR). Student's t test were performed using GraphPad Prism version 8.4.3 software (San Diego, CA). Data were plotted as mean ± standard error of the mean. Statistical significance is indicated where appropriate, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

Chapter 3: Results

3.1 Aim 1: Recall Response Results

3.1.1 There is a higher frequency of NP_{366}-specific CD8+ T cells within the septic spleen; however, these cells are numerically similar compared to the sham condition

To establish immunological memory to study memory IAV-specific CD8+ T cell responses, I had first immunized B6 wild-type mice with PR8 and waited two months prior to performing CLP (or sham) surgery. The purpose of waiting for two months was to ensure that I was dealing with a true memory population as there is overlap between the memory and contraction phase of the immune response[282,408]. Two months after PR8 priming, I had performed CLP surgery (or sham) to induce sepsis in these immunized mice, then waited four days to inject mice with X31. The purpose of waiting four days prior to inducing a recall response was to ensure that these mice were immunosuppressed[400,409,410]. Within the literature, it was reported that the onset of sepsis-induced immunosuppression occurs at day-three post-CLP[409]; however, it was demonstrated that at day-seven post-CLP, there is a recovery of the immune response to secondary infections[410]. Hence, within the CLP model, mice recover from sepsis-induced immunosuppression seven days after the septic insult. Furthermore, within a study by Muenzer et al.[410], the authors discovered that at four-days post-CLP, mice were extremely immunosuppressed as evidenced by their increased mortality to secondary infection and their reduced capacity to mount a protective immune response to secondary infection. Therefore, inducing a recall response at day-four post-CLP is appropriate to study the immunosuppressive effects on the memory IAV-specific CD8+ T cell response. After inducing a recall response, mice were sacrificed seven days later (11 days post-CLP), at the peak memory CD8+ T cell response[411] and subjected to surface and intracellular staining experiments.

The first objective of my study was to examine the frequency and absolute numbers of IAV-specific CD8+ T cells within the spleen at the peak memory CD8+ T cell response during protracted sepsis. To my surprise, I found increased frequencies of NP_{366}-specific CD8+ T cells within the spleen of septic mice by tetramer reagent staining (Figure 11. panel B, p = 0.0003); although, there was no differences in their absolute numbers compared to the sham condition (Figure 11. Panel B, p = 0.6). In terms of septic splenic PA_{224}-specific CD8+ T cells, there were no differences in their frequencies and absolute numbers compared to the sham condition as measured by tetramer reagent staining (Figure 11. Panel B, p = 0.8, 0.8).

In addition to examining the frequencies and absolute numbers of IAV-specific CD8+ T cells by tetramer reagent staining, I had also performed an ICS to detect IFN-γ-producing-IAV-specific CD8+ T cells to quantify their frequencies and absolute numbers within the spleen. To accomplish this, I had stimulated splenocytes *ex vivo* with the cognate peptides for PR8 (NP_{366} and PA_{224}) for five hours, then subsequently stained these cells with anti-IFN-γ and anti-CD8 antibodies to detect the frequencies and absolute numbers of IFN-γ-producing-CD8+ T cells. This process allowed me to specifically detect IAV-specific CD8+ T cells as the stimulation conditions were unique for IAV-specific CD8+ T cells (i.e. cognates peptides for PR8); however, of important note, the ICS assay relies on the functionality of IAV-specific CD8+ T cells rather than their absolute presence within the spleen. Hence, the frequencies and absolute numbers from the ICS may not reflect all the IAV-specific CD8+ T cells present within the spleen. Herein, I demonstrated that the ICS data corroborated my tetramer reagent findings as I discovered that IFN-γ-producing-NP_{366}-specific CD8+ T cells were higher in frequency within the septic spleen (Figure 11. Panel E, p = <0.0001); although, these cells were numerically similar compared to the sham condition (Figure 11. Panel E, p = 0.4). Furthermore, septic splenic IFN-γ-producing-PA_{224}-specific CD8+ T cells demonstrated an upward trend in their frequency (Figure 11. Panel E, p = 0.16); however, these cells did not differ in their absolute numbers compared to sham (Figure 11. Panel E, p = 0.9).

Interestingly, these findings were contrary to my initial hypothesis as I believed that IAV-specific CD8+ T cells within the septic spleen would be reduced in frequency and absolute number at the peak memory CD8+ T cell response in the context of sepsis-induced immunosuppression. To further interrogate the possible reasons for their increased frequencies, albeit, similar absolute numbers, I had decided to examine the frequency and absolute numbers of lymphocytes and CD8+ T cells within the spleen. I discovered a significant decrease in the frequency of lymphocytes within the septic spleen (Figure 11. Panel C, $p = <0.0001$); however, CD8+ T cell frequencies were preserved (Figure 11. Panel C, $p = 0.43$). In terms of absolute numbers, I demonstrated that there was a two-fold reduction in the numbers of both lymphocytes and CD8+ T cells within the septic spleen (Figure 11. Panel F, $p = <0.0001, <0.0001$, respectively). From this information, I may state that septic splenic IAV-specific CD8+ T cells are higher in frequency, although are numerically similar compared to sham due to the lower absolute numbers of CD8+ T cells within the septic spleen.

Taken together, I discovered that septic splenic IAV-specific CD8+ T cells were higher in frequency, albeit they possessed similar absolute numbers compared to the sham condition. These findings may be explained by the two-fold reduction in the absolute numbers of CD8+ T cells within the septic spleen. There are less CD8+ T cells present within the septic spleen, but IAV-specific CD8+ T cells are numerically unaffected by the septic condition; therefore, these cells will be more frequent within the CD8+ T cell compartment. Furthermore, these findings may suggest that septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression could potentially either possess (1) an increased proliferative capacity, (2) a resilience to sepsis-induced apoptosis or (3) an increased migration from different anatomical locations leading to their increased frequency within the septic spleen.

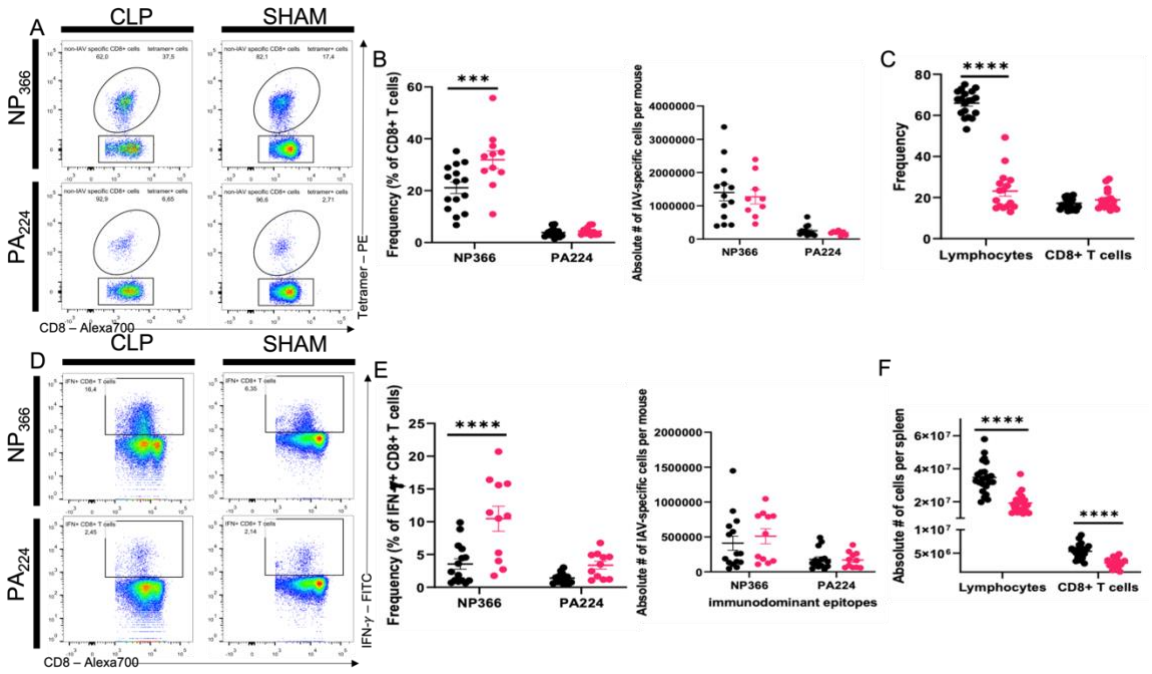

Figure 13. NP_{366}-specific CD8+ T cells within the septic spleen are higher in frequency but are numerically similar compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of splenic IAV-specific CD8+ T cells recognizing two different immunodominant epitopes (NP_{366} and PA_{224}) measured by tetramer reagent. (B) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the spleen of each mouse. (C) Summary plot for the frequency of lymphocytes and CD8+ T cells (gated on lymphocytes) within the spleen of each mouse. (D) Representative flow plots for the frequencies of CD8+ T cells expressing IFN-γ under the stimulation with cognate peptides (NP_{366} and PA_{224}) within the spleen of each mouse. (E) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing IFN-γ under the stimulation with cognate peptides (NP_{366} and PA_{224}) within the spleen of each mouse. (F) Summary plot for the absolute numbers of lymphocytes and CD8+ T cells (gated on lymphocytes) within the spleen of each mouse. This data was pooled from 6 independent experiments: CLP = 1-2 mice/experiment (n = 11); Sham = 2-3 mice/experiment (n = 15). (B, C, E, F) Statistics for significance was based on the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.1.2 IAV-specific CD8+ T cells within the septic spleen appear to be more phenotypically exhausted and activated

My next objective was to phenotypically characterise splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression. To accomplish this, I had immunophenotyped the IAV-specific CD8+ T cells of interest by examining their expression of different exhaustion, activation, death, and survival/maintenance markers using flow cytometry. I performed these experiments by staining freshly isolated splenocytes with different fluorophore-conjugated antibodies targeting the different markers of interest (Table 4) in conjunction with tetramer reagent, then subsequently reading their expression of these markers with a flow cytometer.

In discussing my results, I will first address my findings for the immunophenotyping of the different exhaustion markers I had examined. I discovered that septic splenic IAV-specific CD8+ T cells possessed higher frequencies of cells singly expressing PD-1 and 2B4 (Figure 12. Panel B and E, NP_{366}: p = 0.05, 0.02; PA_{224}: p = <0.0001, 0.01, respectively); however, these cells did not demonstrate any differences in their per-cell expression of PD-1 and 2B4 as shown by their similar geometric mean fluorescence intensity (gMFI) signals compared to the sham condition (Figure 12. Panel B and E, NP_{366}: p = 0.8, 0.9; PA_{224}: p = 0.7, 0.9, respectively). Of important note, gMFI in flow cytometry is a method to quantify the per-cell expression of a specific marker[412]. Similarly to the mean fluorescence intensity (MFI), gMFI calculates the mean fluorescence by adding all the fluorescent signals from cells expressing the marker divided by all cells expressing the marker; however, it differs in that it accounts for non-normal logarithmic data. Typically, in flow cytometry, scientists will analyze the expression of a specific marker using the logarithmic scale (my analyses included); if the data is not a normal distribution, then the arithmetic mean will be useless as the different skews (right or left) will either cause an overestimate or underestimate of the true mean leading to an inaccurate measure of per-cell expression. gMFI compensates for this non-normal logarithmic data. Furthermore, within my analysis, I had calculated the gMFI for the cells that express the marker of interest; therefore, I acquire the per-cell expression of the marker in the cells that are expressing the marker of interest.

Furthermore, I discovered that septic splenic IAV-specific CD8+ T cells possessed an increased frequency of cells singly expressing KLRG1 (Figure 14, Panel B and E, p = 0.007, 0.0008 respectively); however, these cells did not differ in their per-cell expression compared to the sham condition (Figure 14. Panel B and E, p = 0.8, 0.6, respectively). Of important note, KLRG1 is a co-inhibitory marker that gets upregulated in highly proliferative and cytotoxic effector CD8+ T cells, which were stimulated by strong TCR and inflammatory signalling[413,414]. From all this information, I may state that septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response are more phenotypically exhausted compared to sham, which may imply that these cells were more activated.

Currently, the consensus on what defines CD8+ T cell exhaustion is not clear as it has been reported by multiple groups that the presence of an exhaustion marker could either indicate higher[415–418] or lower functionality[404,419–423]; however, recently, it has been suggested that the simultaneous expression of multiple exhaustion markers is a good indicator of CD8+ T cell exhaustion[424,425]. Hence, to ensure robustness within my study, I had also decided to examine the double-expression of the different exhaustion marker combinations within splenic IAV-specific CD8+ T cells. Herein, I discovered that septic splenic IAV-specific CD8+ T cells possessed a higher frequency of cells doubly expressing PD-1+BTLA+ and VISTA+2B4+ (Figure 12. Panel C and F, NP_{366}: p = 0.02, 0.008, PA_{224}: p = 0.004, 0.0001, respectively). Furthermore, in terms of septic splenic NP_{366}-specific CD8+ T cells, these cells demonstrated an increased frequency of cells doubly expressing TIGIT+2B4+ (Figure 12. Panel C, p = 0.01), while displaying an upward trend in the frequency of cells doubly expressing LAG-3+2B4+ (Figure 12. Panel C, p = 0.2). In terms of septic splenic PA_{224}-specific CD8+ T cells, these cells displayed an upward trend in the frequency of cells doubly expressing PD-1+CTLA-4+, TIGIT+VISTA+, TIGIT+2B4+ and LAG-3+2B4+ (Figure 12. Panel F, p = 0.09, 0.06, 0.1, 0.2). From these findings, there is strong evidence to conclude that septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during protracted sepsis are phenotypically more exhausted compared to the sham condition, which may suggest that these cells were more activated.

Next, I will now further discuss my findings for the immunophenotyping of splenic IAV-specific CD8+ T cells for the different activation, death, and survival/maintenance markers at the peak memory CD8+ T cell response during protracted sepsis. I discovered that septic splenic IAV-specific CD8+ T cells demonstrated a reduced frequency of cells expressing CD127 (Figure 13. Panel B and D, $p = 0.002$, <0.0001, respectively). Of important note, CD127 is the IL-7 receptor alpha chain commonly found on memory T cells and it is required for cell survival *in vivo*[228,243]. Furthermore, it has been reported that T cell activation causes the downregulation of CD127[426,427]; therefore, CD127 downregulation may be viewed as an indicator of higher CD8+ T cell activation. Hence, from this finding, I may suggest that septic splenic IAV-specific CD8+ T cells are more phenotypically activated and possess a reduced capacity to maintain themselves *in vivo*. Furthermore, corroborating this finding, I noticed that septic splenic NP_{366}-specific CD8+ T cells possessed an upward trend in the frequency of cells expressing CD95 (Figure 13. Panel B, $p = 0.18$). CD95 is a death marker as cells expressing CD95 can be induced to go through the Fas-FasL apoptotic pathway[428]. Additionally, CD95 expression may also be an indicator of CD8+ T cell activation as it has been demonstrated that chronic immune activation leads to the upregulation of CD95, thereby maintaining effector CD8+ T cell homeostasis[428]. Hence, this finding may suggest that septic splenic NP_{366}-specific CD8+ T cells are more phenotypically activated and are more likely to undergo apoptosis compared to sham.

To conclude, septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression are phenotypically more exhausted and activated compared to their sham counterpart; furthermore, these cells may also possess a reduced capacity to survive *in vivo* within the septic host.

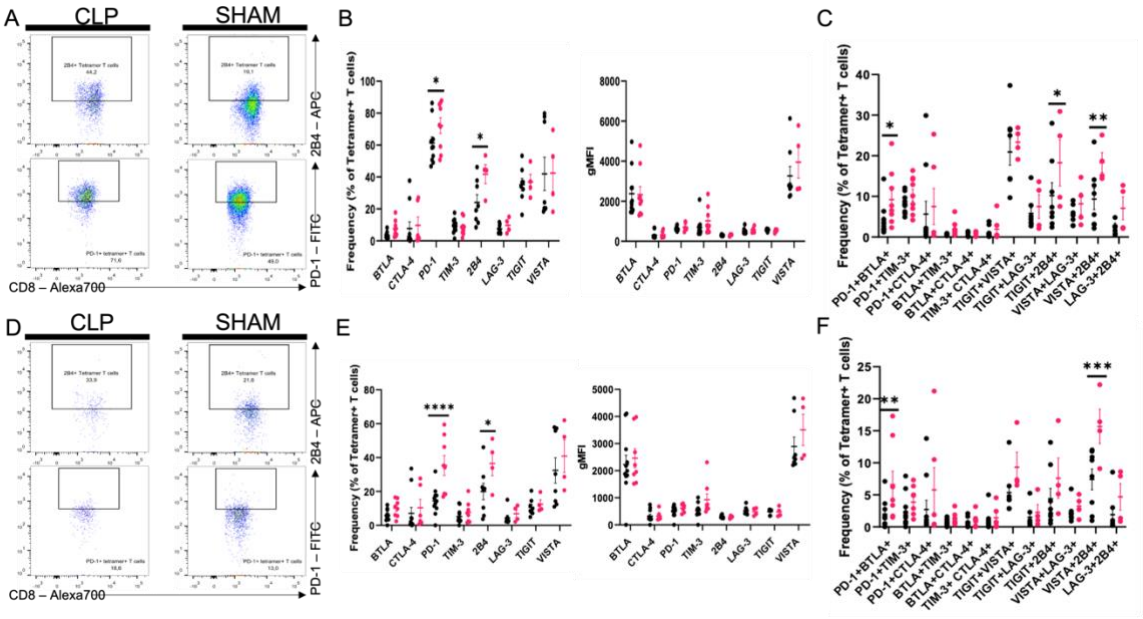

Figure 14. IAV-specific CD8+ T cells within the septic spleen possess higher frequencies of cells singly and doubly expressing the different exhaustion markers. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequencies of NP_{366}-specific CD8+ T cells singly expressing 2B4 and PD-1 within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (C) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (D) Representative flow plots for the frequencies of PA_{224}-specific CD8+ T cells singly expressing 2B4 and PD-1 within the spleen. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (C and F) The combinations of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 5 independent experiments: CLP = 1-2 mice/experiment (n = 9); Sham = 2-3 mice/experiment (n = 11). The data for exhaustion panel 2 was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4);

Sham = 2-3 mice/experiment (n = 8). (B, C, E, F) Statistics for significance was based on the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

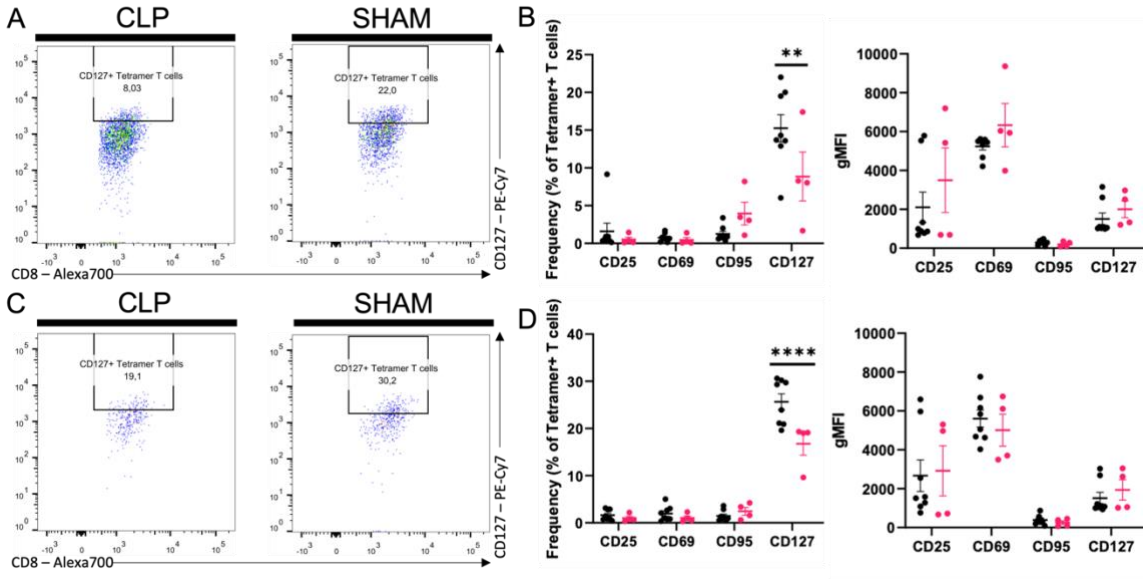

Figure 15. IAV-specific CD8+ T cells within the septic spleen possess lower frequencies of cells expressing CD127. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of NP_{366}-specific CD8+ T cells expressing CD127 within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (C) Representative flow plots for the frequency of PA_{224}-specific CD8+ T cells expressing CD127 within the spleen. (D) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4); Sham = 2-3 mice/experiment (n = 8). (B and D) Statistics for significance was based on the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.3 IAV-specific CD8+ T cells within the septic spleen are more functional as demonstrated by their increased capacity to produce IFN-γ and granzyme B, while maintaining their quality of response

My next objective was to characterize the functionality of septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during protracted sepsis. Previously, within section 3.1.1 of this thesis, I have explained how I performed my ICS experiments, and that applies to this section as it was the same methodology used; however, the only difference is that I included more antibodies against different cytokines and a cytolytic effector molecule, namely granzyme B, IL-2, KLRG1 and TNF-α.

To my surprise, my findings were contrary to my initial hypothesis as I have demonstrated that IAV-specific CD8+ T cells within the septic spleen were more functionally capable in producing the different cytokines and cytolytic effector molecule. Herein, I discovered that IAV-specific CD8+ T cells within the septic spleen possessed significantly higher frequencies of cells capable of producing granzyme B (Figure 14. Panel B and E, $p = 0.01$, 0.001, respectively); however, their per-cell expression of granzyme B was comparable to the sham condition (Figure 14. Panel B and E, $p = 0.9$, 0.9, respectively). This information may suggest that septic splenic IAV-specific CD8+ T cells are more cytotoxic compared to their sham counterpart. Furthermore, NP_{366}-specific CD8+ T cells within the septic spleen demonstrated an increased frequency of cells capable of producing IFN-γ (Figure 14. Panel B, $p<0.0001$); additionally, these cells also appear to produce more IFN-γ on a per-cell basis as illustrated by their increased gMFI levels (Figure 14. Panel B, $p = 0.02$). Moreover, NP_{366}-specific CD8+ T cells within the septic spleen demonstrated a downward trend in the per-cell expression of IL-2 (Figure 14. Panel B, $p = 0.06$). In terms of septic splenic PA_{224}-specific CD8+ T cells, there was no apparent difference in the frequency of cells capable of producing IFN-γ, although, there may be a slight increase (Figure 14. Panel E, $p = 0.2$); however, these cells demonstrated an upward trend in the per-cell expression of IFN-γ (Figure 14. Panel E, $p = 0.07$). Furthermore, septic splenic PA_{224}-specific CD8+ T cells displayed a downward trend in their per-cell expression of IL-2 (Figure 14. Panel E, $p = 0.07$). In terms of the MFI signal for IFN-γ, IAV-specific CD8+ T cells displayed

an increased per-cell expression of IFN-γ (Figure 14. Panel C, p = 0.007, 0.009, respectively). These findings suggest that septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression are more functionally active compared to sham. Furthermore, the decreased per-cell expression of IL-2 may suggest that these cells are beginning to become exhausted as IL-2 is the first cytokine to be downregulated during CD8+ T cell exhaustion[371].

Thereafter, I had also wanted to further characterize their functionality by examining their quality of response through measuring the frequency of cells triple-expressing IFN-γ, IL-2 and TNF-α . I discovered that septic splenic IAV-specific CD8+ T cells had a similar frequency of cells triple expressing IFN-γ, IL-2 and TNF-α compared to the sham condition (Figure 14. Panel F, p = 0.9, 0.5). This finding suggests that the quality of response is not affected during protracted sepsis.

Taken together, septic splenic IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression are more functionally capable in producing granzyme B and IFN-γ, which suggests that these cells are more functional compared to their sham counterpart. Furthermore, septic splenic IAV-specific CD8+ T cells retain their quality of response as demonstrated by the similar frequencies of cells triple-expressing IFN-γ, IL-2 and TNF-α compared to the sham condition.

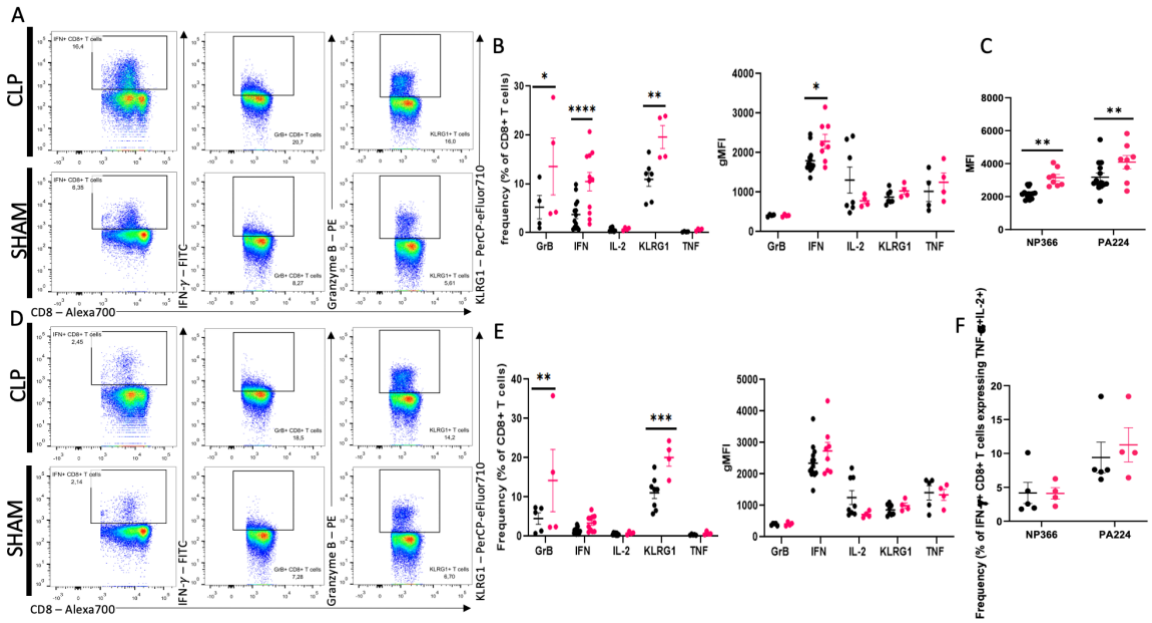

Figure 16. IAV-specific CD8+ T cells within the septic spleen are more functionally capable in producing granzyme B and IFN-γ, while maintaining their polyfunctionality. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequencies of NP_{366}-specific CD8+ T cells expressing granzyme B, IFN-γ, and KLRG1 within the spleen under the stimulation of NP_{366} peptide during a 5-hour stimulation condition. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing granzyme B, IFN-γ, and KLRG1 within the spleen of each mouse. (C) Summary plot for the MFI of IAV-specific CD8+ T cells expressing IFN-γ within the spleen of each mouse under the stimulation with their cognate peptides (NP_{366} and PA_{224}). (D) Representative flow plots for the frequencies of PA_{224}-specific CD8+ T cells expressing granzyme B, IFN-γ, and KLRG1 within the spleen under the stimulation of PA_{224} peptide during a 5-hour stimulation condition. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing granzyme B, IFN-γ, and KLRG1 within the spleen of each mouse. (F) Summary plot for the frequency of polyfunctional IAV-specific CD8+ T cells as defined by the triple-expression of IFN-γ, TNF-α and IL-2. The data for IFN-γ was pooled from 6 independent experiments: CLP = 1-2 mice/experiment (n = 11); Sham = 2-3 mice/experiment (n = 15). The data for the other markers was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4); Sham = 2-3 mice/experiment (n = 8). (B, C, E, F) Statistics for

significance was based on the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.4 Non-IAV-specific CD8+ T cells within the septic spleen appear to be more phenotypically exhausted and activated compared to sham

In my next objective, I had decided to phenotype the non-IAV-specific CD8+ T cells within the septic spleen as this would provide me with (1) potential markers that are unique to IAV-specific CD8+ T cells and (2) a better global understanding on the affects sepsis has on the whole CD8+ T cell pool. To accomplish this, I utilized the data that I had gathered from the IAV-specific CD8+ T cell phenotyping experiments; however, rather than gating on the tetramer positive population, I gated on CD8+ T cells that excluded either NP_{366}- or PA_{224}-specific CD8+ T cells. One potential limitation of gating on either non-NP_{336}- or -PA_{224}-specific CD8+ T cells is that there will be contamination from the other IAV-specific CD8+ T cell. For instance, gating on non-PA_{224}-specific CD8+ T cells will result in contamination from NP_{366}-specific CD8+ T cells, and vice versa. Despite this, I may also use this contamination to my advantage as NP_{366}-specific CD8+ T cells are more plentiful compared to PA_{224}-specific CD8+ T cells during recall responses[429].

I discovered that septic splenic non-IAV-specific CD8+ T cells demonstrated a decreased frequency of cells singly expressing BTLA (Figure 15. Panel A and C, $p = 0.003$, <0.0001, respectively); however, these cells displayed an upward trend in the per-cell expression of BTLA (Figure 15. Panel A and C, $p = 0.09$, 0.1, respectively). Furthermore, septic splenic non-IAV-specific CD8+ T cells had higher frequencies of cells singly expressing PD-1 and 2B4 (Figure 15. Panel A and C, non-NP_{366}: $p = 0.01$, 0.04; non-PA_{224}: $p = <0.0001$, 0.008, respectively); however, only septic splenic non-NP_{366}-specific CD8+ T cells displayed a downward trend in the per-cell expression of 2B4 (Figure 15. Panel A, $p = 0.1$), while non-PA_{224}-specific CD8+ T cells did not demonstrate this trend (Figure 15. Panel C, $p = 0.7$). Moreover, non-IAV-specific CD8+ T cells within the septic spleen had an increased per-cell expression of TIM-3 as illustrated by their increased gMFI levels compared to the sham condition (Figure 15. Panel A and C, $p = 0.03$, 0.002, respectively). This information suggests that non-IAV-specific CD8+ T cells within the septic spleen are phenotypically more exhausted compared to sham, which may also imply that they are more activated as well.

Just as I had previously done for the IAV-specific CD8+ T cells of interest, I decided to also examine the co-expression of the different exhaustion markers within the septic splenic non-IAV-specific CD8+ T cells. I found that there were significantly higher frequencies of cells doubly expressing PD-1+BTLA+ and VISTA+2B4+ (Figure 15. Panel B and D, non-NP_{366}: p = 0.02, <0.0001; non-PA_{224}: p = 0.02, <0.0001, respectively), while non-NP_{336}-specific CD8+ T cells expressed higher frequencies of VISTA+LAG-3+ (Figure 15. Panel B, p = 0.05) and non-PA_{224}-specific CD8+ T cells expressed higher levels of TIGIT+VISTA+ (Figure 15. Panel D, p = 0.04). Taken together, it is evident that non-IAV-specific CD8+ T cells at the peak memory CD8+ T cell response are phenotypically more exhausted compared to their sham counterpart, which may also suggest that they have been more activated.

From these findings, there is some evidence to suggest that 2B4 upregulation may be unique to IAV-specific CD8+ T cells during the recall response in the context of sepsis-induced immunosuppression. Although there was a higher frequency of non-IAV-specific CD8+ T cells singly expressing 2B4, there was a trend towards decreased per-cell expression of 2B4. As previously alluded to, there will be contamination from the different IAV-specific CD8+ T cells in the non-IAV-specific CD8+ T cell compartments. Observing the gMFI data from the non-NP_{366}-specific CD8+ T cell compartment, the trend of lower per-cell expression of 2B4 is more pronounced compared to the non-PA_{224}-specific CD8+ T cell compartment, which makes sense as there is a higher frequency of NP_{366}-specific CD8+ T cells contaminating the non-PA_{224}-specific CD8+ T cell compartment leading to a higher signal. Furthermore, in terms of the frequency of non-IAV-specific CD8+ T cells expressing 2B4, there is a more pronounced upregulation within the non-PA_{224}-specific CD8+ T cell compartment compared to the non-NP_{366}-specific CD8+ T cell compartment. These findings may suggest (1) that the increased frequency of 2B4 within non-IAV-specific CD8+ T cells could be just due to the contribution from the IAV-specific CD8+ T cells and (2) that the upregulation of 2B4 may be unique to IAV-specific CD8+ T cells during the recall response in the context of sepsis-induced immunosuppression.

In addition to examining the exhaustion phenotype within non-IAV-specific CD8+ T cells, I had also decided to look at the expression of the different activation, death, and survival/maintenance markers within this population.

Interestingly, I discovered that septic splenic non-IAV-specific CD8+ T cells did not differ in their frequency and per-cell expression of CD127 (Figure 16. Panel A and B, non-NP_{366}: p = 0.45, 0.4; non-PA_{224}: 0.86, 0.77, respectively). This finding may suggest (1) that the downregulation of CD127 seen within the IAV-specific CD8+ T cell populations may be unique to these IAV-specific CD8+ T cells during the recall response in the context of protracted sepsis and (2) that these non-IAV-specific CD8+ T cells may not possess a dampened ability to survive within the post-septic environment. Additionally, another interesting point of observation, septic splenic non-IAV-specific CD8+ T cells possessed a reduced per-cell expression of CD69 (Figure 16. Panel A and B, p = 0.03, 0.01, respectively), which may indicate that these cells are less active or have less ability to retain themselves within the tissue; however, this finding may not be meaningful as there were a low frequency of cells expressing this marker. Furthermore, this finding may be a by-product of the homeostatic proliferation mechanisms that occur as homeostatic proliferated CD8+ T cells will not express CD69[217,430].

Taken together, septic splenic non-IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during protracted sepsis do not experience the same downregulation of CD127 as was seen within the IAV-specific CD8+ T cell compartment, which may suggest (1) that the downregulation of CD127 is unique to IAV-specific CD8+ T cells and (2) that non-IAV-specific CD8+ T cells may have no inherent problem in their *in vivo* survival.

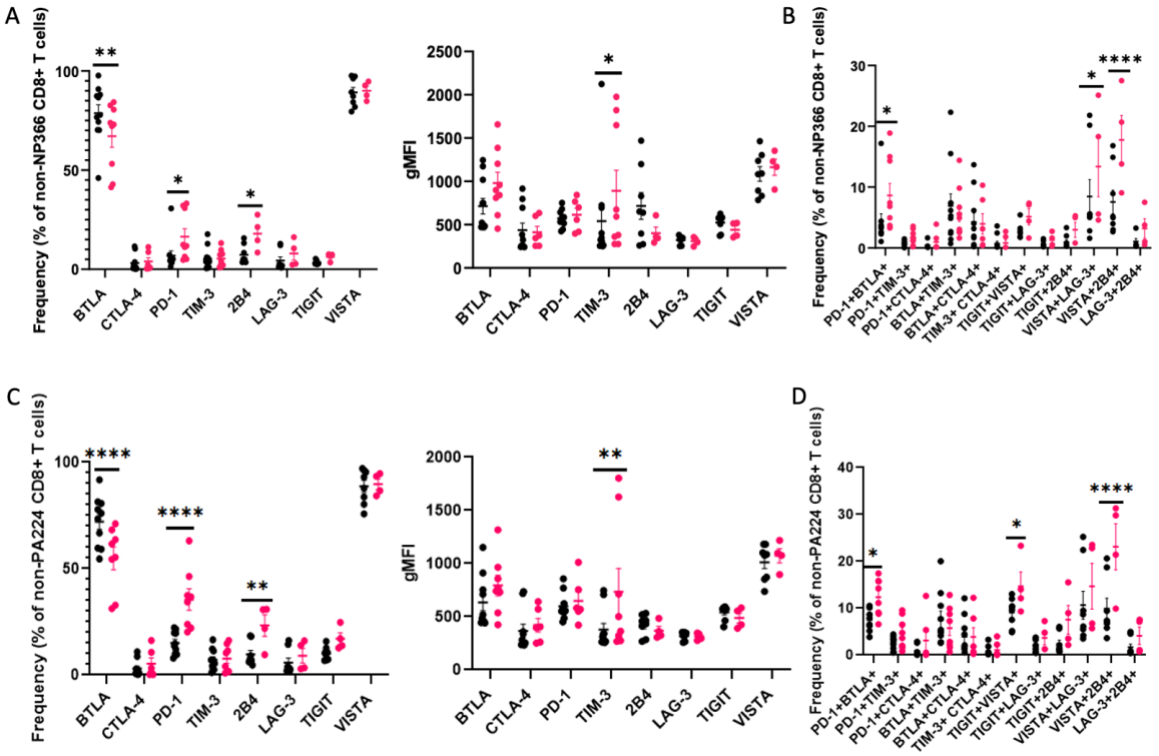

Figure 17. Non-IAV-specific CD8+ T cells within the septic spleen appear to be more phenotypically exhausted compared to their sham counterpart. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers examined within the spleen of each mouse. (B) Summary plot for the frequencies of non-NP_{366}-specific CD8+ T cells doubly expressing the different combinations of exhaustion markers within the spleen of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers examined within the spleen of each mouse. (D) Summary plot for the frequencies of non-PA_{224}-specific CD8+ T cells doubly expressing the different combinations of exhaustion markers within the spleen of each mouse. (B and D) The combinations of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 5 independent experiments: CLP = 1-2 mice/experiment (n = 9); Sham = 2-3 mice/experiment (n = 11). The data for exhaustion panel 2 was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4); Sham = 2-3 mice/experiment (n = 8).

(A-D) Statistics for significance was based on the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

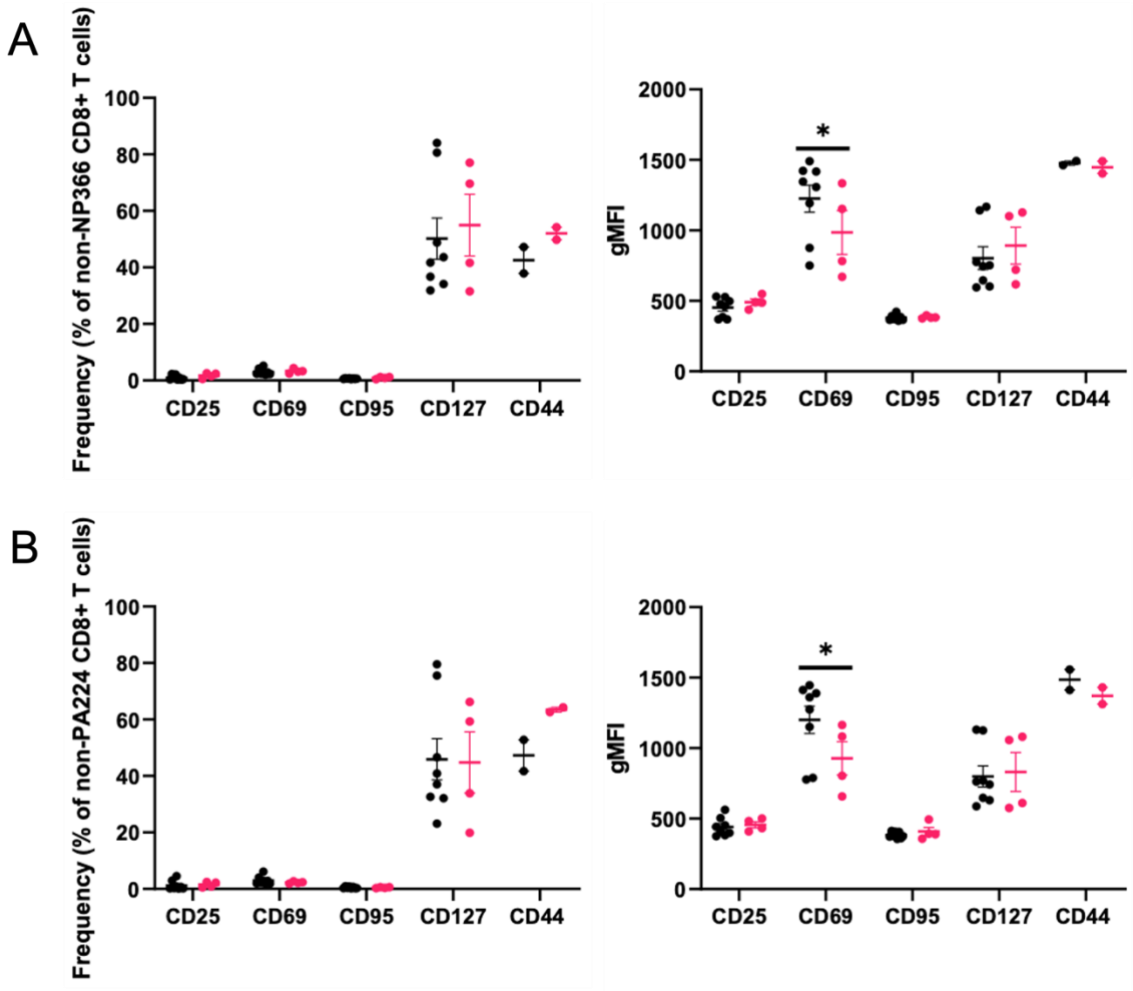

Figure 18. Non-IAV-specific CD8+ T cells within the septic spleen possess similar frequencies of cells expressing CD127. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4); Sham = 2-3 mice/experiment (n = 8). (A and B) Statistics for significance was based on the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.5 There is a higher frequency of activated CD8+ T cells within the septic spleen; however, there is a numerical loss of central memory CD8+ T cells

Next, I had wanted to enrich my study by examining the effect that sepsis has on the different splenic memory CD8+ T cell subsets as this would provide me with (1) a better global understanding on the effect sepsis has on the different memory CD8+ T cell subsets and (2) a better understanding on the effects sepsis has on IAV-specific CD8+ T cells. To accomplish this objective, I used antibodies against the cell markers CD44 and CD62L to examine the different memory CD8+ T cell subsets, which has been commonly used to differentiate between effector and central memory CD8+ T cells[282]. CD44 is an activation marker that gets upregulated during naïve T cell activation and is maintained on the memory T cells that are generated[282]. CD62L is an L-selectin that differentiates between central and effector memory CD8+ T cells; central memory CD8+ T cells possess CD62L, while effector memory CD8+ T cells lack CD62L[282]. Of important note, there are some limitations to examining these different memory CD8+ T cell subsets with the definition I have utilized, which will be extensively discussed within section 4.3.1. One such limitation that needs to be discussed is that my definition may encompass other cell populations. One such example of contamination is within the CD44+CD62L- populations; this population will be composed of effector memory and effector CD8+ T cells. Hence, I must take a special consideration in examining my data for this.

Within the spleen of septic mice, I discovered that there was a trend towards increased frequencies of activated CD8+ T cells as measured by their expression of CD44 (Figure 17. Panel A, $p = 0.16$); however, there was a slight decrease in their absolute numbers (Figure 17, Panel A, $p = 0.24$). These findings reflect the expression of all CD8+ T cells expressing CD44; however, I had decided to also examine the septic splenic CD8+ T cells that highly express CD44, which was termed $CD44^{HI}$. I discovered that there was a higher frequency of CD8+ T cells which highly expressed CD44 (Figure 17. Panel B, $p = 0.01$). This information suggests that there is a higher frequency of activated CD8+ T cells within the septic spleen compared to the sham condition.

To further characterize the different memory CD8+ T cell subsets, I had examined the expression of CD62L on CD44+CD8+ T cells. In my data, I noticed a shift in frequency from central to effector memory CD8+ T cells as illustrated by the frequency shift in both central and effector memory CD8+ T cells (Figure 17. Panel C, p = 0.01, 0.01, respectively). To further interrogate this shift in phenotype, I decided to look at the absolute numbers of central and effector memory CD8+ T cells within the spleen. I demonstrated that there was a numerical loss of central memory CD8+ T cells within the septic spleen; however, effector memory CD8+ T cells were numerically similar compared to sham (Figure 17. Panel C, p = 0.05, 0.9, respectively). From this information, it could be postulated that the frequency shift in the different memory CD8+ T cell subsets is due to the numerical loss of central memory CD8+ T cells. This has important implications as these different cells types have a differential localization within the host[431]. For instance, central memory CD8+ T cells roam through the circulation and secondary lymphoid tissues, while effector memory CD8+ T cells preferential roam through the peripheral tissues. Considering this shift, this may suggest that memory CD8+ T cells during the recall response in the context sepsis-induced immunosuppression would be more prevalent within the peripheral tissues.

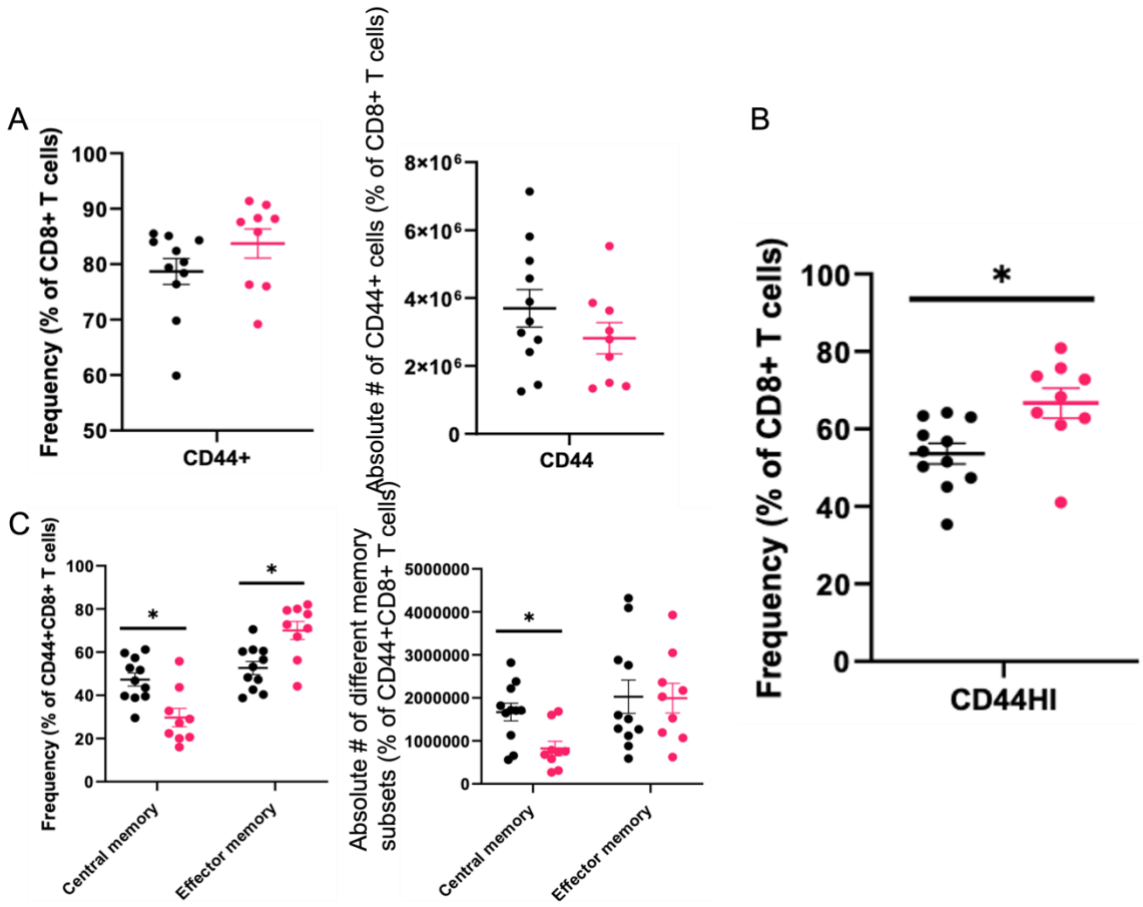

Figure 19. There is a higher frequency of effector memory CD8+ T cells due to the numerical depletion of central memory CD8+ T cells. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and absolute number (right) of activated CD8+ T cells as measured by the expression of CD44 within the spleen of each mouse. (B) Summary plot for the frequency of activated CD8+ T cells as measured by the high expression of CD44 within the spleen of each mouse. (C) Summary plots for the frequency (left) and absolute number (right) of the different memory CD8+ T cell subsets (central and effector memory) gated on all CD44+CD8+ T cells based on the expression of CD62L within the spleen of each mouse. The data was pooled from 5 independent experiments: CLP = 1-2 mice/experiment (n = 9); Sham = 2-3 mice/experiment (n = 11). (A-C) Statistics for significance was based on the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.6 Within the septic spleen, central memory CD8+ T cells possess higher frequencies of BTLA and 2B4, while effector memory CD8+ T cells possess higher levels of PD-1; however, both memory CD8+ T cell populations express similar levels of CD127 compared to sham

Next, I had wanted to study the phenotype of the different memory CD8+ T cell subsets during the recall response in the context of sepsis-induced immunosuppression, in hopes of (1) elucidating potential markers that are unique to IAV-specific CD8+ T cells and (2) acquiring a better understanding on the effect sepsis has on the different memory CD8+ T cell subsets. Of important note, the IAV-specific CD8+ T cells under study would be either classified as central or effector memory CD8+ T cells; however, at the peak memory CD8+ T cells response, these cells would be mostly characterized as effector memory CD8+ T cells with a smaller proportion being central memory CD8+ T cells[282,432,433]. Hence, when examining the effector memory CD8+ T cell compartment, I can make inferences regarding the IAV-specific CD8+ T cells of interest.

First, I will discuss the results that were gathered for the exhaustion phenotyping of the different memory CD8+ T cell subsets. In terms of the septic splenic central memory CD8+ T cells, I discovered that these cells possessed higher frequencies of cells expressing BTLA and 2B4 (Figure 18. Panel B, $p = 0.001$, 0.02, respectively); however, the results for 2B4 may not be significant as the data was quite heterogeneous. Furthermore, these cells demonstrated an upward trend in their frequencies of cells doubly expressing the different combinations of exhaustion markers, but the only double-expressor that reached significance was VISTA+2B4+ (Figure 18. Panel C, $p = 0.001$). Within the septic splenic effector memory CD8+ T cells, there was a higher frequency of cells expressing PD-1 (Figure 18. Panel E, $p = 0.001$), while their co-expression profile of the different exhaustion markers is similar to sham (Figure 18. Panel F). This information suggests that the different memory CD8+ T cell subsets may have an increased exhaustion status compared to their sham counterpart.

Additionally, just as the non-IAV-specific CD8+ T cell compartment, there is evidence to suggest that the expression of 2B4 may be unique to IAV-specific CD8+ T cells during the recall response in the context of sepsis-induced immunosuppression. Examining the effector memory CD8+ T cell data closer, which the majority of the IAV-specific CD8+ T cells would be classified as, there was no upregulation of 2B4. This makes sense as the signal for 2B4 from IAV-specific CD8+ T cells would be dampened due to the higher presence of other effector memory and effector CD8+ T cells. Hence, this finding provides further evidence to suggest that 2B4 upregulation is unique to IAV-specific CD8+ T cells during the recall response in the context of protracted sepsis.

Thereafter, I had also wanted to study the expression of the different activation, death, and survival/maintenance markers within the different memory CD8+ T cell subsets at the peak memory CD8+ T cell response. I found that for both central and effector memory CD8+ T cells, there was no differences in the frequency of cells expressing CD95 and CD127 (Figure 19. Panel A and B, central memory CD8+ T cell: $p = 0.9, 0.6$; effector memory CD8+ T cell: $p = 0.9, 0.8$); however, in terms of the effector memory CD8+ T cells, the variance of the data was large, so there may in fact be a reduction in the expression of CD127, but more replicates are necessary to elucidate this. From this information, it could be suggested that the downregulation of CD127 and upregulation of CD95 may be unique to IAV-specific CD8+ T cells. Furthermore, the similar frequencies of CD127 for the different memory CD8+ T cell subsets may suggest that these cells are still capable of maintaining themselves and surviving *in vivo*; however, more experiments are necessary to prove this thought.

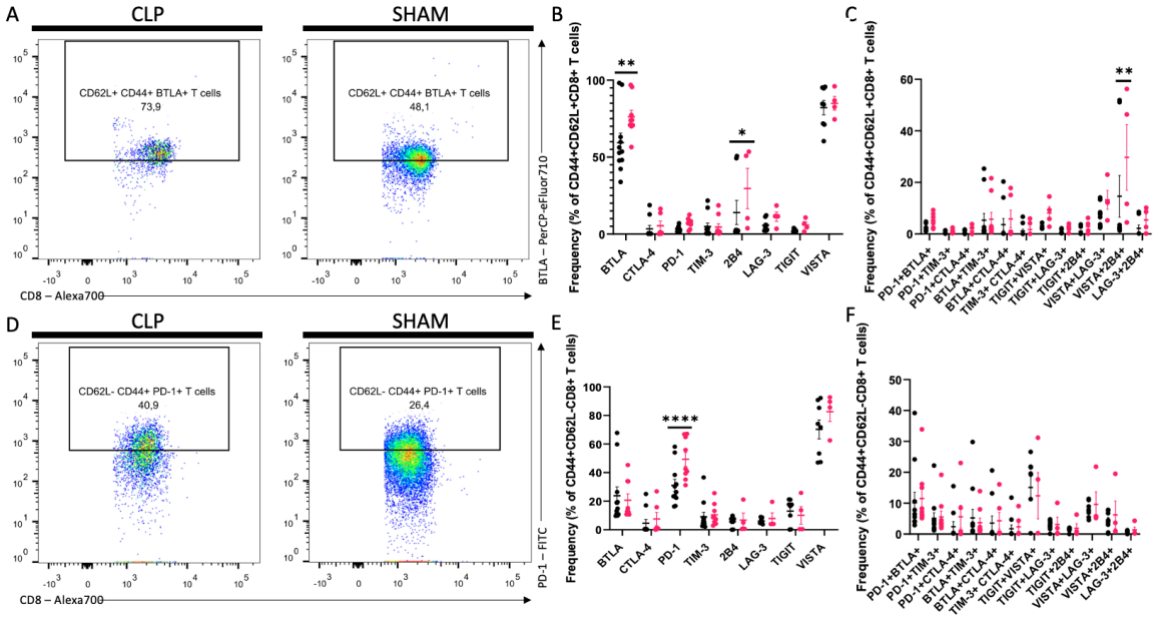

Figure 20. Within the septic spleen, central memory CD8+ T cells express higher frequencies of cells expressing BTLA and 2B4, while effector memory CD8+ T cells have higher frequencies of PD-1. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of central memory CD8+ T cells singly expressing BTLA within the spleen. (B) Summary plot for the frequency of central memory CD8+ T cells singly expressing the different exhaustion markers examined within the spleen of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different combinations of exhaustion markers within the spleen of each mouse. (D) Representative flow plots for the frequency of effector memory CD8+ T cells singly expressing PD-1 within the spleen. (E) Summary plot for the frequency of effector memory CD8+ T cells singly expressing the different exhaustion markers examined within the spleen of each mouse. (F) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different combinations of exhaustion markers within the spleen of each mouse. (C and F) The combination of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 5 independent experiments: CLP = 1-2 mice/experiment (n = 9); Sham = 2-3 mice/experiment (n = 11). The data for exhaustion panel 2 was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4);

Sham = 2-3 mice/experiment (n = 8). (B, C, E, F) Statistics for significance was based on the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

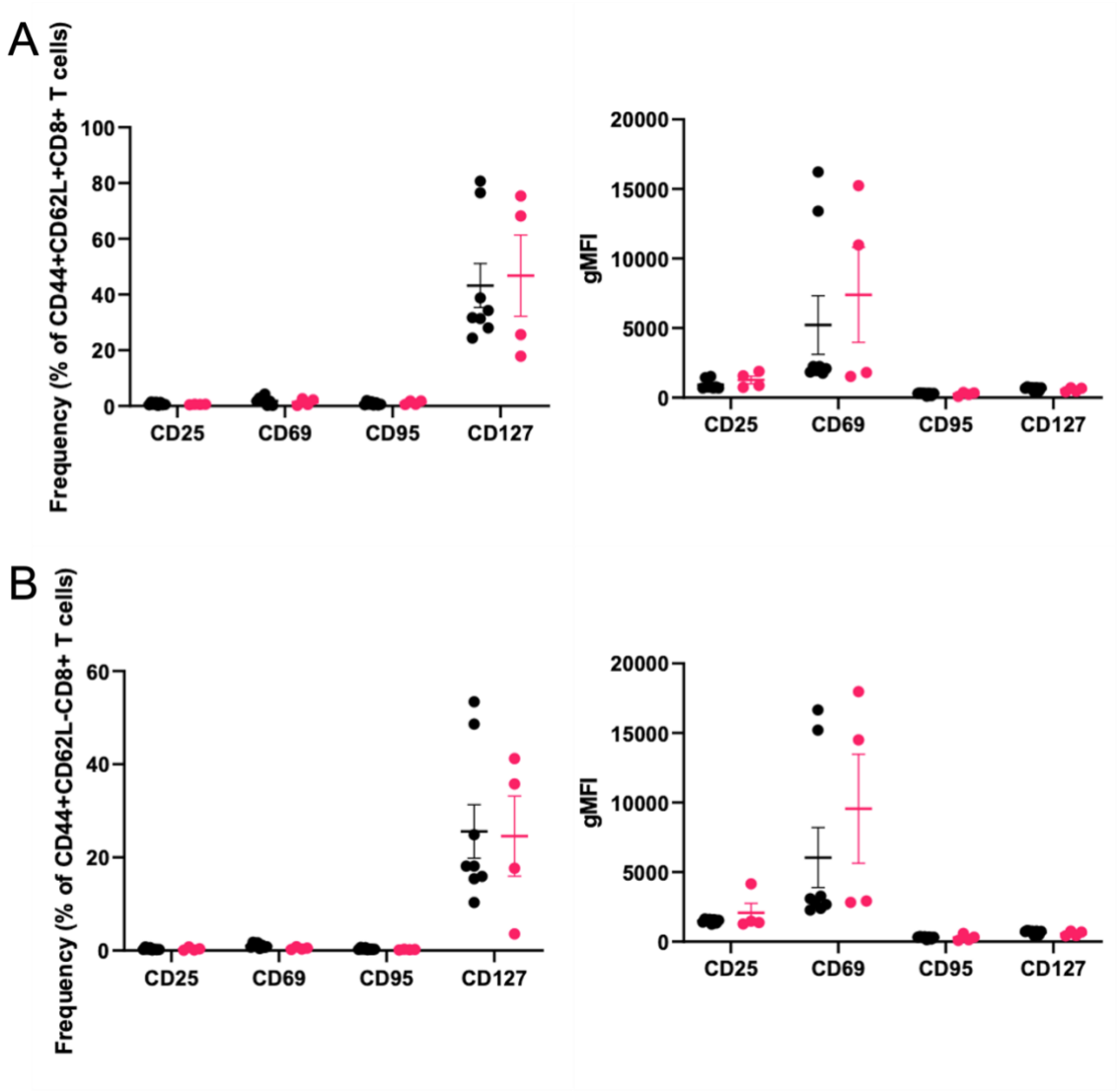

Figure 21. Central and effector memory CD8+ T cells within the septic spleen possess similar frequencies of cells expressing CD127 compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of central memory CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of effector memory CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 independent experiments: CLP = 1-2 mice/experiment (n = 4); Sham = 2-3 mice/experiment (n = 8). (A and B) Statistics for significance was based on the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.7 There is a decreased frequency of IAV-specific CD8+ T cells within the septic PECs with an associated dampened ability to produce IFN-γ

In addition to examining the IAV-specific CD8+ T cell responses within the spleen, I had also wanted to examine their responses within the PECs. In terms of the spleen, examining IAV-specific CD8+ T cell responses provides information regarding the systemic immune response to IAV in the context of sepsis-induced immunosuppression, but I had also wanted to examine the local immune response to IAV during sepsis. The PECs is the location of the onset of sepsis and studying this tissue would provide me with the local immune response to IAV during sepsis-induced immunosuppression.

Herein, I discovered that septic NP_{366}-specific CD8+ T cells within the PECs were lower in frequency as measured by tetramer reagent (Figure 20. Panel B, $p = 0.04$); additionally, these cells demonstrated a weak trend towards reduced absolute numbers (Figure 20. Panel B, $p = 0.4$), but this is not conclusive as the variance of the data was too large. In terms of septic PA_{224}-specific CD8+ T cells, there was no apparent trend of reduced frequencies and absolute numbers measured by tetramer reagent (Figure 20. Panel B, $p = 0.6, 0.7$, respectively); although, the averages were lower for this population. Furthermore, I had also performed an ICS to detect IAV-specific CD8+ T cells capable of producing IFN-γ under the stimulation with their cognate peptides. I found that IAV-specific CD8+ T cells had a weak trend towards reduced frequencies of cells capable of producing IFN-γ (Figure 20. Panel E, $p = 0.3, 0.5$, respectively); although, the variance within the NP_{366}-specific CD8+ T cell compartment was too large to make conclusive remarks. This information suggests that septic IAV-specific CD8+ T cells within the PECs may have a reduced presence, and this could suggest that they may have a dampened response within this tissue.

Thereafter, I had also wanted to phenotypically assess these IAV-specific CD8+ T cells within the PECs at the peak memory CD8+ T cell response during protracted sepsis. Of important note, due to the limited number of cells collected from the PECs, I had only immunophenotyped a few exhaustion markers as I had used the majority of the cells for the functional assays. Within the PECs, I discovered that septic IAV-specific CD8+ T cells possessed similar frequencies and per-cell expression (data not shown) of all the different exhaustion markers examined compared to sham (Figure 20. Panel C and F). Furthermore, I also demonstrated that these cells did not have a significant difference in their double expression of the possible combinations of the different exhaustion markers (data not shown). This information suggests that septic IAV-specific CD8+ T cells within the PECs may be similarly exhausted compared to sham; however, further studies need to be performed as the replicate number for the immunophenotyping experiments was too low to make any definitive conclusions (n=2).

Furthermore, I wanted to also examine the IAV-specific CD8+ T cell functionality at the peak memory CD8+ T cell response in the context of protracted sepsis by quantifying their IFN-γ producing capabilities under the stimulation with cognate peptides. Herein, I discovered that there was a weak trend towards reduced frequencies of IAV-specific CD8+ T cells capable of producing IFN-γ (Figure 20. Panel E, $p = 0.3, 0.5$, respectively); however, I did find that the per-cell production of IFN-γ was reduced as demonstrated by the lower gMFI signal (Figure 20. Panel E, $p = 0.1, 0.02$, respectively). Taken together, septic IAV-specific CD8+ T cells within the PECs appear to have a dampened ability to produce IFN-γ, which would indicate that they are less functionally competent. Furthermore, the septic PECs may also have a reduced frequency of IAV-specific CD8+ T cells capable of producing IFN-γ.

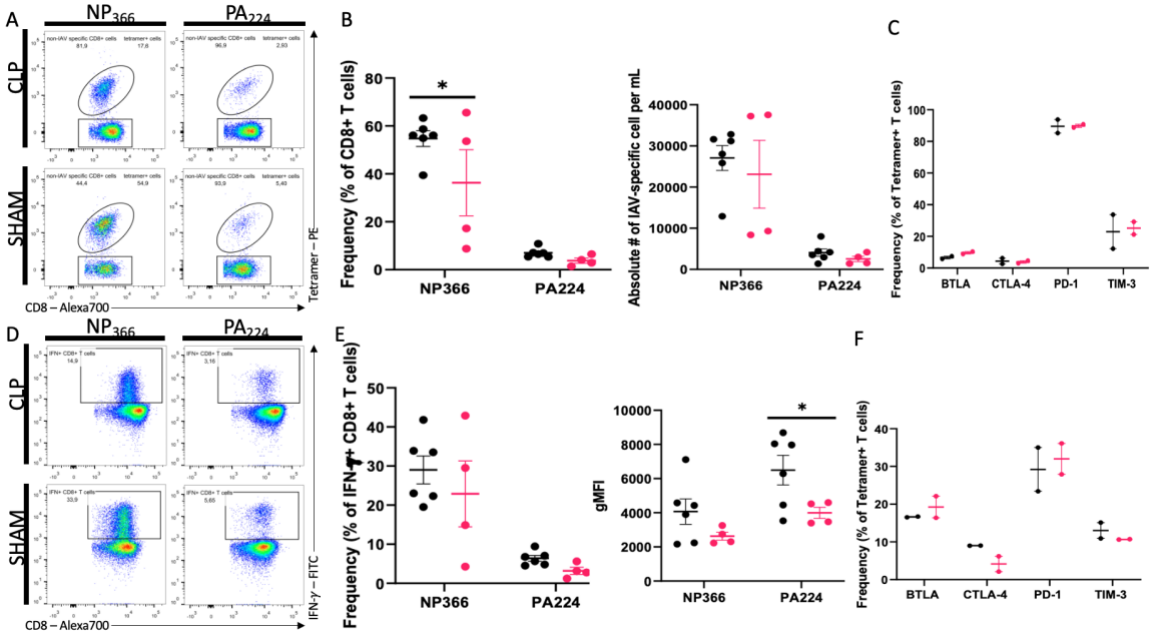

Figure 22. IAV-specific CD8+ T cells within the septic PECs may be less frequent and possess reduced numbers; furthermore, these cells possess a dampened functionality. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells measured by tetramer reagent within the PECs. (B) Summary plots for the frequency (left) and absolute number (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the PECs of each mouse. Note: absolute numbers were reported as the number of cells per mL of peritoneal fluid recovered. (C) Summary plot for the frequency of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (D) Representative flow plots for the frequency of IAV-specific CD8+ T cells capable of producing IFN-γ under the stimulation with their cognate peptides (NP_{366} and PA_{224}) within the PECs. (E) Summary plots for the frequency (left) and gMFI (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the stimulation with their cognate peptides (NP_{366} and PA_{224}) within the PECs of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. The data for the tetramer and ICS were pooled from 2 independent experiments: CLP = 2 mice/experiment (n = 4); Sham = 2-4 mice/experiment (n = 6). The data for the exhaustion panel 1 was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B and E) Statistics for significance was based on the unpaired student's t test with *$p<0.05$,

**p<0.01, ***p<0.001, ****p<0.0001. (C and F) No statistics were performed due to the low replicate number (n=2).

3.1.8 Non-IAV-specific CD8+ T cells within the septic PECs demonstrate a trend of increased frequencies of cells expressing BTLA, while having reduced PD-1

Next, just as I had previously done for the spleen, I had wanted to examine the exhaustion phenotypes within the non-IAV-specific CD8+ T cell compartment.

The results I found were quite interesting, I discovered that septic non-IAV-specific CD8+ T cells possessed an upward trend in the frequency and per-cell expression of BTLA (Figure 21. Panel B and E), which may suggest that these cells are (1) less activated within the septic condition and (2) are less able to respond to pathogens as BTLA is considered a negative regulator of T cell activation[434]. Furthermore, these septic non-IAV-specific CD8+ T cells demonstrated a trend towards reduced frequencies of cells singly expressing PD-1 (Figure 21. Panel B and E), which may suggest that these cells are not as activated compared to sham as the expression of PD-1, in addition to their exhaustion, has been shown to be a sign of T cell activation and activity[282,431,435,436]. Moreover, I had also examined the co-expression of the different exhaustion markers and demonstrated that double-expressors of PD-1 and BTLA were reduced by roughly 4-fold, which provides more evidence that they are not as activated compared to their sham counterpart (Figure 21. Panel C and F).

Taken together, these findings suggest that within the septic PECs, non-IAV-specific CD8+ T cells may be less activated and able to respond to infection as demonstrated by their increased expression of the negative regulator BTLA, while possessing reduced frequencies of cells expressing PD-1, which is commonly upregulated during T cell activation. Hence, these cells may be less exhausted and activated; however, these findings are not conclusive as the replicate number was too low to make any strong assertions.

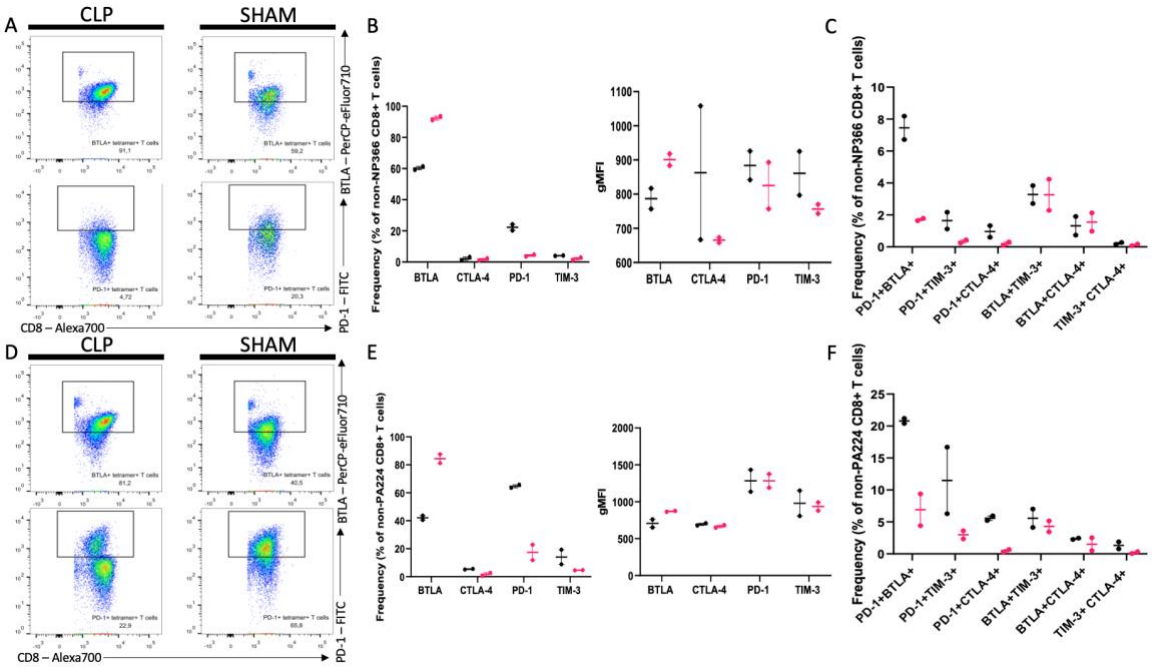

Figure 23. Non-IAV-specific CD8+ T cells within the septic PECs demonstrate a trend towards higher frequencies and per-cell expression of BTLA, while having diminished frequencies of PD-1. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of non-NP_{366}-specific CD8+ T cells singly expressing BTLA and PD-1 within the PECs. (B) Summary plots for the frequency (left) and gMFI (right) of non-NP_{336}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (C) Summary plot for the frequency of non-NP_{336}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. (D) Representative flow plots for the frequency of non-PA_{224}-specific CD8+ T cells singly expressing BTLA and PD-1 within the PECs. (E) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (F) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. The data was pooled from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B, C, D, E) No statistics were performed as the replicate number is too low.

3.1.9 IAV-specific CD8+ T cells within the septic lungs appear to be higher in frequency and absolute numbers, while possessing a lower per-cell expression of BTLA

Thereafter, considering that I discovered a differential response between the spleen and PECs, I had wanted to extend my findings to other tissues (i.e. lungs and liver). I decided to examine the IAV-specific CD8+ T cell responses within the lungs because a common source of death after the septic insult is secondary pneumonia[63]. Hence, the responses within this tissue are extremely important to study to broaden the knowledge on how sepsis effects the different tissues and their antigen specific CD8+ T cell responses. Currently, there are no studies that have examined antigen specific CD8+ T cell responses within the lungs; therefore, my research is novel.

I discovered that NP_{366}-specific CD8+ T cells demonstrated a strong trend towards higher frequencies and absolute numbers within the septic lungs at the peak memory CD8+ T cell response (Figure 22. Panel A and B), which may suggest that there is an enhanced response from IAV-specific CD8+ T cells within the septic lungs. Furthermore, I had also examined the exhaustion phenotype of these IAV-specific CD8+ T cells. I found that these cells possessed similar frequencies of single- and double-expression of the different exhaustion markers examined (Figure 22. Panel D, C, E, F); however, these cells did possess a lower per-cell expression of BTLA, which may suggest that they are more activated as BTLA is a negative regulator of T cell activation and gets downregulated during T activation[434].

From all this information, it may be suggested that IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during protracted sepsis are more plentiful within the septic condition. Furthermore, IAV-specific CD8+ T cells may be more activated; however, these results are not conclusive as the replicate number was too low.

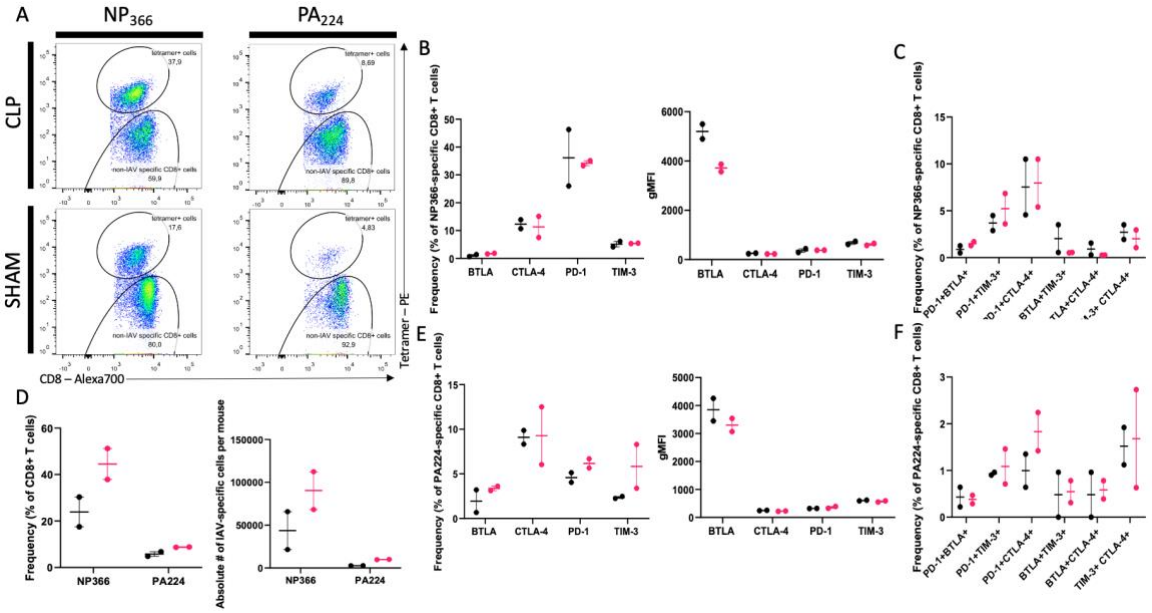

Figure 24. NP_{366}-specific CD8+ T cells within the septic lungs appear to be higher in frequency and absolute numbers, while both IAV-specific CD8+ T cells demonstrate a trend of diminished per-cell expression of BTLA. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells measured by tetramer reagent within the lungs. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (C) Summary plots for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (D) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the lungs of each mouse. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (F) Summary plots for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B-F) No statistics were performed as the replicate number is too low.

3.1.10 Non-IAV-specific CD8+ T cells within the septic lungs appear to have a diminished frequency and per-cell expression of BTLA

Next, I had wanted to examine the exhaustion phenotype of the non-IAV-specific CD8+ T cells as this would provide a better understanding on the effect sepsis has on the whole CD8+ T cell pool within the lungs.

I discovered that septic non-IAV-specific CD8+ T cells demonstrated a trend towards reduced frequencies and per-cell expression of BTLA (Figure 23. Panel B and E), while displaying reduced frequencies of co-expression of the different exhaustion markers with BTLA (Figure 23. Panel C and F). Furthermore, septic non-IAV-specific CD8+ T cells demonstrated similar frequencies and per-cell expression of the other exhaustion markers examined compared to sham.

Taken together, these findings suggest that septic non-IAV-specific CD8+ T cells may be more activated as illustrated by their decreased expression of BTLA; however, they have a similar exhaustion profile compared to sham. Despite these findings, they are not conclusive as the replicate number was too low to make any definitive conclusions.

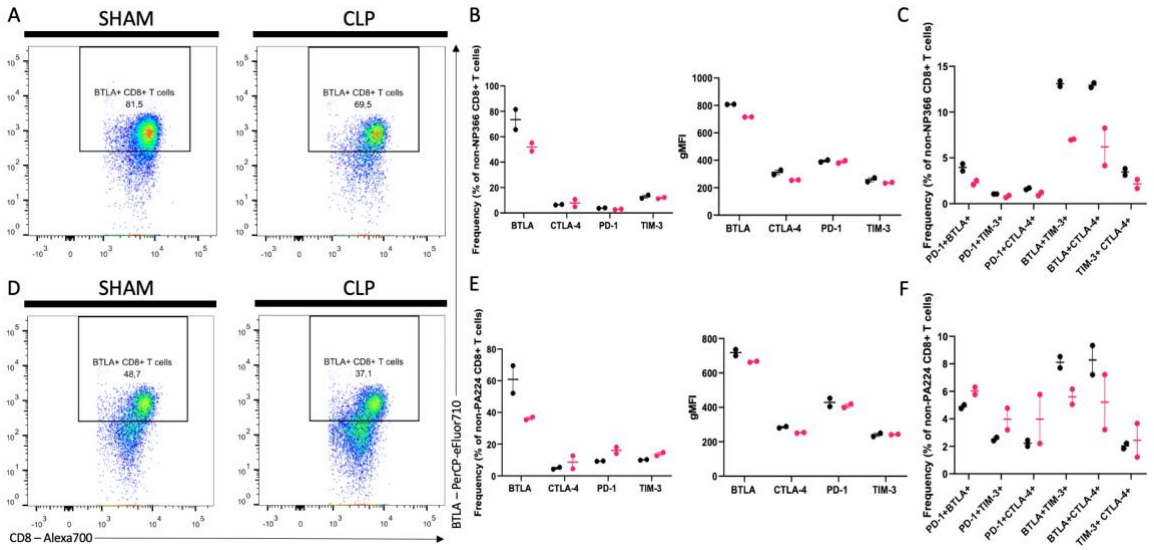

Figure 25. Non--IAV-specific CD8+ T cells within the septic lungs appear to have a reduced frequency and per-cell expression of BTLA, while possessing a similar exhaustion profile compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of non-NP_{366}-specific CD8+ T cells singly expressing BTLA within the lungs. (B) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (C) Summary plot for the frequency of non-NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (D) Representative flow plots for the frequency of non-PA_{224}-specific CD8+ T cells singly expressing BTLA within the lungs. (E) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (F) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B, C, E, F) No statistics were performed as the replicate number is too low.

3.1.11 Within the septic lungs, there appears to be a phenotypic shift from central to effector memory CD8+ T cells due to the increase in absolute numbers of effector memory CD8+ T cells

Herein, I discovered that there was a trend towards higher frequencies and absolute numbers of activated CD8+ T cells within the septic lungs as measured by the expression of CD44 (Figure 24. Panel B). Activated CD8+ T cells within the septic lungs appear to have a 20% increase in frequency and roughly a four-fold increase in their absolute numbers compared to sham. In terms of the different memory CD8+ T cell populations, I found that there was a shift in frequency from central to effector memory CD8+ T cells, which appears to be due to the numerical increase of effector memory CD8+ T cells within the septic condition (Figure 24. Panel E).

In terms of their phenotypic differences, central memory CD8+ T cells display no differences in the single- and double-expression of the different exhaustion markers examined (Figure 24. Panel C and D). In terms of the effector memory CD8+ T cell population, these cells tended to singly express lower frequencies of BTLA (Figure 24. Panel F); however, for the other exhaustion markers examined, both single- and double-expression were similar in frequency compared to sham (Figure 24. Panel F and G).

Taken together, this information suggests that there is either (1) an influx of effector memory CD8+ T cells into the septic lungs or (2) an increased ability of effector memory CD8+ T cells to perform *in situ* proliferation ultimately leading to the higher presence of effector memory CD8+ T cells within the septic lungs. These findings also suggest that there is a higher level of activated CD8+ T cells within the lungs of septic mice compared to sham. Furthermore, effector memory CD8+ T cells within the septic lungs may be more activated as illustrated by their reduced frequencies of BTLA. Despite these findings, I may not provide definitive conclusions as the replicate number was too low.

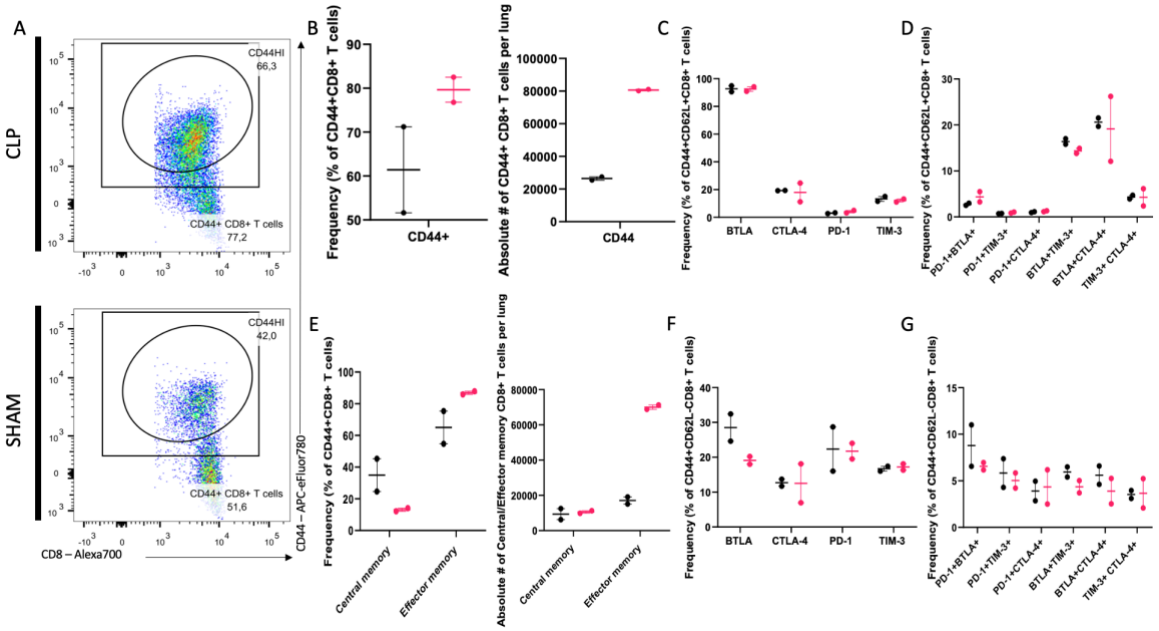

Figure 26. There is a trend towards higher frequencies and absolute numbers of activated CD8+ T cells with an associated phenotypic shift from central to effector memory due to the higher absolute numbers of effector memory CD8+ T cells within the septic lungs. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of CD8+ T cells expressing CD44 within the lungs. (B) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing CD44 within the lungs of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (D) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (E) Summary plot for the frequency (left) and absolute numbers (right) of the different memory CD8+ T cell subsets examined (central and effector memory) within the spleen of each mouse. (F) Summary plot for the frequency of effector memory CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (G) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B-G) No statistics were performed as the replicate number is too low.

3.1.12 IAV-specific CD8+ T cells within the septic liver appear to be higher in frequency and absolute numbers, while displaying a differential expression pattern of exhaustion

Next, I had also wanted to examine the peak memory IAV-specific CD8+ T cell response during sepsis-induced immunosuppression within the liver. The liver is an important organ to study in the context of abdominal sepsis as the initiation of sepsis occurs within the peritoneal cavity and the liver is highly intertwined with the gut as it possesses a blood network dedicated to receiving blood from the gut. Hence, it is important to study the liver in the context of abdominal sepsis.

Within the septic liver, I discovered that there appeared to be a higher frequency and absolute number of IAV-specific CD8+ T cells (Figure 25. Panel D), which may suggest that there is either (1) a higher influx of these cells from the circulatory system or (2) these cells possess a higher proliferative capacity *in situ*.

In terms of their phenotypic differences, septic hepatic NP_{366}-specific CD8+ T cells demonstrated a trend towards higher frequencies of cells expressing PD-1 (Figure 25. Panel B and C). These findings may suggest that (1) these cells are more exhausted, (2) these cells are more activated[282,431,435,436] and (3) the septic liver may possess more tissue-resident memory CD8+ T cells as it has been shown that intrahepatic CD8+ T cells that reside within this tissue possess higher frequencies of PD-1 compared to intrahepatic CD8+ T cells that do not reside within the liver[437]. In terms of septic hepatic PA_{224}-specific CD8+ T cells, there was a trend towards reduced frequencies of cells expressing CTLA-4 (Figure 25. Panel E and F). These findings may suggest that septic PA_{224}-specific CD8+ T cells within the liver could be less functional compared to their sham counterpart as CTLA-4 is transiently expressed early during the immune response; at the peak memory CD8+ T cell response, antigen-specific CD8+ T cells that express CTLA-4 tend to be more functional compared to antigen-specific CD8+ T cells that have lost its expression[438]. Despite these findings, the conclusions are not absolute due to the low number of replicates.

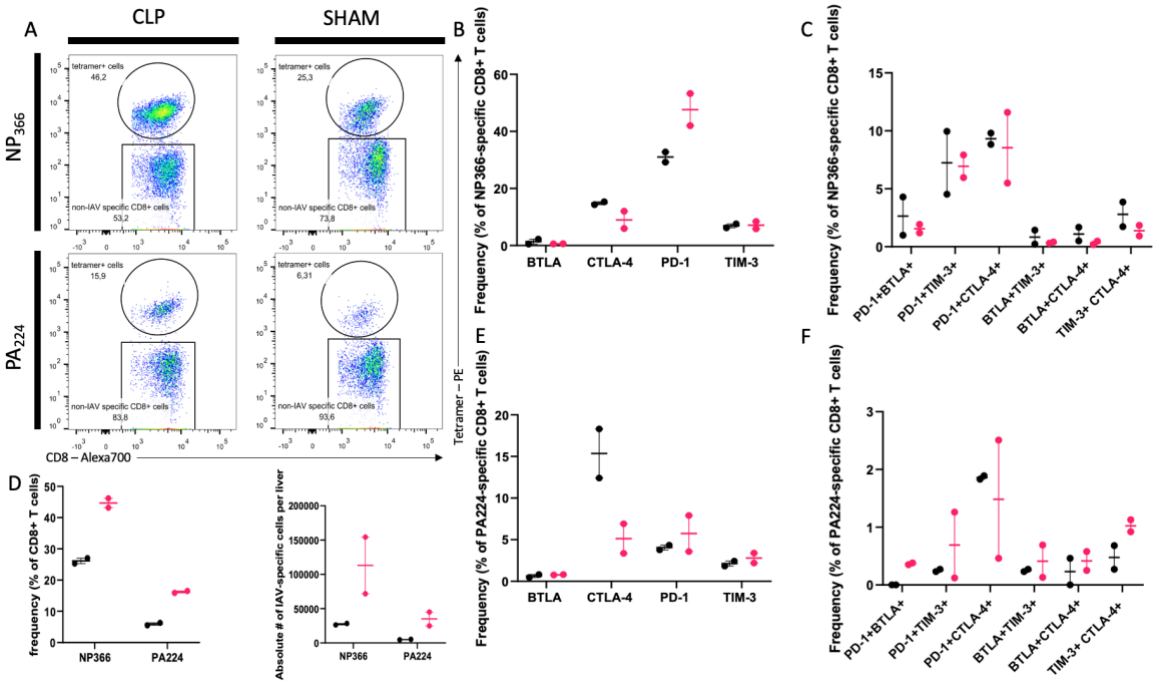

Figure 27. IAV-specific CD8+ T cells appear to be higher in frequency and absolute numbers within the septic liver, while NP_{366}-specific CD8+ T cells demonstrate an upward trend in the frequencies of PD-1 and PA_{224}-specific CD8+ T cells display a downward frequency trend of CTLA-4. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells measured by tetramer reagent within the liver. (B) Summary plot for the frequency of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (C) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (D) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells within the liver of each mouse. (E) Summary plot for the frequency of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B-F) No statistics were performed because the replicate number was too low.

3.1.13 Non-IAV-specific CD8+ T cells within the septic liver appear to possess lower frequencies and per-cell expression of BTLA

Thereafter, just as I had done for the other tissues, I also wanted to examine the non-IAV-specific CD8+ T cell population within the liver to acquire a better understanding on the effects protracted sepsis has on the whole CD8+ T cell compartment within this tissue.

I discovered that septic non-IAV-specific CD8+ T cells within the liver demonstrated a trend towards reduced frequencies and per-cell expression of BTLA (Figure 26. Panel B and E), which may suggest that these cells are more activated within the septic liver as BTLA is a negative regulator of T cell activation and is subsequently downregulated during CD8+ T cell activation[434]. Hence, considering that BTLA was expressed at lower levels within the septic liver, this is an indication that they may be more activated. Furthermore, there was no other notable differences in the frequency and per-cell expression of the other exhaustion markers examined (Figure 26. Panel B and E); however, for non-NP_{366}-specific CD8+ T cells, there was a decrease in the frequency of cells doubly expressing different exhaustion markers associated with BTLA (Figure 26. Panel C), but this might just be due to the reduced expression of BTLA.

Taken together, there is some evidence to suggest that septic non-IAV-specific CD8+ T cells are more phenotypically activated compared to sham; however, these results are not conclusive and further experiments need to be performed as the number of replicates was too low to provide any definitive conclusions.

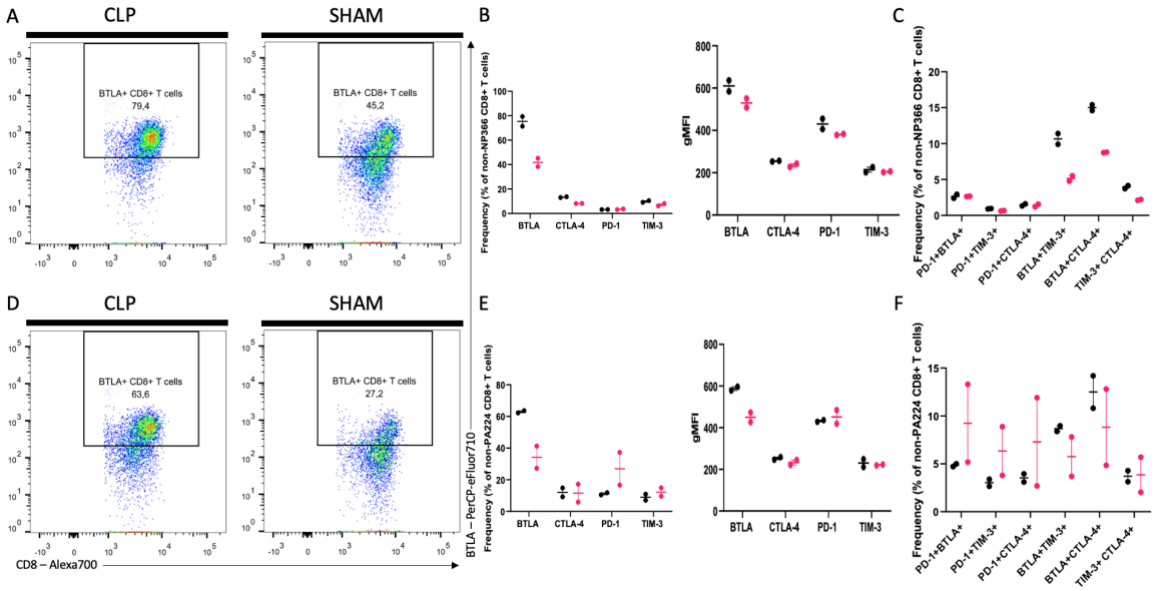

Figure 28. Non-IAV-specific CD8+ T cells within the septic liver appear to possess lower frequencies and per-cell expression of BTLA. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of non-NP_{366}-specific CD8+ T cells singly expressing BTLA within the liver. (B) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (C) Summary plot for the frequency of non-NP_{336}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (D) Representative flow plots for the frequency of non-PA_{224}-specific CD8+ T cells singly expressing BTLA within the liver. (E) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (F) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B, C, E, F) No statistics were performed as the replicate number is too low.

3.1.14 Within the septic liver, there appears to be a higher frequency and absolute number of activated CD8+ T cells with an associated phenotypic shift from central to effector memory due to the loss of central memory CD8+ T cells and the gain of effector memory CD8+ T cells

Thereafter, I had decided to examine the bulk memory CD8+ T cell compartment within the septic liver. I discovered that there was an upward trend in the frequency and absolute number of activated CD8+ T cells as measured by the expression of CD44 (Figure 27. Panel B). To further characterize this increased presence of activated CD8+ T cells, I decided to look at the frequencies and absolute numbers of the different memory CD8+ T cell subsets. Herein, I noted that there appeared to be a phenotypic shift in frequency from central to effector memory CD8+ T cells within the septic liver, which appeared to be due to the numerical reduction of central memory CD8+ T cells and the numerical gain of effector memory CD8+ T cells (Figure 27. Panel E). From this information and taking into consideration that effector memory CD8+ T cells have a poor ability to proliferate[431], it appears as though their gain in numbers is due to the their increased influx into the liver.

In terms of their phenotypic differences, the different septic hepatic memory CD8+ T cells did not show a trend or difference in their single- and double-expression of the different exhaustion markers examined (Figure 27. Panel C, D, F, G).

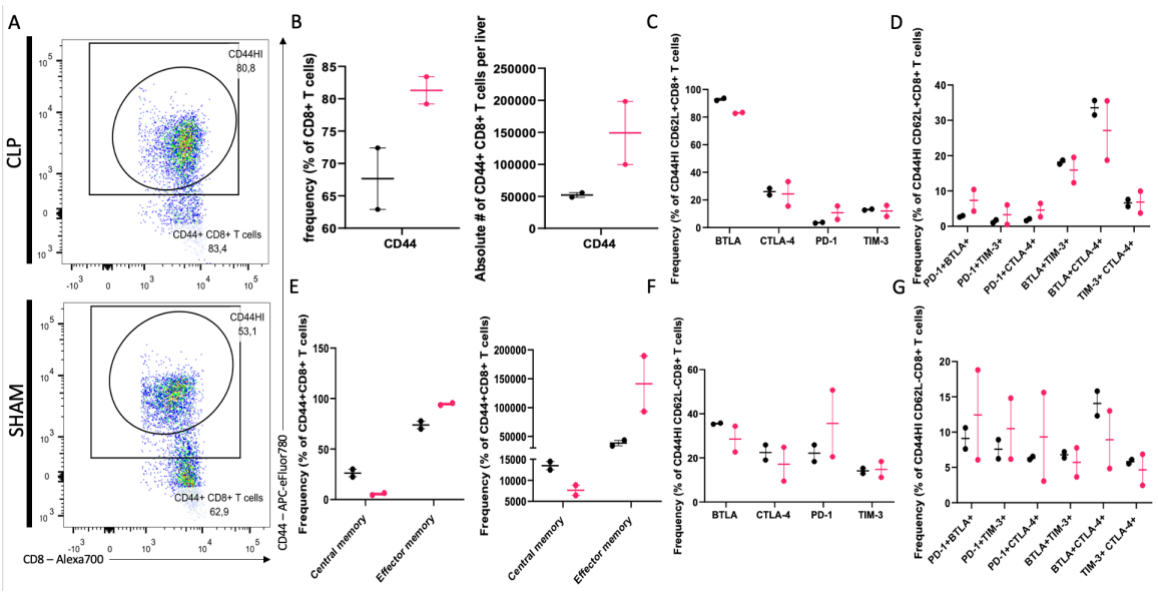

Figure 29. Within the septic liver, there appears to be a higher frequency and absolute number of activated CD8+ T cells with an associated phenotypic shift from central to effector memory due to the loss of central memory CD8+ T cells and the gain of effector memory CD8+ T cells. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of CD8+ T cells expressing CD44 within the liver. (B) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing CD44 within the liver of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (D) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (E) Summary plot for the frequency (left) and absolute numbers (right) of the different memory CD8+ T cell subsets (central and effector memory) within the liver of each mouse. (F) Summary plot for the frequency of effector memory CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (G) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. The data was from 1 independent experiment: CLP = 2 mice; Sham = 2 mice. (B-G) No statistics were performed as the replicate number was too low.

3.2 Aim 2: Primary Response Results

3.2.1 IAV-specific CD8+ T cells within the septic spleen are higher in frequency, but are numerically similar compared to sham; however, there is an increased frequency and absolute number of IFN-γ-producing-IAV-specific CD8+ T cells

In my second aim, I had wanted to examine IAV-specific CD8+ T cell responses within the spleen (among other tissues) at the peak primary CD8+ T cell response during protracted sepsis. This aim would provide me with important information, such as the naïve antigen specific CD8+ T cell response during sepsis-induced immunosuppression; however, it would also provide me with information regarding the differences between the recall and primary response in the context of sepsis-induced immunosuppression. Currently, within the literature, scientists have only focused on one or the other, so this study is important as I provide information on to be able to compare the results. In order to accomplish this aim, I performed CLP surgery on naïve B6 mice, then subsequently administered PR8 four days later to induce a primary response. Seven days post-infection, at the peak primary CD8+ T cell response[411], I sacrificed the mice and performed the phenotypic and functional experiments.

I first decided to examine the frequencies and absolute numbers of IAV-specific CD8+ T cells within spleen. Through the tetramer reagent experiments, I discovered that NP_{366}-specific CD8+ T cells were higher in frequency within the septic spleen compared to sham, while PA_{224}-specific CD8+ T cells only demonstrated an upward trend towards higher frequencies (Figure 28. Panel B, p = 0.0001, 0.26, respectively). Despite these findings, IAV-specific CD8+ T cells were numerically similar between the two conditions (Figure 28. Panel B, p = 0.7, 0.8, respectively). Furthermore, in addition to the tetramer experiments, I had also performed ICS experiments to detect IAV-specific CD8+ T cells capable of producing IFN-γ under the stimulation with their cognate peptides. I demonstrated that there was a higher frequency and absolute number of IAV-specific CD8+ T cells capable of producing IFN-γ (Figure 28. Panel D, Frequency: p = <0.0001, 0.002; Absolute numbers: p = 0.0001, 0.04, respectively).

Taken together, these findings suggest that IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression are more frequent within the septic spleen; however, they are numerically similar compared to sham. I have also discovered that CD8+ T cells within the septic spleen were numerically reduced (data not shown). These two pieces of information suggest that IAV-specific CD8+ T cells either (1) have an increased proliferative capacity, (2) are resistant to sepsis-induced apoptosis or (3) these cells have a higher capacity to migrate from different anatomical locations to the spleen. Furthermore, I also discovered that there was a higher frequency and absolute number of IFN-γ-producing-IAV-specific CD8+ T cells within the septic, which may suggest that out of all the IAV-specific CD8+ T cells present in the spleen, within the septic condition, there is more IAV-specific CD8+ T cells that are capable of producing IFN-γ compared to sham.

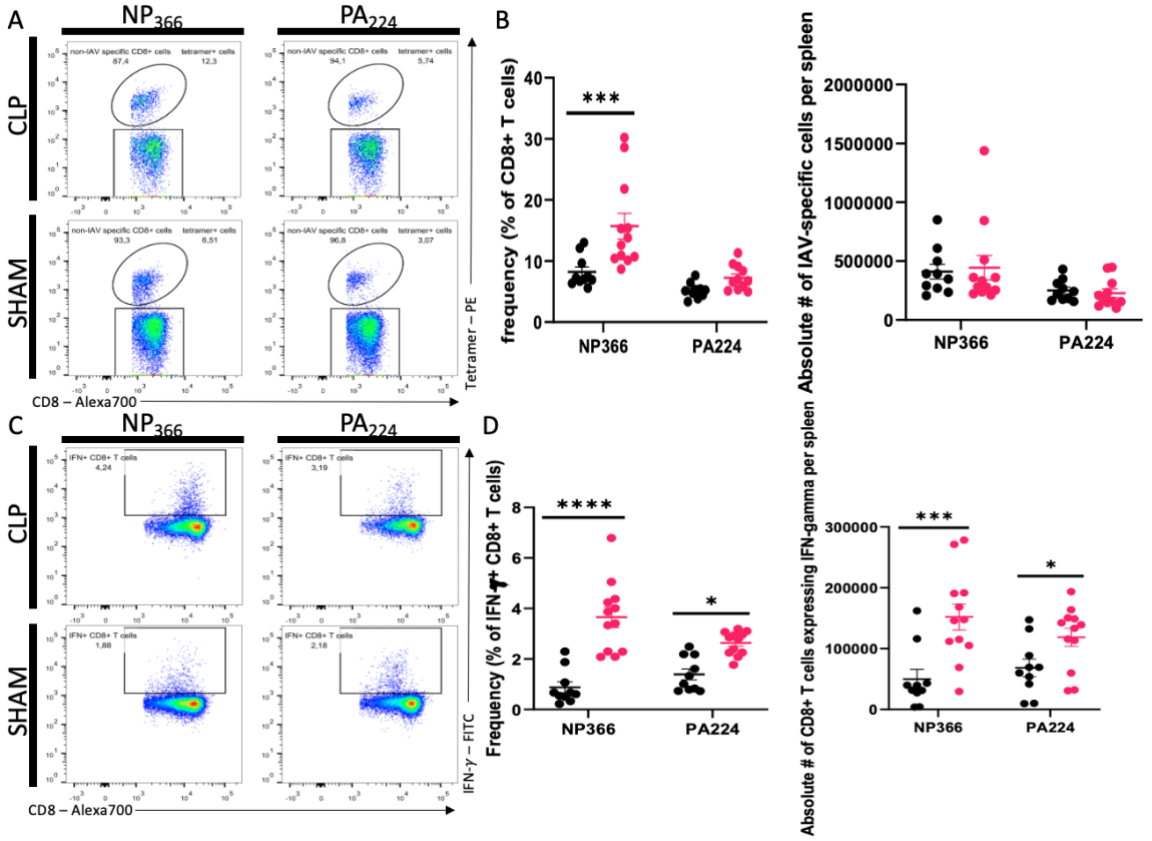

Figure 30. IAV-specific CD8+ T cells within the septic spleen are higher in frequency, although are numerically similar compared to sham; furthermore, there is a higher frequency and absolute number of IFN-γ-producing-IAV-specific CD8+ T cells within the septic spleen. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells measured by tetramer reagent within the spleen. (B) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the spleen of each mouse. (C) Representative flow plots for the frequency of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with cognate peptides (NP_{366} and PA_{224}) within the spleen. (D) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with cognate peptides (NP_{366} and PA_{224}) within the spleen of each mouse. The data was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10). (B and D) Statistics for

significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.2 Septic splenic IAV-specific CD8+ T cells are more phenotypically exhausted and activated compared to sham

Next, I had wanted to phenotypically characterize IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. I had first examined their exhaustion phenotype by looking at the expression of nine different exhaustion markers, namely PD-1, BTLA, CTLA-4, TIM-3, VISTA, TIGIT, 2B4, LAG-3 and KLRG1.

Herein, I discovered that septic splenic IAV-specific CD8+ T cells possessed higher frequencies of cells singly expressing 2B4 (Figure 29. Panel B and E, p = 0.003, 0.0005, respectively); however, their per-cell expression of 2B4 was similar compared to sham (Figure 29. Panel B and E, p = 0.9, 0.7, respectively). Furthermore, I also discovered that NP_{366}-specific CD8+ T cells had significantly increased frequencies of TIGIT expression, while PA_{244}-specific CD8+ T cells only demonstrated an upward trend (Figure 29. Panel B and E, p = 0.01, 0.06, respectively); however, these IAV-specific CD8+ T cells may have a reduced per-cell expression of TIGIT (Figure 29. Panel B and E, p = 0.4, 0.2, respectively), but the data has a high variance so this might not be significant. Additionally, IAV-specific CD8+ T cells demonstrated a significant increase in the frequency of cells expressing KLRG1 (Figure 31. Panel C and E, p = <0.0001, <0.0001, respectively). Moreover, one other interesting observation that I noted was that PA_{224}-specific CD8+ T cells demonstrated a downward trend in the frequency of cells singly expressing PD-1 (Figure 29. Panel E, p = 0.2), which was associated with a decreased frequency of cells doubly expressing PD-1+TIM-3+ (Figure 29. Panel F, p = 0.02). This finding may suggest that septic splenic PA_{224}-specific CD8+ T cells are less exhausted and activated compared to their septic splenic NP_{366}-specific CD8+ T cell counterpart and the sham splenic PA_{224}-specific CD8+ T cells.

In addition to examining the single expression of the different exhaustion markers, I had also decided to look at the frequencies of IAV-specific CD8+ T cells doubly expressing the different exhaustion marker combinations as this would be more indicative of CD8+ T cell exhaustion[424,425]. In terms of NP_{366}-specific CD8+ T cells, these cells demonstrated a significant increased frequency of cells doubly expressing TIGIT+VISTA+ and TIGIT+2B4+ (Figure 29. Panel C, p = 0.01, 0.02, respectively), while showing an upward trend for VISTA+LAG-3+, VISTA+2B4+ and LAG-3+2B4+ (Figure 29. Panel C, p = 0.2, 0.09, 0.3, respectively). In terms of PA_{224}-specific CD8+ T cells, these cells possessed a significant reduction in the frequency of cells doubly expressing PD-1+TIM-3+ (Figure 29. Panel F, p = 0.02); however, these cells demonstrated an upward trend in the frequencies of cells doubly expressing TIGIT+VISTA+, TIGIT+2B4+, VISTA+2B4+ and LAG-3+2B4+ (Figure 29. Panel F, p = 0.1, 0.06, 0.3, 0.4, respectively).

Taken together, it appears that septic splenic IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression are more phenotypically exhausted compared to their sham counterpart; however, septic splenic NP_{366}-specific CD8+ T cells may be more exhausted compared to septic splenic PA_{224}-specific CD8+ T cells as they possessed a more pronounced exhaustion profile.

In addition to examining the different exhaustion markers, I had also assessed the expression of different markers for activation, death, and survival/maintenance. I had examined the expression of CD25, CD69, CD44, CD127, and CD95. Of important note, CD127 is expressed on naïve CD8+ T cells to maintain and promote survival[251]. During naïve CD8+ T cell activation through cognate antigen exposure, CD127 gets downregulated and the naïve CD8+ T cells that maintain their expression of CD127 generate long-lived memory CD8+ T cells[426,439–441]. Hence, when examining the expression of CD127, I may make inferences on their activation state and their ability to generate a long-lived memory CD8+ T cell pool.

Herein, I discovered that septic splenic IAV-specific CD8+ T cells had a reduced frequency of cells expressing CD44 (Figure 30. Panel A and B, $p = 0.04, 0.02$, respectively) with an associated downward trend in their per-cell expression of CD44 (Figure 30. Panel A and B, $p = 0.5, 0.4$, respectively). Despite these findings, it may not be biologically relevant as the frequency of cells expressing CD44 only differed by a few percent. Furthermore, septic splenic PA_{224}-specific CD8+ T cells demonstrated a significant decrease in the frequency of cells expressing CD127 (Figure 30. Panel B, $p = <0.0001$), while septic splenic NP_{366}-specific CD8+ T cells only displayed a strong downward trend in the frequency of cells expressing CD127 (Figure 30. Panel A, $p = 0.07$).

Taken together, in collaboration with the exhaustion profiles, I can suggest that septic splenic IAV-specific CD8+ T cells appear to be more activated compared to sham. Additionally, these cells may have a dampened ability to produce a robust memory CD8+ T cell population.

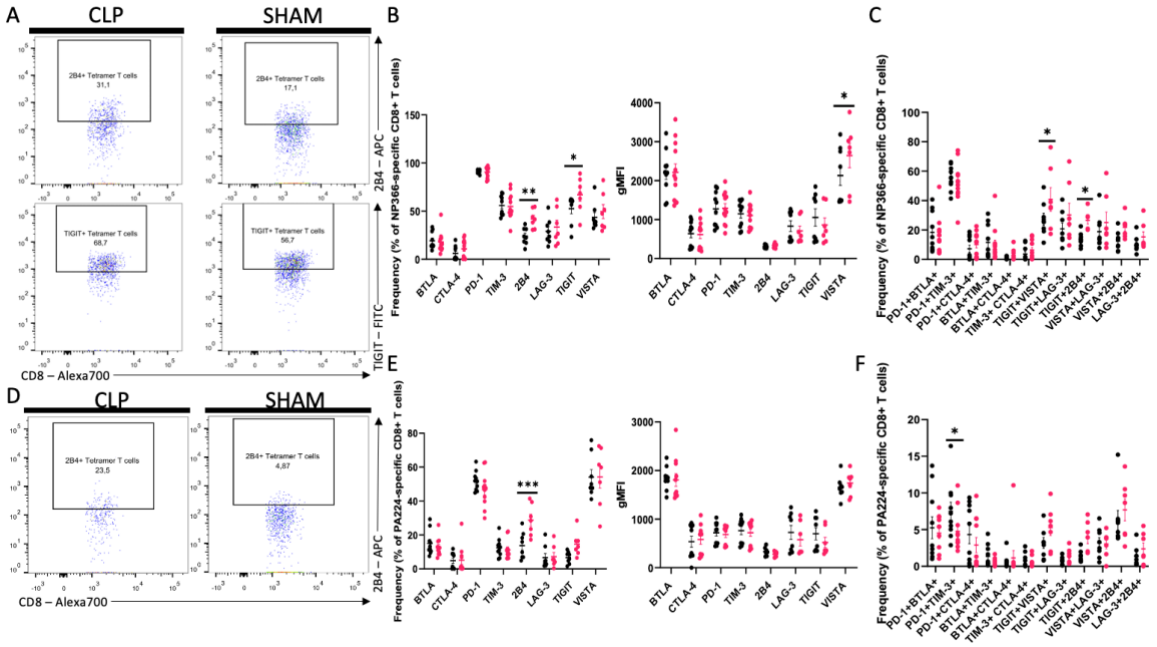

Figure 31. IAV-specific CD8+ T cells within the septic spleen possess higher frequencies of cells expressing 2B4, while NP_{366}-specific CD8+ T cells express higher frequencies of TIGIT and PA_{224}-specific CD8+ T cells only showing a trend. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of NP_{366}-specific CD8+ T cells singly expressing TIGIT and 2B4 within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (C) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (D) Representative flow plots for the frequency of PA_{224}-specific CD8+ T cells singly expressing 2B4 within the spleen. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (C and F) The combination of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10). The data for exhaustion

panel 2 was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 8). (B, C, E, F) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

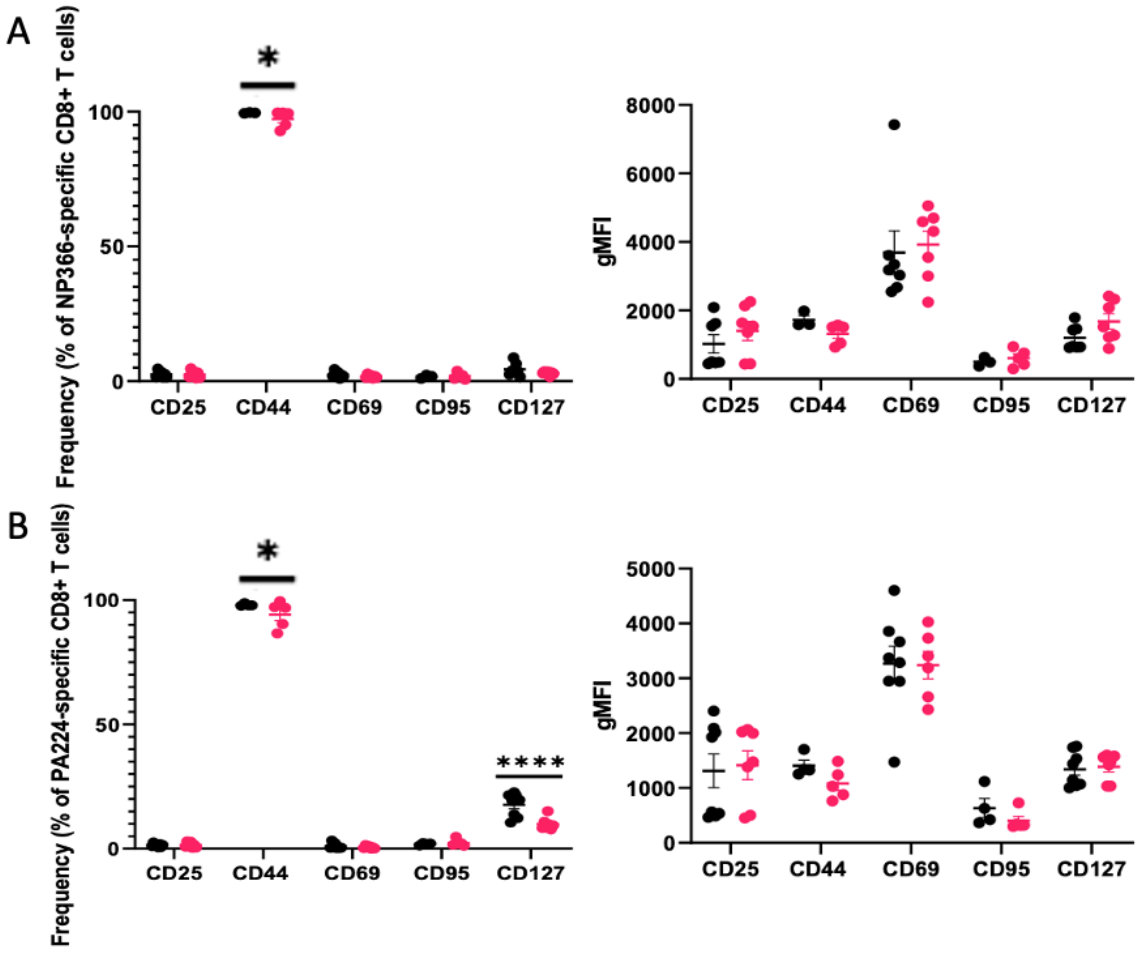

Figure 32. Within the septic spleen, PA_{224}-specific CD8+ T cells possess lower frequencies of cells expressing CD127, while NP_{336}-specific CD8+ T cells only demonstrated a downward trend. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 8). (A and B) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.3 Septic splenic IAV-specific CD8+ T cells are more functionally capable of producing granzyme B, IFN-γ and IL-2; however, these cells may have a dampened quality of response

Next, I had wanted to assess the functionally of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. In order to accomplish this, I stimulated splenocytes *ex vivo* with the cognate peptides for the IAV-specific CD8+ T cells (NP_{366} and PA_{224}), then subsequently performed an ICS to detect the production of different cytokines, a cytolytic effector molecule and a surface marker for co-inhibition.

Within the septic spleen, IAV-specific CD8+ T cells capable of producing IFN-γ were higher in frequency (Figure 31. Panel B, p<0.0001, 0.002, respectively) and absolute numbers (Figure 31. Panel B, p = 0.0001, 0.04, respectively). Furthermore, the MFI data demonstrated that septic splenic IAV-specific CD8+ T cells produced more IFN-γ on a per-cell basis (Figure 31. Panel B, p = 0.004, 0.02, respectively). From this information, it could be stated that within the septic spleen at the peak primary CD8+ T cell response, there is a higher frequency and absolute number of IAV-specific CD8+ T cells capable of producing IFN-γ with an associated increase in their per-cell expression of IFN-γ.

Additionally, I had also decided to further characterize their functionality by broadening the cytokines examined. I discovered that IAV-specific CD8+ T cells within the septic spleen had significantly higher frequencies of cells expressing granzyme B (Figure 31. Panel C and E, p<0.0001, <0.0001, respectively); however, their per-cell expression of granzyme B was comparable to sham (Figure 31. Panel C and E, p = 0.8, 0.8, respectively). Furthermore, I demonstrated that the per-cell expression of IL-2 was significantly higher in the septic splenic IAV-specific CD8+ T cells (Figure 31. Panel C and E, p = 0.02, 0.05, respectively). Moreover, septic splenic IAV-specific CD8+ T cells also demonstrated an upward trend in the per-cell expression of TNF-α (Figure 31. Panel C and E, p = 0.3 , 0.1). This information suggests that septic splenic IAV-specific CD8+ T cells are more functionally capable than their sham counterpart.

Next, I had also wanted to examine the quality of response in IAV-specific CD8+ T cells by assessing their polyfunctionality measured by the triple-expressing of IFN-γ, TNF-α, and IL-2. Septic splenic NP_{366}-specific CD8+ T cells demonstrated a downward trend in the frequency of triple-expressors indicative of reduced polyfunctionality (Figure 31. Panel D, $p = 0.4$), while PA_{224}-specific CD8+ T cells also displayed the same trend, albeit not to the same degree (Figure 31. Panel D, $p = 0.5$). Despite their apparent increased ability to produce cytokines and cytolytic effector molecules, their quality of response may be dampened within the septic spleen.

Taken together, it appears as though septic splenic IAV-specific CD8+ T cells are more functionally capable compared to sham as demonstrated by their increased expression of IFN-γ, granzyme B and IL-2. Furthermore, considering the role of IL-2, these findings provide some evidence to show that septic splenic IAV-specific CD8+ T cells may possess a higher capability to proliferate within the septic condition. Despite these findings, I demonstrated that septic splenic IAV-specific CD8+ T cells may have a dampened quality of response as they possessed reduced frequencies of cells triple-expressing IFN-γ, TNF-α, and IL-2.

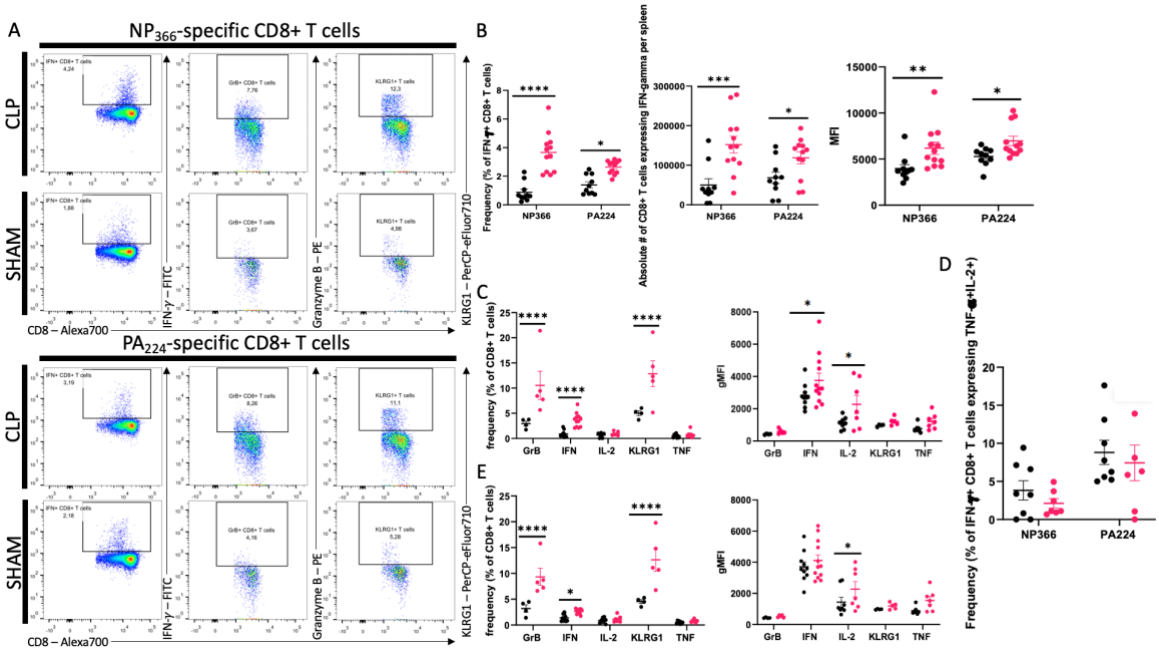

Figure 33. IAV-specific CD8+ T cells within the septic spleen appear to be more functionally active as demonstrated by their increased capabilities in producing IFN-γ, granzyme B and IL-2; however, their quality of response may be dampened. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells capable of producing IFN-γ and granzyme B under the *ex vivo* stimulation with their cognates peptides (NP_{366} and PA_{224}), while additionally assessing for the surface expression of the co-inhibitory marker KLRG1 within the spleen. (B) Summary plots for the frequency (left), absolute numbers (middle) and MFI (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the spleen of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing the different cytokines, cytolytic effector molecule and a surface marker for co-inhibition within the spleen of each mouse. (D) Summary plot for the frequency of IAV-specific CD8+ T cells that triple-express IFN-γ, TNF-α and IL-2 to determine the polyfunctionality of these cells within the spleen of each mouse. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing the different cytokines, cytolytic effector molecule and a surface marker for co-inhibition within the spleen of each mouse. The data for IFN-γ was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10).

The data for TNF-α and IL-2 was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 8). The data for granzyme B and KLRG1 was pooled from 2 independent experiments: CLP = 2-3 mice/experiments (n = 5); Sham = 2 mice/experiment (n = 4). (B-E) Statistics for significance was determined using the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.2.4 Non-IAV-specific CD8+ T cells within the septic spleen appear to be more exhausted and activated compared to sham

Next, just as I have previously done, I had wanted to examine the phenotype of non-IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during protracted sepsis. This would provide me with (1) a better global understanding on the effects sepsis has on the whole CD8+ T cell pool and (2) a better understanding on the effects sepsis has on the IAV-specific CD8+ T cells as there may be markers that are unique to this cell population.

Septic splenic non-IAV-specific CD8+ T cells demonstrate an upward trend in the frequency of cells singly expressing PD-1 and 2B4 (Figure 32. Panel A and C, Non-NP_{366}: p = 0.3, 0.1; Non-PA_{224}: p = 0.06, 0.08, respectively). In terms of septic splenic non-PA_{224}-specific CD8+ T cells, these cells possess an upward trend in their frequency of cells singly expressing TIM-3 and TIGIT (Figure 32. Panel C, p = 0.3, 0.06, respectively). This information suggests that septic splenic non-IAV-specific CD8+ T cells may be more phenotypically exhausted compared to their sham counterpart, which may also suggest that these cells were more activated.

Furthermore, I had also wanted to characterize their co-expression of the different exhaustion marker combinations as this would provide more robust evidence on their exhaustion profile. Non-IAV-specific CD8+ T cells within the septic spleen displayed a significant increase in the frequency of cells doubly expressing VISTA+2B4+ and TIGIT+VISTA+ (Figure 32. Panel B and D, Non-NP_{366}: p = 0.0008, 0.04; Non-PA_{224}: p = 0.01, 0.002, respectively), while non-NP_{366}-specific CD8+ T cells doubly expressed higher frequencies of BTLA+CTLA-4+ and VISTA+LAG-3+ (Figure 32. Panel B, p = 0.008, 0.03, respectively) and non-PA_{224}-specific CD8+ T cells doubly expressed higher frequencies of PD-1+TIM-3+ and TIGIT+2B4+ (Figure 32. Panel D, p = 0.001, 0.001, respectively). These findings are strong evidence to show that septic splenic non-IAV-specific CD8+ T cells are phenotypically more exhausted compared to sham, which may also suggest that these cells were more activated.

There is some evidence to suggest that TIGIT and 2B4 may be unique to IAV-specific CD8+ T cells at the peak primary CD8+ T cell response in the context of sepsis-induced immunosuppression. Within the non-PA_{224}-specific CD8+ T cell compartment, there will be contamination from NP_{366}-specific CD8+ T cells, and vice-versa; however, I may use this contamination to my benefit as there will be more NP_{366}-specific CD8+ T cells contaminating the non-PA_{224}-specific CD8+ T cell compartment. Examining the frequencies for these markers, I noticed that non-PA_{224}-specific CD8+ T cells had a stronger trend in the expression of 2B4 and TIGIT, while non-NP_{366}-specific CD8+ T cells had a weaker trend or no trend at all. This information suggests that both TIGIT and 2B4 may be unique to IAV-specific CD8+ T cells as the signal is most likely being contributed by the IAV-specific CD8+ T cells contaminating this population.

Next, I had also wanted to examine their activation phenotype by looking at the expression of different activation, death, and survival/maintenance markers. Herein, I discovered that non-IAV-specific CD8+ T cells within the septic spleen had an increased frequency of cells expressing CD44, while demonstrating a reduced frequency of cells expressing CD127 (Figure 33. Panel B and C, Non-NP_{366}: $p = 0.0002, 0.002$; Non-PA_{224}: $p = 0.0007, 0.0007$, respectively). From this information, it could be suggested that within the septic spleen, there is a higher frequency of activated CD8+ T cells as demonstrated by their increased expression of CD44[282] and reduced expression of CD127[426,427]. Furthermore, non-IAV-specific CD8+ T cells may have a reduced capacity to produce a robust memory CD8+ T cell pool or are reduced in their capacity to maintain themselves within the septic host as CD127 is required for T cell homeostasis and survival[439–441]. Furthermore, I also demonstrated that non-IAV-specific CD8+ T cells had a decreased per-cell expression of CD69 (Figure 33. Panel B and C, $p = 0.01, 0.01$, respectively). This information might suggest a few things: First, these cells are less activated; however, this seems unlikely as the other findings suggest otherwise. Secondly, they may have a reduced capacity to retain themselves within tissues[442]. Thirdly, which is the most probable, it is just a result from homeostatic proliferative mechanisms as naïve CD8+ T cells have been shown to adopt a memory-like phenotype with the upregulation of CD44, but do not induce upregulation of CD69 and CD25[185,217,443].

Taken together, it is evident that non-IAV-specific CD8+ T cells within the septic spleen at the peak primary CD8+ T cell response during protracted sepsis are phenotypically more exhausted compared to sham as they demonstrated higher co-expression of the different exhaustion marker combinations. Furthermore, non-IAV-specific CD8+ T cells within the septic spleen appear to be more phenotypically activated as they possess a higher frequency of cells that express CD44, while having a reduced frequency of cells expressing CD127.

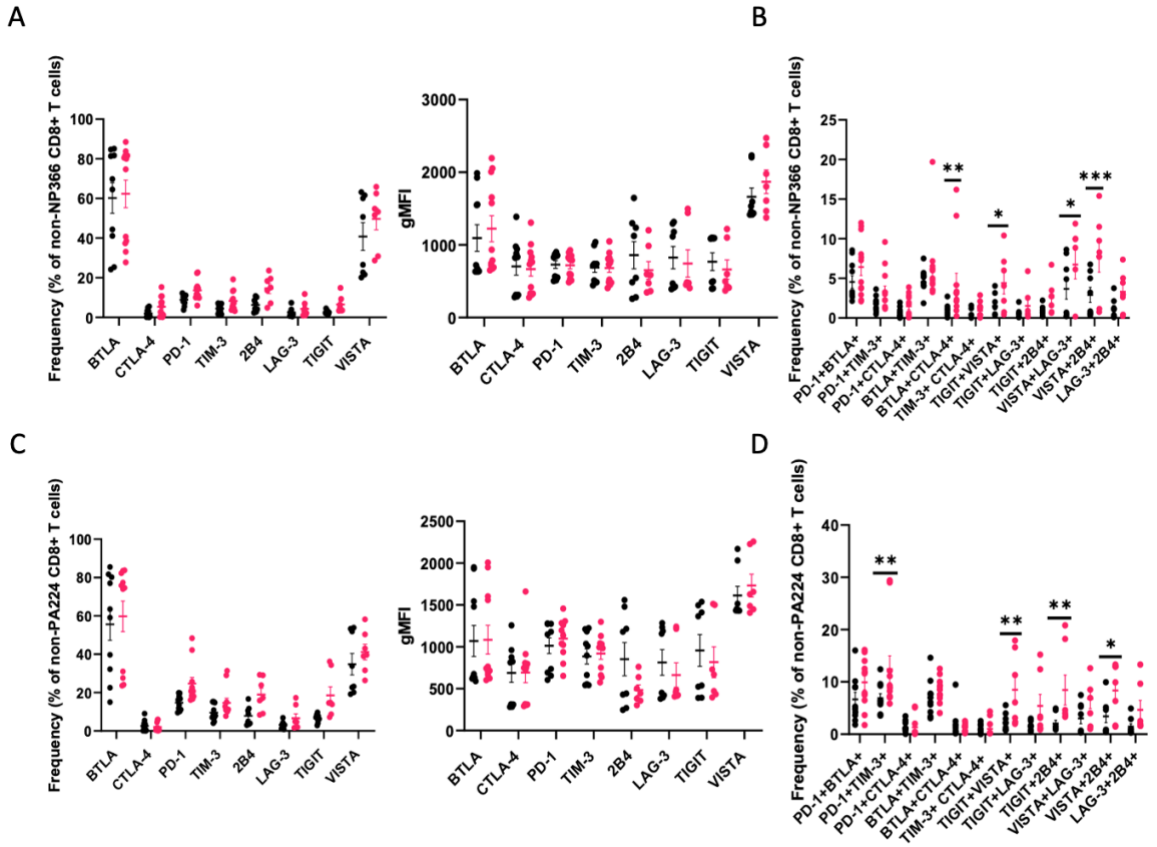

Figure 34. Non-IAV-specific CD8+ T cells within the septic spleen are phenotypically more exhausted compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (B) Summary plot for the frequency of non-NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (D) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (B and D) The combination of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10). The data for exhaustion panel 2 was pooled from 3 different

independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 8). (A-D) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

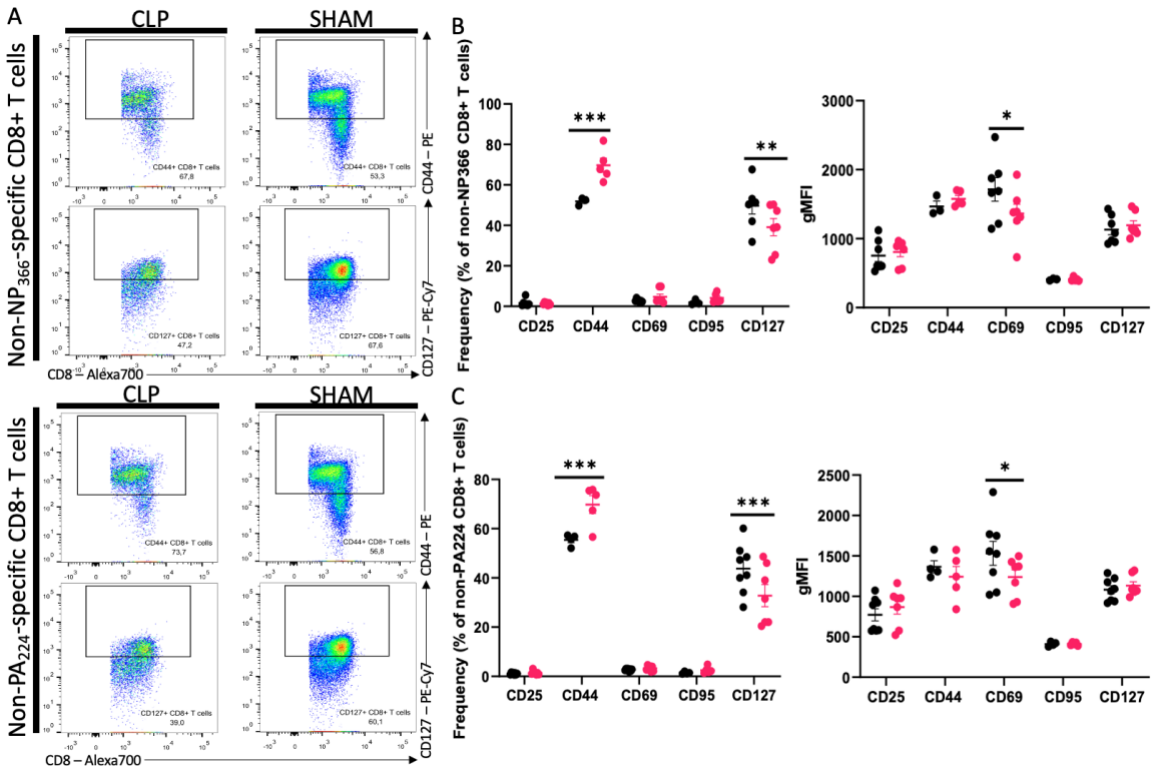

Figure 35. Non-IAV-specific CD8+ T cells within the septic spleen possess higher frequencies of cells expressing CD44 and reduced frequencies of CD127. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of non-IAV-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 8). (B and C) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.5 There is a higher frequency of activated CD8+ T cells within the septic spleen; however, these cells are numerically reduced due to the loss of central memory CD8+ T cells

Next, I had also wanted to examine the bulk memory CD8+ T cell compartment at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. Of important note, the IAV-specific CD8+ T cells of interest are not memory CD8+ T cells; however, I can still study these populations as the cells of interest would all be classified as effector memory CD8+ T cells, in which effector CD8+ T cells are part of this population. Hence, when examining the memory CD8+ T cell subsets, I can focus on the effector memory CD8+ T cells to provide us with information regarding the IAV-specific CD8+ T cells of interest.

Within the septic spleen, I discovered that there was a higher frequency of activated CD8+ T cells as measured by the expression of CD44 (Figure 34. Panel B, $p = 0.0001$); however, there was a reduced absolute number of activated CD8+ T cells within the septic condition (Figure 34. Panel B, $p = 0.005$). This information suggests that activated CD8+ T cells are more frequent within the septic spleen; however, they are numerically reduced. To further characterize this cell loss, I decided to look at the frequencies and absolute numbers of the different memory CD8+ T cell subsets. I found that there was a phenotypic shift in the frequency from central to effector memory CD8+ T cells (Figure 34. Panel D, $p = 0.0004$), in which this shift was due to the loss of central memory CD8+ T cells (Figure 34. Panel D, $p<0.0001$). Furthermore, this cell loss of central memory CD8+ T cells also reflects the loss within the activated CD8+ T cells. This finding is very important as this suggests that there is an alteration in the composition of the CD8+ T cell compartment due to sepsis. This alteration has significant effects on the localization of these cells, for instance, it has been shown that effector memory CD8+ T cells preferentially localize within peripheral tissues[431]. Hence, this phenotypic shift may result in increased amounts of CD8+ T cells within the peripheral tissue, rather than circulating through the blood and secondary lymphoid tissues.

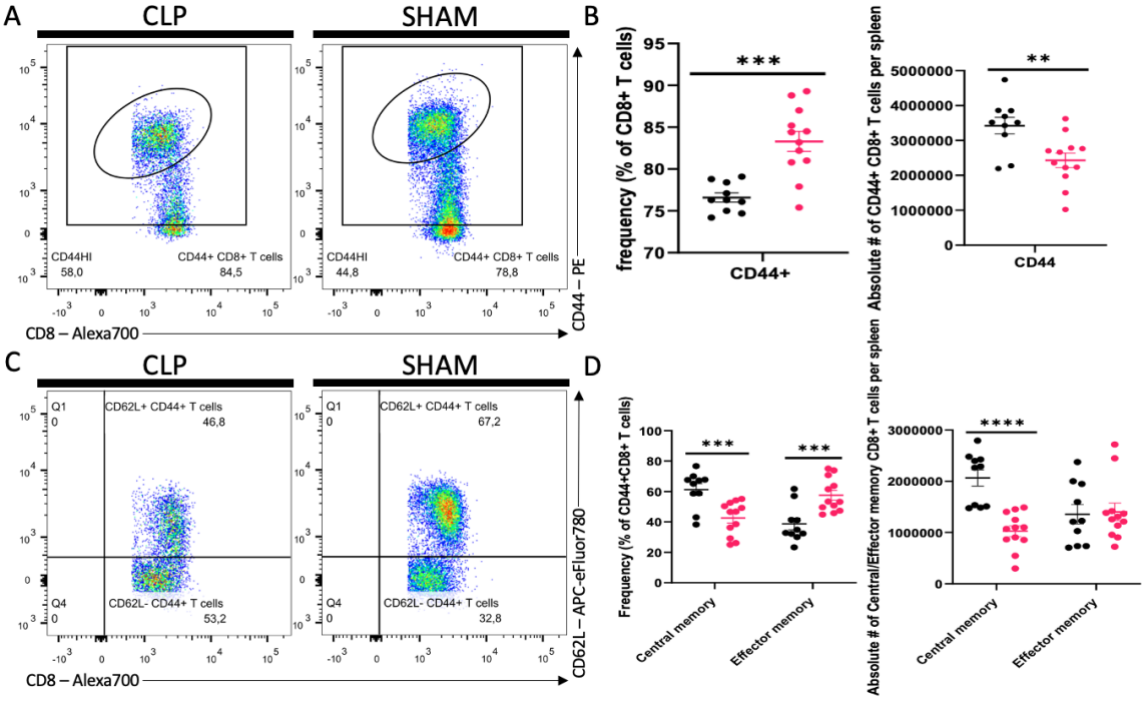

Figure 36. There is a higher frequency of activated CD8+ T cells within the septic spleen; however, these cells are numerically decreased due to the loss of central memory CD8+ T cells. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of CD8+ T cells expressing CD44 within the spleen. (B) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing CD44 within the spleen of each mouse. (C) Representative flow plots for the frequency of central and effector memory CD8+ T cell populations measured by the expression of CD62L gated on CD44+ CD8+ T cells within the spleen. (D) Summary plots for the frequency (left) and absolute numbers (right) of central and effector memory CD8+ T cell populations measured by the expression of CD62L gated on CD44+ CD8+ T cells within the spleen of each mouse. The data was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10). (B and D) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.6 Within the septic spleen, memory CD8+ T cells are phenotypically more exhausted, while effector memory CD8+ T cells possess lower frequencies of cells expressing CD127

In addition to examining the frequencies and absolute numbers of the different memory CD8+ T cell subsets, I had also decided to examine their phenotypic differences at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. In doing so, it could potentially reveal markers that are unique to IAV-specific CD8+ T cells and would provide a better understanding on the effect sepsis has on the whole CD8+ T cell compartment.

I will first discuss my findings for the exhaustion phenotyping experiments. In terms of central memory CD8+ T cells within the septic spleen, there were an increased frequency of cells singly expressing BTLA (Figure 35. Panel B, $p = 0.0007$). Furthermore, septic splenic central memory CD8+ T cells possessed higher frequencies of cells doubly expressing TIGIT+VISTA+, TIGIT+2B4+ and VISTA+2B4+ (Figure 35. Panel C, $p = 0.04, 0.04, 0.0002$, respectively). From this information, it could be suggested that central memory CD8+ T cells within the septic spleen are more phenotypically exhausted compared to sham, which may also imply that these cells were more activated. Furthermore, these cells may possess a dampened ability to respond to infection as they possessed an increased frequency of cells expressing BTLA, which is a negative regulator of T cell activation[434]. To explain these discrepancies, I could suggest that the cells that are activated will be more activated compared to sham leading to higher levels of exhaustion; however, there are less cells being activated within the septic condition as there is a higher frequency of these cells expressing BTLA.

In terms of effector memory CD8+ T cells within the septic spleen, I discovered that there were a higher frequency of cells singly expressing 2B4 with an upward trend in the frequency of cells singly expressing PD-1 and TIGIT (Figure 35. Panel D, p = 0.05, 0.2, 0.1, respectively). Furthermore, septic splenic effector memory CD8+ T cells possessed higher frequencies of cells doubly expressing PD-1+TIM-3+, TIGIT+2B4+ and VISTA+2B4+ (Figure 35. Panel E, p = 0.02, 0.05, 0.03, respectively). From this information, I may state that effector memory CD8+ T cells within the septic spleen at the peak primary CD8+ T cell response in the context of sepsis-induced immunosuppression are more phenotypically exhausted compared to sham, which may also suggest that these cells were more activated. Furthermore, there is evidence to suggest that the upregulation of TIGIT, but not 2B4, may be unique to IAV-specific CD8+ T cells. In terms of 2B4, there is a significant increase in frequency of effector memory CD8+ T cells expressing this marker, which illustrates that this marker might not be specific for IAV-specific CD8+ T cells of interest; however, it could also just be due to the contribution from the IAV-specific CD8+ T cells. In terms of TIGIT, effector memory CD8+ T cells display a trend towards higher frequencies of cells expressing TIGIT, while IAV-specific CD8+ T cells had a significant upregulation of TIGIT, which is evidence to suggest that TIGIT might be unique to the IAV-specific CD8+ T cells of interest. Furthermore, the trend might just be due to the contribution of IAV-specific CD8+ T cells to this signal.

Next, I had also decided to examine the expression of the different activation, death, and survival/maintenance markers within the different memory CD8+ T cell subsets to acquire a better understanding on their activation status.

In terms of septic splenic central memory CD8+ T cells, there was no differences in the frequency or per-cell expression of the different activation, death, and survival/maintenance markers of interest (Figure 36. Panel A).

In terms of the septic splenic effector memory CD8+ T cell population, it was demonstrated that there was a reduced frequency of cells expressing CD127 and a downward trend for the frequency of cells expressing CD69 (Figure 36. Panel C, $p = 0.0007$, 0.3, respectively); however, these markers were not affected in their per-cell expression (Figure 36. Panel C, $p = 0.9$, 0.6, respectively). From this information, in regard to the CD127 expression, it could be suggested that septic splenic effector memory CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression are (1) more phenotypically activated compared to sham[426,427], (2) less able to maintain themselves *in vivo*[439–441] and (3) less able to produce a robust memory CD8+ T cell pool[439–441]. Furthermore, in regard to the expression of CD69, as previously mentioned, this potential decrease in frequency may be due to the homeostatic mechanisms at play during the course of sepsis.

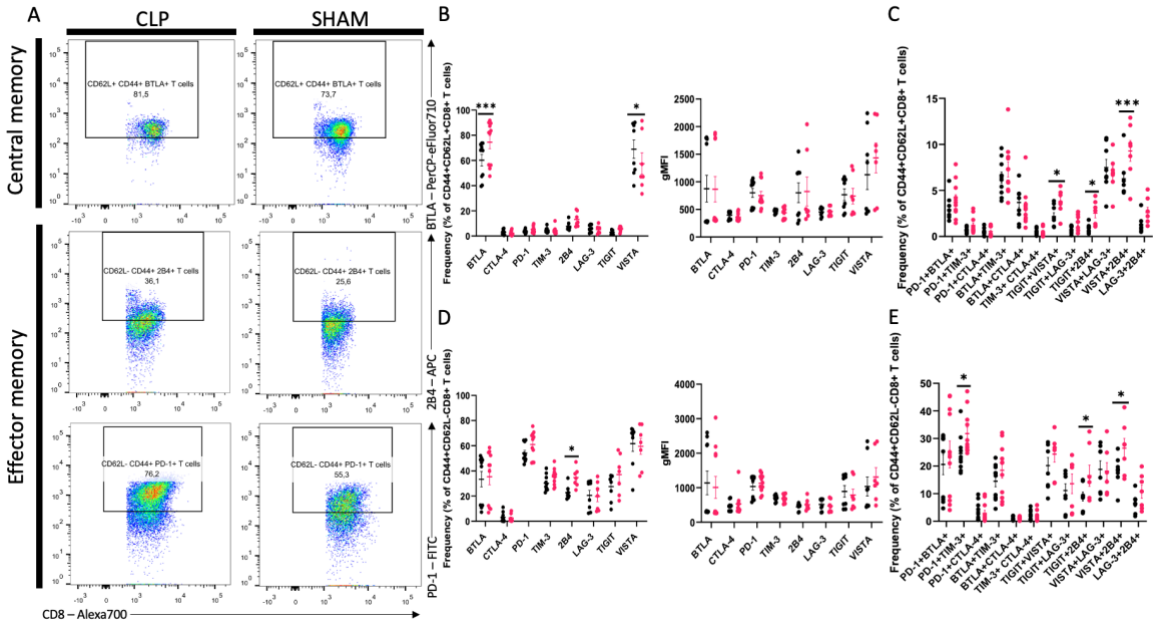

Figure 37. Memory CD8+ T cells within the septic spleen are more phenotypically exhausted compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of central memory CD8+ T cells singly expressing BTLA and of effector memory CD8+ T cells singly expressing 2B4 and PD-1 within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of central memory CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (D) Summary plots for the frequency (left) and gMFI (right) of effector memory CD8+ T cells singly expressing the different exhaustion markers within the spleen of each mouse. (E) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the spleen of each mouse. (C and E) The combination of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data for exhaustion panel 1 was pooled from 4 different independent experiments: CLP = 2-4 mice/experiment (n = 12); Sham = 2-3 mice/experiment (n = 10). The data for exhaustion panel 2 was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3

mice/experiment (n = 8). (B-E) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

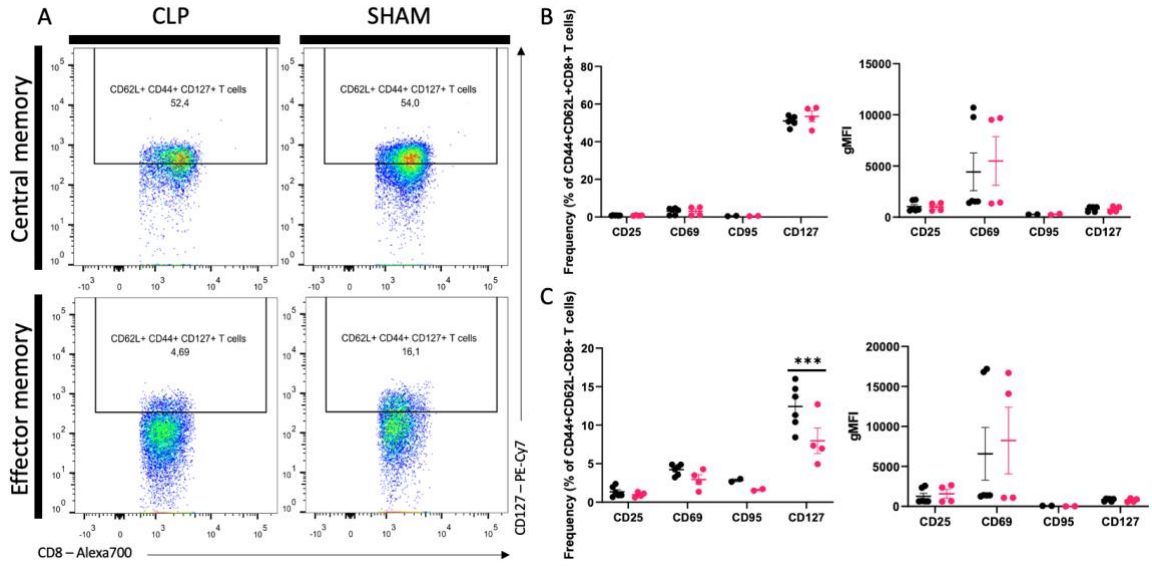

Figure 38. Effector memory CD8+ T cells, but not central memory CD8+ T cells, possess lower frequencies of cells expressing CD127 within the septic spleen. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of central and effector memory CD8+ T cells expressing CD127 within the spleen. (B) Summary plots for the frequency (left) and gMFI (right) of central memory CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of effector memory CD8+ T cells expressing the different activation, death, and survival/maintenance markers within the spleen of each mouse. The data was pooled from 3 different independent experiments: CLP = 2-3 mice/experiment (n = 7); Sham = 2-3 mice/experiment (n = 3). (B and C) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.7 IAV-specific CD8+ T cells within the septic PECs are similar in frequency, but demonstrate a downward trend in absolute numbers; additionally, these cells appear to be phenotypically less exhausted compared to sham

Next, I had wanted to examine the peak primary IAV-specific CD8+ T cell response during protracted sepsis within the PECs to study the local immune response within the peritoneal cavity as this is the site of sepsis initiation. My first objective was to examine the frequency and numbers of IAV-specific CD8+ T cells within the PECs; additionally, I had also decided to immunophenotype these cells by examining their expression of different exhaustion markers.

Herein, I discovered within the septic PECs that there was a similar frequency of IAV-specific CD8+ T cells compared to sham as measured by tetramer reagent (Figure 37. Panel C, p = 0.6, 0.9, respectively); however, there was a downward trend in the absolute numbers of these cells within the septic condition (Figure 37. Panel C, p = 0.3, 0.09, respectively). Furthermore, I had also performed an ICS to detect for IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{22}). I demonstrated that there was an upward trend in the frequency and absolute numbers of septic IFN-γ-producing-NP_{366}-specific CD8+ T cells (Figure 38. Panel B, p = 0.07, 0.3, respectively); however, there were no differences in the frequency and absolute numbers of septic IFN-γ-producing-PA_{224}-specific CD8+ T cells compared to sham (Figure 38. Panel B. p = 0.8, 0.8, respectively). Taken together, these findings suggest that there are numerically less IAV-specific CD8+ T cells within the septic PECs; however, within the septic condition, there are more IFN-γ-producing-IAV-specific CD8+ T cells, which may suggest that there is a higher percentage of IAV-specific CD8+ T cells capable in producing IFN-γ.

Thereafter, I had decided to examine the phenotypic changes of IAV-specific CD8+ T cells within the PECs by assessing the expression of different exhaustion markers at the peak primary CD8+ T cell response during protracted sepsis. Herein, I discovered that septic IAV-specific CD8+ T cells demonstrated a reduced frequency of cells singly expressing BTLA, PD-1 and TIM-3 (Figure 37. Panel D and E, NP_{366}: p = 0.001, 0.02, 0.01; PA_{224}: p = 0.006, 0.01, 0.2, respectively); however, the per-cell expression of these markers was quite different. For instance, septic IAV-specific CD8+ T cells demonstrated a significant increase in the per-cell expression of BTLA (Figure 37. Panel D and E, p = <0.0001, 0.04), while CTLA-4 and TIM-3 displayed an upward trend in their per-cell expression (Figure 37. Panel D and E, NP_{366}: p = 0.08, 0.08; PA_{224}: p = 0.4, 0.3, respectively). Furthermore, septic NP_{366}-specific CD8+ T cells demonstrated a downward trend in the per-cell expression of PD-1, while septic PA_{224}-specific CD8+ T cells did not (Figure 37. Panel D and E, p = 0.08, 0.7, respectively). In addition to examining the single-expression of the different exhaustion markers, I had also wanted to assess the co-expression of the different exhaustion marker combinations within the IAV-specific CD8+ T cell populations as this would provide more robust evidence on their exhaustion phenotype. I discovered that septic IAV-specific CD8+ T cells possessed lower frequencies of cells doubly expressing PD-1+BTLA+, PD-1+TIM-3+ and BTLA+TIM-3+ (Figure 37. Panel F and G, NP_{366}: p<0.0001, 0.0004, <0.0001; PA_{224}: p = 0.0002, 0.04, 0.01, respectively). Taken together, from these findings I may suggest a few conclusions. Firstly, considering the role of BTLA as a negative regulator of T cell activation[434], I may be able to suggest (1) that there are a higher frequency of activated IAV-specific CD8+ T cells within the septic PECs as evidenced by their reduced frequencies of cells expressing BTLA and (2) that the septic IAV-specific CD8+ T cells that express BTLA are more resistant to T cell activation as they possess a higher per-cell expression of BTLA. Secondly, I may be able to suggest that the activated septic IAV-specific CD8+ T cells are phenotypically less exhausted compared to their sham counterpart as these cells possessed a reduced frequency of cells singly and doubly expressing the different exhaustion, which may also imply that these cells were less activated compared to sham.

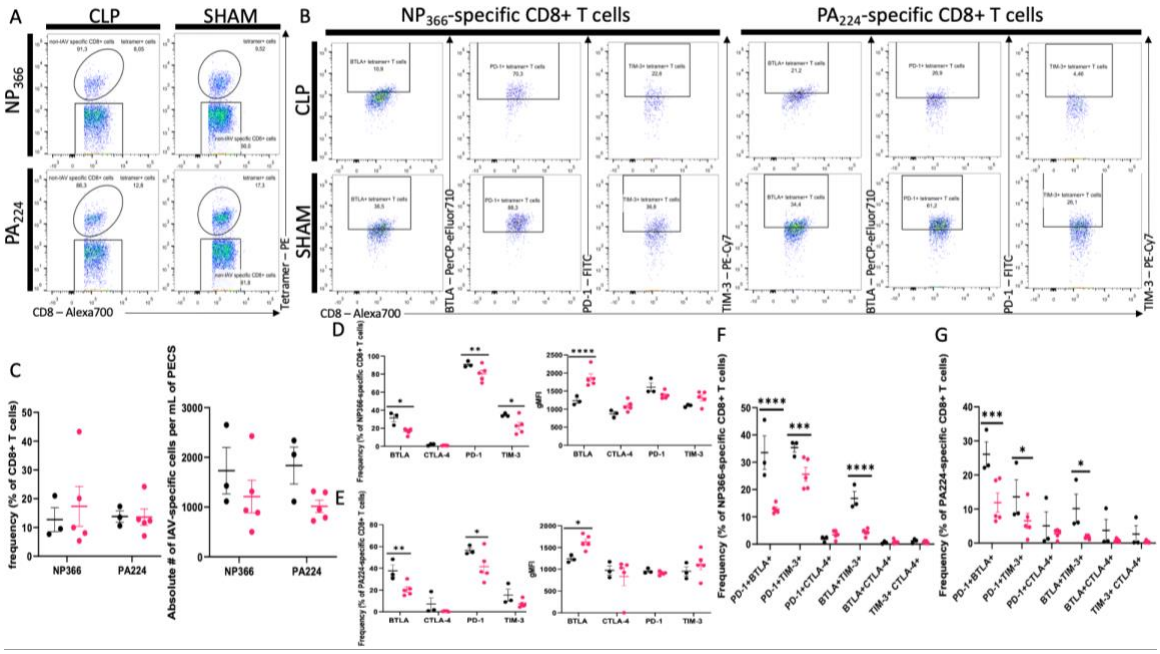

Figure 39. IAV-specific CD8+ T cells within the septic PECs are similar in frequency, although, demonstrate a trend of reduced absolute numbers compared to sham; additionally, these cells possess significantly lower frequencies of single- and double-expression of the different exhaustion markers. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells measured by tetramer reagent within the PECs. (B) Representative flow plots for the frequency of IAV-specific CD8+ T cells singly expressing the exhaustion markers BTLA, PD-1 and TIM-3 within the PECs. (C) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the PECs of each mouse. Note: IAV-specific CD8+ T cell numbers were reported as the number of cells per volume of PECs recovered during isolation (cells/mL of PECs recovered). (D) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (F) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. (G) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. (F and G) The

combination of double-expressors was limited due to the panel design. Exhaustion panel 1 consisted of PD-1, BTLA, TIM-3 and CTLA-4, whereas exhaustion panel 2 consisted of TIGIT, LAG-3, 2B4 and VISTA. The data was pooled from 1 independent experiment: CLP = 5 mice; Sham = 3 mice. (C-G) Statistics for significance was determined using the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.2.8 IFN-γ-producing-IAV-specific CD8+ T cells within the septic PECs may be higher in frequency and absolute numbers; however, per-cell expression of IFN-γ is dampened

Next, I had also assessed the functionality of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response within the PECs during sepsis-induced immunosuppression to acquire a better understanding on how sepsis effects the functionality of these cells. Herein, I discovered that septic NP_{366}-specific CD8+ T cells displayed an upward trend in the frequency of cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptide (NP_{366}) (Figure 38. Panel B, p = 0.07), while also demonstrating a weak upward trend in their absolute numbers (Figure 38. Panel B, p = 0.3). In terms of septic PA_{224}-specific CD8+ T cells, there were no differences in the frequency and absolute numbers of these cells capable of producing IFN-γ compared to the sham condition (Figure 38. Panel B. p = 0.8, 0.8, respectively). Furthermore, I had also decided to examine the per-cell expression of IFN-γ, which would provide information on the productive capabilities on a per-cell basis. I discovered that septic IAV-specific CD8+ T cells demonstrated a reduced per-cell expression of IFN-γ compared to sham (Figure 38. Panel B, p = 0.05, 0.001, respectively), which would indicate that these cells are less functionally competent in their ability to produce IFN-γ on a per-cell basis.

Taken together, within the septic PECs, there may be a higher presence of IFN-γ-producing-NP_{366}-specific CD8+ T cells compared to sham. As previously discussed (section 3.2.7), there may be a higher presence of IFN-γ-producing-IAV-specific CD8+ T cells among all IAV-specific CD8+ T cells present in the septic PECs. This may suggest that there are more activated IAV-specific CD8+ T cells in the septic PECs, which I had also suggested within the exhaustion phenotyping experiments. Despite this, septic IAV-specific CD8+ T cells produce less IFN-γ on a per-cell basis, which would indicate that these cells are less functional; additionally, this finding may also suggest that the activated IAV-specific CD8+ T cells are less activated compared to sham, which was also suggested within the exhaustion phenotyping experiments (section 3.2.7).

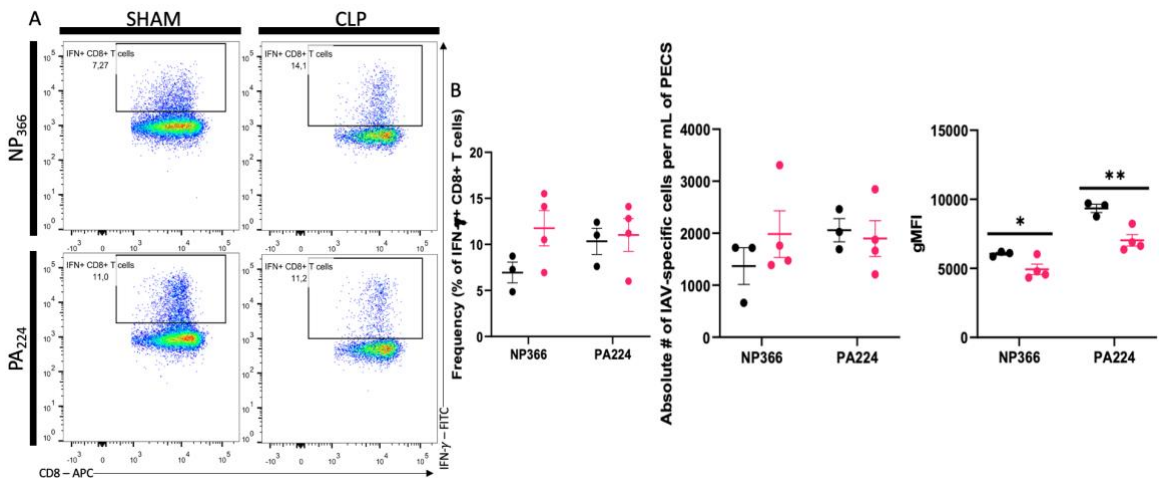

Figure 40. IFN-γ-producing-NP_{366}-specific CD8+ T cells within the septic PECs displayed an upward trend in frequency and absolute numbers; however, both IAV-specific CD8+ T cells demonstrated a reduced per-cell expression of IFN-γ. Sham is denoted in black dots, while CLP are red dots. (A) Representative flow plots for the frequency of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the PECs. (B) Summary plots for the frequency (left), absolute numbers (middle) and gMFI (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the PECs of each mouse. The data was pooled from 1 independent experiment: CLP = 4 mice; Sham = 3 mice. (B) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.9 Non-IAV-specific CD8+ T cells within the septic PECs appear to be less phenotypically exhausted compared to sham

In addition to studying the IAV-specific CD8+ T cells within the septic PECs, I had also wanted to the examine the exhaustion phenotype of non-IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during protracted sepsis to acquire a better understanding on the effects sepsis has on the whole CD8+ T cell pool.

Non-IAV-specific CD8+ T cells within the septic PECs appear to have a similar frequency of cells singly expressing the different exhaustion markers; although, they might have a slight reduction in their expression (Figure 39. Panel A and C, BTLA, PD-1 and TIM-3, non-NP_{366}: p = 0.6, 0.2, 0.7; non-PA_{224}: p = 0.5, 0.5, 0.7, respectively). Furthermore, septic non-IAV-specific CD8+ T cells demonstrated an increased per-cell expression of CTLA-4 (Figure 39. Panel A and C, p = 0.02, 0.03, respectively); however, this finding may not be significant as the frequency of non-IAV-specific CD8+ T cells expressing CTLA-4 was relatively small. Moreover, septic non-NP_{366}-specific CD8+ T cells possessed significantly higher per-cell expression of TIM-3, while septic non-PA_{224}-specific CD8+ T cells only demonstrated a weak upward trend (Figure 39. Panel A and C, p = 0.03, 0.5, respectively). Additionally, septic non-IAV-specific CD8+ T cells demonstrated a slight increase in the per-cell expression of BTLA and PD-1, although it was not an apparent trend (Figure 39. Panel A and C, non-NP_{366}: p = 0.6, 0.4; non-PA_{224}: p = 0.9, 0.7). This information suggests that non-IAV-specific CD8+ T cells within the septic condition may have a lower frequency of cells that are exhausted, which would indicate that there is a lower frequency of these cells that have been activated.

Furthermore, I had also wanted to examine the co-expression of the different exhaustion marker combinations within the non-IAV-specific CD8+ T cells, which would provide me with stronger evidence of their exhaustion phenotype. Herein, I discovered that non-IAV-specific CD8+ T cells within the septic PECs demonstrated an across-the-board downward trend in the frequencies of cells doubly expressing the different exhaustion marker combinations. For instance, in terms of septic non-NP_{366}-specific CD8+ T cells, there was a weak downward trend in the frequency of cells doubly expressing PD-1+BTLA+ and BTLA+TIM-3+ (Figure 39. Panel B, p = 0.3, 0.4, respectively). In terms of septic non-PA_{224}-specific CD8+ T cells, there was a downward trend in the frequency of cells doubly expressing PD-1+BTLA+ and BTLA+TIM-3+ (Figure 39. Panel D, p = 0.3, 0.3, respectively). From this information, it appears as though non-IAV-specific CD8+ T cells within the septic PECs are less phenotypically exhausted compared to sham, which may potentially signify that the cells expressing these exhaustion markers were in fact less activated compared to their sham counterpart as they have a reduced co-expression of the different exhaustion markers.

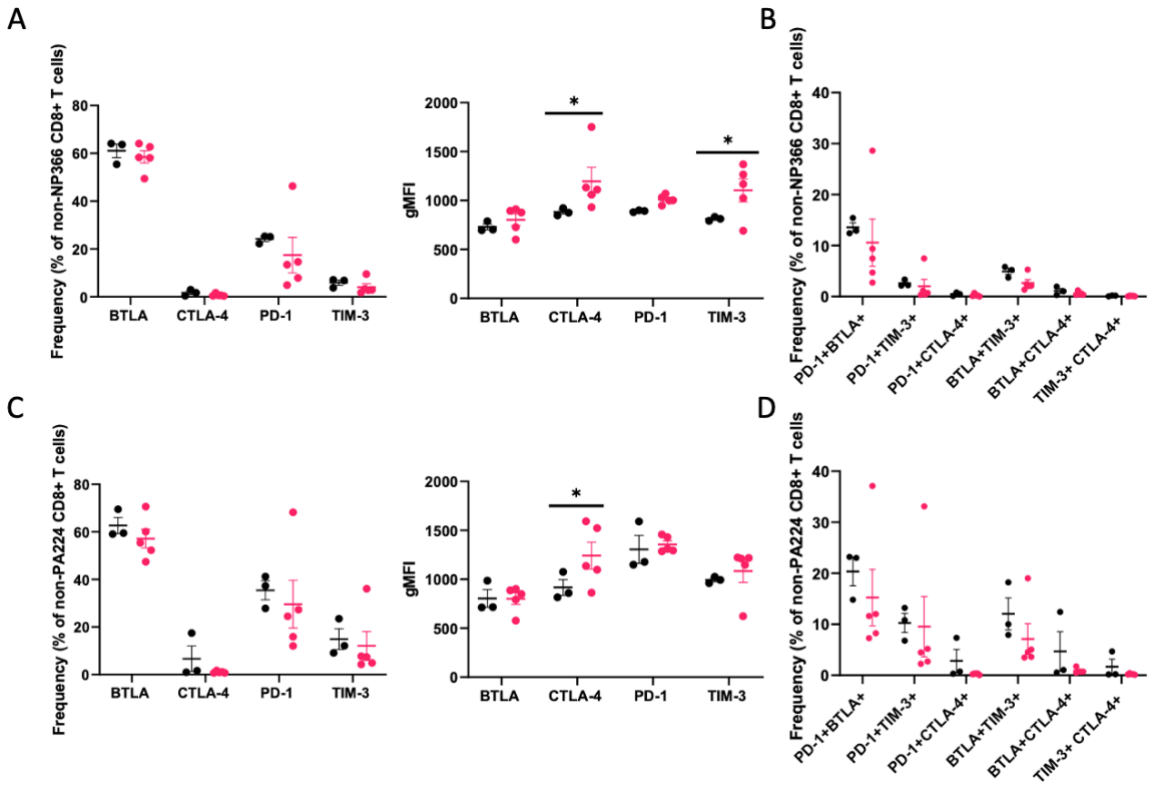

Figure 41. Non-IAV-specific CD8+ T cells within the septic PECs appear to have a reduced single- and double-expression of the different exhaustion markers. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (B) Summary plot for the frequency of non-NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the PECs of each mouse. (D) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the PECs of each mouse. The data was pooled from 1 independent experiment: CLP = 5 mice; Sham = 3 mice. (A-D) Statistics for significance was determined using the unpaired student's t test with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.10 IAV-specific CD8+ T cells within the septic lungs may be similar in frequency and absolute numbers compared to sham, but may be more phenotypically exhausted

Next, in addition to examining the IAV-specific CD8+ T cell responses within the spleen and PECs, I had also decided to examine their responses within the lungs, just as I had done within the recall response. This information would provide me with (1) how IAV-specific CD8+ T cells responses are affected by sepsis-induced immunosuppression within the lungs and (2) an ability to compare the responses seen within the recall response in the lungs to the primary response in the lungs.

First, I had wanted to assess the frequency and absolute numbers of IAV-specific CD8+ T cells at the peak primary CD8+ T cell responses during sepsis-induced immunosuppression within the lungs. Herein, I discovered that IAV-specific CD8+ T cells within the septic lungs appeared to have a similar frequency and absolute number compared to sham (Figure 40. Panel A); however, due to the low number of replicates, I am not able to provide any definitive conclusions based on these findings.

Additionally, I had also decided to examine the exhaustion phenotype of IAV-specific CD8+ T cells within the lungs. I discovered that septic IAV-specific CD8+ T cells demonstrated an upward trend in the frequency of cells singly expressing 2B4, with a weaker upward trend in the frequency of cells singly expressing TIGIT (Figure 40. Panel C and E). Furthermore, septic IAV-specific CD8+ T cells also demonstrated an upward trend in the per-cell expression of VISTA (Figure 40. Panel C and E). Moreover, septic IAV-specific CD8+ T cells within the lungs demonstrated an upward trend in the frequency of cells expressing KLRG1 (Figure 41. Panel B and D, $p = 0.08$, 0.1, respectively). These findings suggest that IAV-specific CD8+ T cells at the peak primary CD8+ T cell response within the lungs during sepsis-induced immunosuppression may be more phenotypically exhausted compared to sham, which may imply that they are more activated; however, the replicate number was too low to make any definitive conclusions and further experimental confirmation is needed.

Furthermore, to provide stronger evidence on whether IAV-specific CD8+ T cells are phenotypically more exhausted compared to sham, I had also examined the frequency of these cells co-expressing the different exhaustion marker combinations. I discovered that IAV-specific CD8+ T cells within the septic lungs appeared to be more phenotypically exhausted compared to sham as these cells tended to possess higher frequencies of cells doubly expressing the different exhaustion marker combinations. In terms of NP_{366}-specific CD8+ T cells, it was demonstrated within the septic condition that they possessed a higher frequency of cells doubly expressing TIGIT+VISTA+, TIGIT+2B4+, VISTA+2B4+ and LAG-3+2B4+ (Figure 40. Panel D). In terms of septic PA_{224}-specific CD8+ T cells, I discovered that these cells possessed a higher frequency of cells doubly expressing TIGIT+VISTA+, TIGIT+LAG-3+, TIGIT+2B4+, VISTA+LAG-3+, VISTA+2B4+ and LAG-3+2B4+ (Figure 40. Panel F). These findings may suggest that IAV-specific CD8+ T cells within the septic lungs are phenotypically more exhausted compared to sham, which may also suggest that these cells were more activated compared to sham; however, further confirmation of these findings are necessary as the number of replicates was too low to make any definitive conclusions

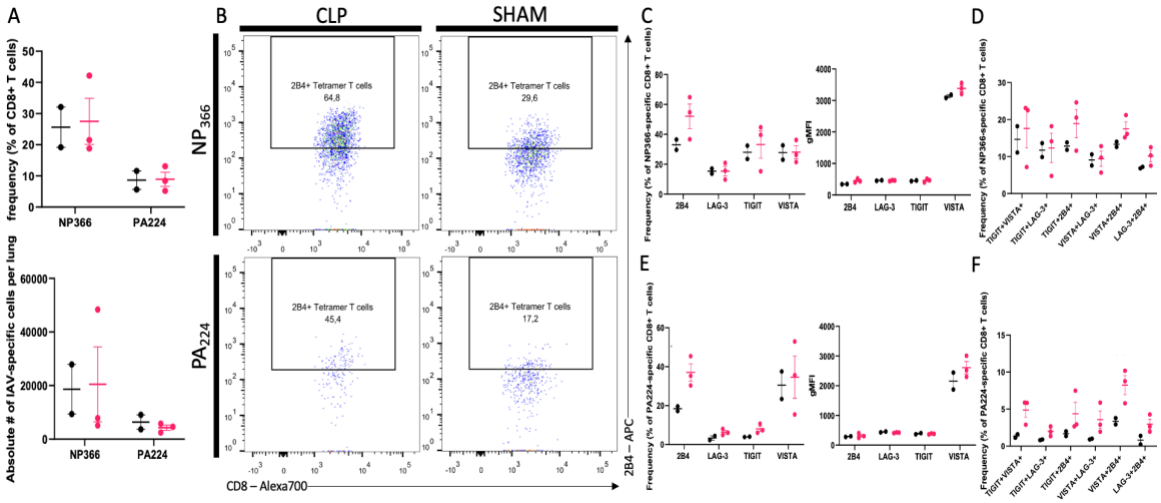

Figure 42. IAV-specific CD8+ T cells within the septic lungs may be similar in frequency and absolute numbers compared to sham; however, these cells may be more phenotypically exhausted. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (top) and absolute numbers (bottom) of IAV-specific CD8+ T cells measured by tetramer reagent within the lungs of each mouse. (B) Representative flow plots for the frequency of IAV-specific CD8+ T cells singly expressing the exhaustion marker 2B4 within the lungs. (C) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (D) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (E) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A, C-F) No statistics were performed due to the low replicate number.

3.2.11 IFN-γ-producing-IAV-specific CD8+ T cells within the septic lungs are similar in frequency and absolute numbers compared to sham; however, these cells may be more functional, while their quality of response differs

Next, I had wanted to examine the functionality of IAV-specific CD8+ T cells within the lungs at peak primary CD8+ T cell response during protracted sepsis. I discovered that septic IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) were similar in frequency and absolute numbers compared to sham (Figure 41. Panel A, NP_{366}: p = 0.9, 0.5; PA_{224}: p = 0.6, 0.5, respectively); however, these cells may have a higher presence as there was a slight increase in their frequency and absolute numbers, but the data was quite heterogeneous. Furthermore, I had also decided to examine their per-cell expression of IFN-γ and I discovered that both IAV-specific CD8+ T cells demonstrated an upward trend in their per-cell expression of IFN-γ (Figure 41. Panel A, p = 0.5, 0.1, respectively). From all this information, it may be suggested that IAV-specific CD8+ T cells capable of producing IFN-γ within the septic lungs appear to have a similar frequency and absolute numbers compared to sham; however, these cells may have an increased per-cell expression of IFN-γ, which suggests that on a per-cell basis, these cells are functionally more capable in producing IFN-γ.

In addition to examining the capability of IAV-specific CD8+ T cells to produce IFN-γ, I had decided to examine the production of different cytokines and cytolytic effector molecules to broaden the functionality assays. Herein, I discovered that septic IAV-specific CD8+ T cells may have an upward trend in the frequency of cells capable of producing granzyme B (Figure 41. Panel B and D, p = 0.3, 0.5, respectively); however, the trend was weak. Furthermore, septic IAV-specific CD8+ T cells may also possess an upward trend in their per-cell expression of TNF-α (Figure 41. Panel B and D, p = 0.1, 0.3, respectively). This information suggests that IAV-specific CD8+ T cells within the septic lungs may possess an increased functionality compared to sham at the peak primary CD8+ T cell response during protracted sepsis.

Furthermore, I had decided to examine the polyfunctionality of IAV-specific CD8+ T cells within the lungs by looking at the frequency of cells triple-expressing IFN-γ, TNF-α and IL-2. This would provide me with information regarding their quality of response. Interestingly, their polyfunctionality differed depending on the IAV-specific CD8+ T cell of interest. Septic NP_{366}-specific CD8+ T cells displayed a downward trend in the frequency of cells triple-expressing IFN-γ, TNF-α and IL-2 (Figure 41. Panel C. p = 0.4), which would indicate that these cells are dampened in their quality of response. In terms of septic PA_{224}-specfiic CD8+ T cells, these cells demonstrated an upward trend in the frequencies of cells triple-expressing IFN-γ, TNF-α and IL-2 (Figure 41. Panel C. p = 0.2), which may suggest that these cells have an increased quality of response compared to sham.

Taken together, within the septic lungs, IAV-specific CD8+ T cells may be more functionally capable than sham as they demonstrated an upward trend in their per-cell expression IFN-γ and TNF-α, while displaying an upward trend in the frequency of cells expressing granzyme B; however, there quality of response differed depending on the IAV-specific CD8+ T cells of interest.

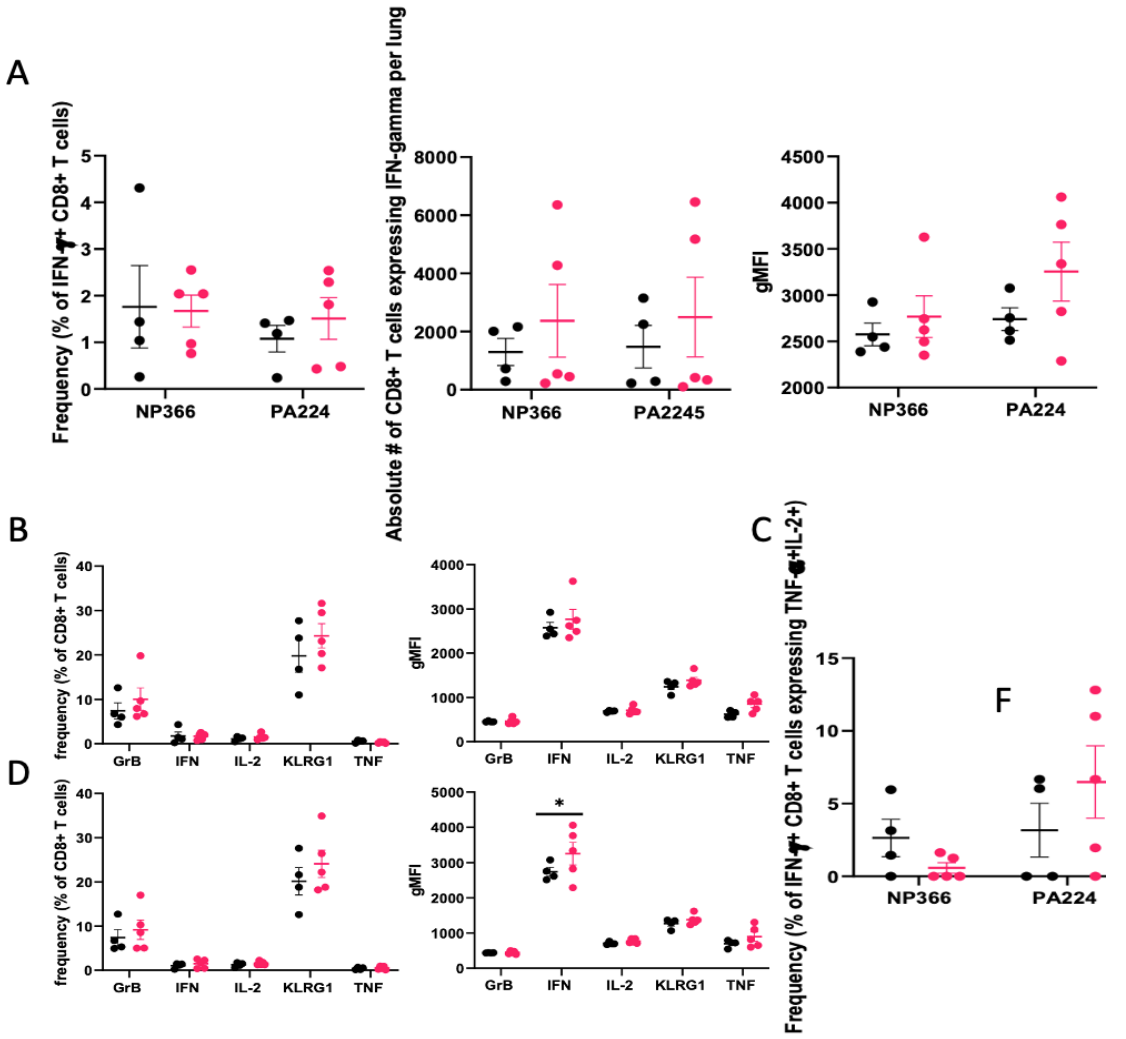

Figure 43. IFN-γ-producing-IAV-specific CD8+ T cells within the septic lungs are similar in frequency and absolute numbers compared to sham; however, these cells may be more functional, while their quality of response differs. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left), absolute numbers (middle) and gMFI (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the lungs of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing the different cytokines, a cytolytic effector molecule and a surface marker of co-inhibition under the *ex vivo* stimulation with its cognate peptide (NP_{366}) within the lungs of each mouse. (C) Summary plot for the frequency of IAV-specific CD8+ T cells triple-expressing TNF-α, IFN-γ and IL-2 under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the lungs of

each mouse. (D) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing the different cytokines, a cytolytic effector molecule and a surface marker of co-inhibition under the *ex vivo* stimulation with its cognate peptide (PA_{224}) within the lungs of each mouse. The data was pooled from 2 independent experiments: CLP = 2-3 mice/experiment (n = 5); Sham = 2 mice/experiment (n = 4). (A-D) Statistics for significance was determined using the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.2.12 Non-IAV-specific CD8+ T cells within the septic lungs may be more phenotypically exhausted compared to their sham counterpart

Just as I had previously done, I wanted to examine the exhaustion phenotype of non-IAV-specific CD8+ T cells within the septic lungs at the peak primary CD8+ T cells response during sepsis-induced immunosuppression. Herein, I discovered that non-IAV-specific CD8+ T cells within the septic lungs demonstrated an upward trend in the frequency of cells singly expressing 2B4 and VISTA (Figure 42. Panel A and C), while these cells displayed an upward trend in the per-cell expression of VISTA (Figure 42. Panel A and C). This information may suggest that non-IAV-specific CD8+ T cells within the septic lungs are phenotypically more exhausted compared to sham, which may also suggest that these cells were more activated.

Additionally, I had also examined the co-expression of the different exhaustion marker combinations within the non-IAV-specific CD8+ T cell compartment to acquire more robust information on their exhaustion state. In terms of the septic non-NP_{366}-specific CD8+ T cells, I discovered that there was an upward trend in the frequency of cells doubly expressing VISTA+LAG-3+, VISTA+2B4+ and LAG-3+2B4+ (Figure 42. Panel B). In terms of the septic non-PA_{224}-specific CD8+ T cells, there was an upward trend in the frequency of cells doubly expressing TIGIT+VISTA+, VISTA+LAG-3+, VISTA+2B4+ and LAG-3+2B4+ (Figure 42. Panel D). From this information, there is evidence to suggest that non-IAV-specific CD8+ T cells within the septic lungs are more phenotypically exhausted compared to sham, which may also imply that they are more activated.

Taken together, non-IAV-specific CD8+ T cells within the septic lungs at the peak primary CD8+ T cell response during sepsis-induced immunosuppression may be more phenotypically exhausted compared to sham as illustrated by their increased single- and double-expression of the different exhaustion markers, which may also suggest that these cells were more activated. Despite these findings, due to the low number of replicates, I may not make any definitive conclusions as more replicates are needed to confirm these observations.

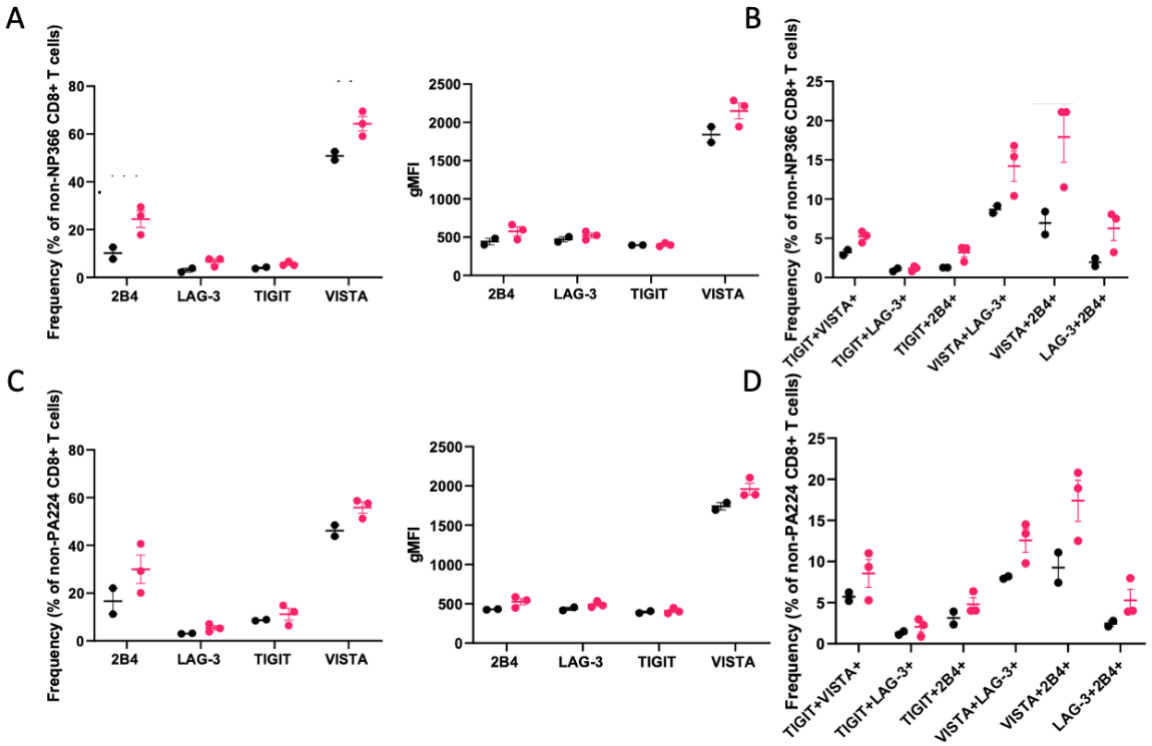

Figure 44. Non-IAV-specific CD8+ T cells within the septic lungs may be more phenotypically exhausted compared to sham. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (B) Summary plot for the frequency of non-NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (D) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A-D) No statistics were performed due to the low replicate number.

3.2.13 There may be a similar frequency and absolute number of activated CD8+ T cells, central and effector memory CD8+ T cells within the septic lungs compared to sham; additionally, they may demonstrate an increased exhaustion state

In addition to studying non- and IAV-specific CD8+ T cells, I had also wanted to assess the frequency, absolute numbers, and exhaustion phenotype of the different memory CD8+ T cells subsets within the septic lungs at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. Within the septic lungs, I discovered that there may be a similar frequency and absolute number of activated CD8+ T cells as measured by the expression of CD44 (Figure 43. Panel A). To further characterize these activated CD8+ T cells, I had examined the frequency and absolute numbers of the different memory CD8+ T cell subsets by examining the expression of CD62L. It was demonstrated that within the septic condition, there may be a similar frequency and absolute number of central and effector memory CD8+ T cells compared to sham (Figure 43. Panel B). These findings suggest that there may be no differences in the presence of activated CD8+ T cells, central and effector memory CD8+ T cells within the septic lungs, which was quite different compared to the recall response; however, due to the low replicate number, more experiments are needed to confirm these observations.

Next, I had assessed the exhaustion phenotype of the different memory CD8+ T cell subsets. In terms of the septic central memory CD8+ T cells, these cells did not display any differences in the single expression of the different exhaustion markers examined (Figure 43. Panel C); however, these cells did demonstrate an upward trend in the frequency of cells doubly expressing VISTA+LAG-3+ and VISTA+2B4+ (Figure 43. Panel D). In terms of the septic effector memory CD8+ T cells, these cells demonstrated an upward trend in the frequency of cells singly expressing 2B4 (Figure 43. Panel E) and doubly expressing VISTA+2B4+ (Figure 43. Panel F). These findings may suggest that the different memory CD8+ T cell subsets within the septic lungs at the peak primary CD8+ T cell response during protracted sepsis are phenotypically more exhausted compared to sham, which also insinuates that these cells were more activated; however, due to the low number of replicates, more experiments are required to confirm these results.

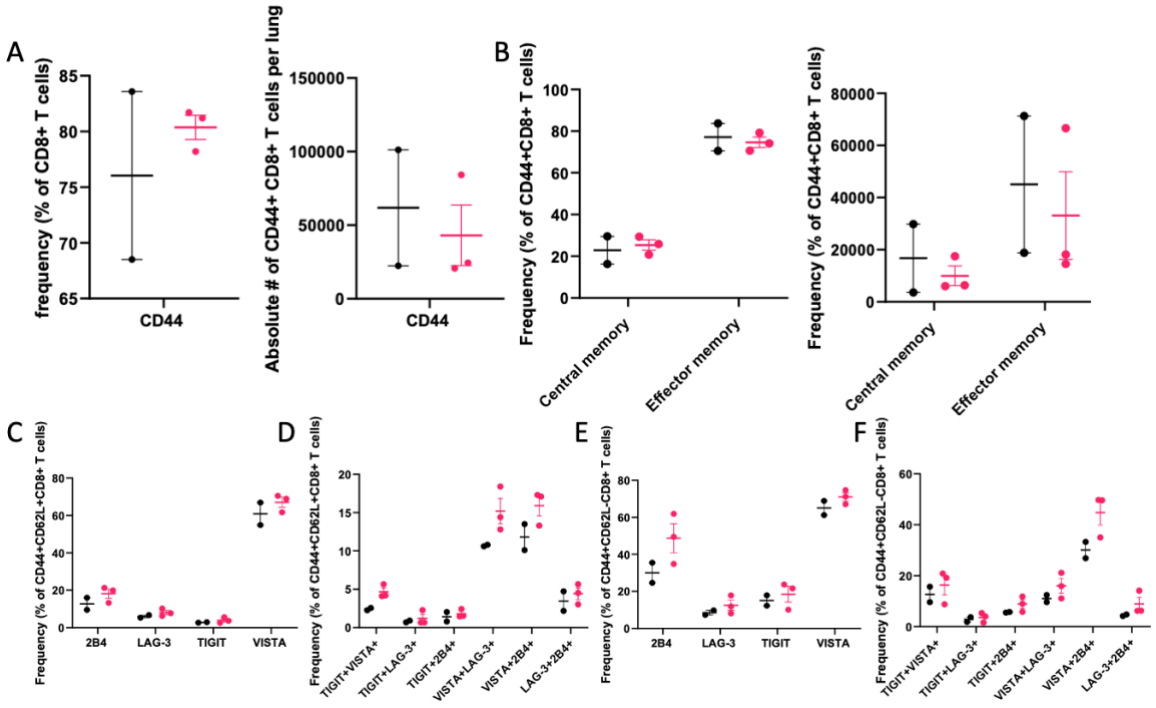

Figure 45. Within the septic lungs, there may be a similar frequency and absolute number of activated CD8+ T cells, central and effector memory CD8+ T cells compared to sham; however, they demonstrate signs of a higher exhaustion status. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing CD44 within the lungs of each mouse. (B) Summary plots for the frequency (left) and absolute numbers (right) of central and effector memory CD8+ T cells measured by the expression of CD62L gated on CD44+CD8+ T cells within the lungs of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (D) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. (E) Summary plot for the frequency of effector memory CD8+ T cells singly expressing the different exhaustion markers within the lungs of each mouse. (F) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the lungs of each mouse. The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A-F) No statistics were performed due to the low replicate number.

3.2.14 IAV-specific CD8+ T cells within the septic liver may be similar in frequency compared to sham, but NP_{366}-specific CD8+ T cells appear to be more numerically plentiful; additionally, septic hepatic IAV-specific CD8+ T cells may be more phenotypically exhausted

Next, just as I had previously done within the recall response, I had wanted to examine the frequency, absolute numbers, exhaustion phenotype and functionality of IAV-specific CD8+ T cells within the septic liver at the peak primary CD8+ T cell response during protracted sepsis. Herein, I demonstrated that septic IAV-specific CD8+ T cells are similar in frequency compared to sham as measured by tetramer reagent (Figure 44. Panel A); however, NP_{366}-specific CD8+ T cells appear to be more numerically plentiful, while PA_{224}-specific CD8+ T cells are similar in their absolute numbers compared to sham as measured by tetramer reagent (Figure 44. Panel A). Moreover, I had also examined the frequencies and absolute numbers of IFN-γ-producing-IAV-specific CD8+ T cells within the septic liver and I discovered that there was a slight increase in their frequencies and absolute numbers (Figure 45. Panel A, NP_{366}: p = 0.5, 0.5; PA_{224}: p = 0.4, 0.5, respectively). These findings may suggest that within the septic condition, IAV-specific CD8+ T cells in the liver are of similar frequency compared to sham; however, NP_{366}-specific CD8+ T cells may be numerically more plentiful, but IFN-γ-producing-NP_{366}-specific CD8+ T cells appear to be relatively similar in number compared to sham. Hence, septic hepatic NP_{366}-specific CD8+ T cells may possess a reduced frequency of cells capable of producing IFN-γ.

Thereafter, I had examined the exhaustion phenotype of IAV-specific CD8+ T cells within the septic liver. In terms of septic NP_{366}-specific CD8+ T cells, there was an upward trend in the frequency of cells singly expressing 2B4, LAG-3, TIGIT and VISTA (Figure 44. Panel B); however, only VISTA demonstrated an upward trend in the per-cell expression of this marker (Figure 44. Panel B). Furthermore, these cells possessed a significant increase in the frequency of cells singly expressing KLRG1 (Figure 45. Panel B, p = 0.04). In terms of septic PA_{224}-specific CD8+ T cells, these cells possessed a significant increase in the frequency of cells singly expressing KLRG1 (Figure 45. Panel D, p = 0.05) and

displayed an upward trend in the per-cell expression of VISTA (Figure 44. Panel C). These findings may suggest that IAV-specific CD8+ T cells within the septic liver at the peak primary CD8+ T cell response during sepsis-induced immunosuppression are phenotypically more exhausted compared to their sham counterpart, which may also imply that these cells are more activated; however, these are not definitive conclusions as the number of replicates is too low to make any strong assertions.

Furthermore, to provide more robust insight into their exhaustion status, I had also examined the frequency of IAV-specific CD8+ T cells co-expressing the different exhaustion marker combinations. In terms of septic NP_{366}-specific CD8+ T cells, there was an upward trend in the frequency of cells doubly expressing TIGIT+VISTA+, TIGIT+LAG-3+ and VISTA+LAG-3+ (Figure 44. Panel E). In terms of septic PA_{224}-specific CD8+ T cells, there was an upward trend in the frequency of cells doubly expressing TIGIT+VISTA+, TIGIT+LAG-3+ and VISTA+LAG-3+ (Figure 44. Panel F). From this information, I may be able to suggest that septic IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression within the liver are phenotypically more exhausted compared to sham, which may also imply that these cells were more activated; however, the replicate number is too low to make any definitive conclusions.

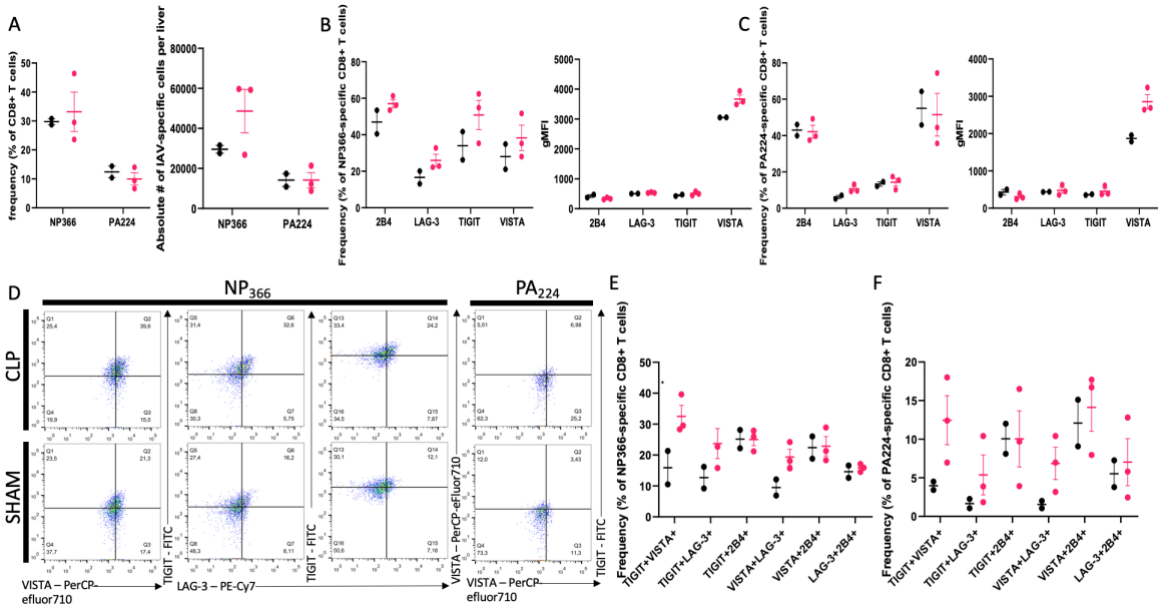

Figure 46. Within the septic liver, IAV-specific CD8+ T cells appear to be similar in frequency, but NP_{366}-specific CD8+ T cells may be numerically increased compared to sham; additionally, IAV-specific CD8+ T cells appear to be more phenotypically exhausted. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and absolute numbers (right) of IAV-specific CD8+ T cells measured by tetramer reagent within the liver of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (D) Representative flow plots for the frequency of IAV-specific CD8+ T cells doubly expressing TIGIT+VISTA+, TIGIT+LAG-3+ and VISTA+LAG-3+ within the liver. (E) Summary plot for the frequency of NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (F) Summary plot for the frequency of PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A-C, E-F) No statistics were performed as the replicate number was too low.

3.2.15 IFN-γ-producing-IAV-specific CD8+ T cells within the septic liver may be increased in frequency and absolute numbers, while potentially having an increased per-cell expression of IFN-γ; however, their quality of response is dampened

Next, I wanted to assess the IAV-specific CD8+ T cell functionality within the septic liver at the peak primary CD8+ T cell response during protracted sepsis. I discovered that septic IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides demonstrated a weak upward trend in their frequency and absolute numbers (Figure 45. Panel A, NP_{366}: p = 0.5, 0.5; PA_{224}: p = 0.4, 0.5, respectively); however, the data had a big variance so this trend might not be significant. Furthermore, septic IAV-specific CD8+ T cells may produce more IFN-γ on a per-cell basis as these cells demonstrated an upward trend in their MFI signals compared to sham (Figure 45. Panel A, p = 0.4, 0.3, respectively). This information suggests that IAV-specific CD8+ T cells capable of producing IFN-γ within the septic liver may be increased in frequency and absolute number, while demonstrating an increased per-cell expression of IFN-γ; however, due to the high variance within the data, more experiments are needed to confirm these preliminary observations.

Furthermore, I had also decided to examine the expression of other cytokines and cytolytic effector molecules within septic hepatic IAV-specific CD8+ T cells to broaden my functionality assays. Herein, I discovered that for all the other functional markers examined, namely granzyme B, IL-2 and TNF-α, septic hepatic IAV-specific CD8+ T cells demonstrated no differences in the frequency and per-cell expression of these markers compared to sham (Figure 45. Panel B and D). Additionally, I had decided to also examine the quality of response by assessing the frequencies of IAV-specific CD8+ T cells triply expressing IFN-γ, TNF-α and IL-2. I demonstrated that septic PA_{224}-specific CD8+ T cells displayed a significant decrease in the frequency of cells triple expressing IFN-γ, TNF-α and IL-2, while NP_{366}-specific CD8+ T cells only demonstrated a downward trend (Figure 45. Panel C, p = 0.02, 0.2, respectively), which may suggest that these cells possess a dampened polyfunctionality.

Taken together, IAV-specific CD8+ T cells within the septic liver may be more functionally capable in producing IFN-γ; however, these cells possess a dampened quality of response as they possess lower frequencies of cells that are polyfunctional.

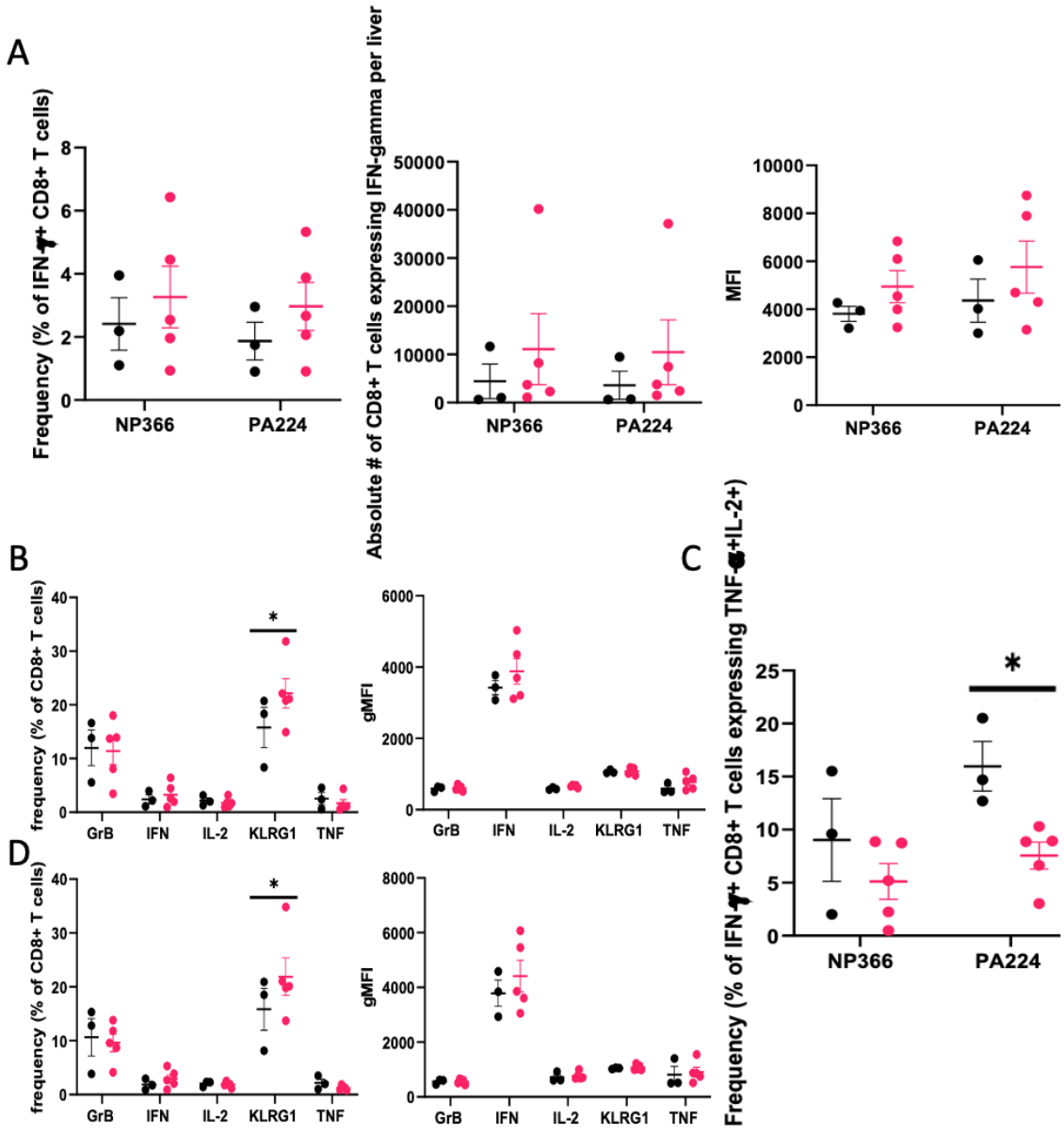

Figure 47. IFN-γ-producing-IAV-specific CD8+ T cells within the septic liver may be higher in frequency and absolute numbers, while potentially producing more IFN-γ on a per-cell basis; however, they have a dampened quality of response. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left), absolute numbers (middle) and MFI (right) of IAV-specific CD8+ T cells capable of producing IFN-γ under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the liver of each mouse. (B) Summary plots for the frequency (left) and gMFI (right) of NP_{366}-specific CD8+ T cells expressing the different cytokines, a cytolytic effector molecule and a surface marker of co-inhibition under the *ex vivo* stimulation with

its cognate peptide (NP_{366}) within the liver of each mouse. (C) Summary plot for the frequency of IAV-specific CD8+ T cells triple-expressing TNF-α, IFN-γ and IL-2 under the *ex vivo* stimulation with their cognate peptides (NP_{366} and PA_{224}) within the liver of each mouse. (D) Summary plots for the frequency (left) and gMFI (right) of PA_{224}-specific CD8+ T cells expressing the different cytokines, a cytolytic effector molecule and a surface marker of co-inhibition under the *ex vivo* stimulation with its cognate peptide (PA_{224}) within the liver of each mouse. The data was pooled from 2 independent experiments: CLP = 2-3 mice/experiment (n = 5); Sham = 2 mice/experiment (n = 4). (A-D) Statistics for significance was determined using the unpaired student's t test with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.2.16 Non-IAV-specific CD8+ T cells within the septic liver demonstrate a differential pattern of exhaustion

Thereafter, I wanted to examine the exhaustion profile of non-IAV-specific CD8+ T cells within the septic liver at the peak primary CD8+ T cell response during sepsis-induced immunosuppression, just as I had previously done for the other tissues. I discovered that septic non-IAV-specific CD8+ T cells demonstrated an upward trend in the frequency and per-cell expression of VISTA (Figure 46. Panel A and C). In terms of the septic non-NP_{366}-specific CD8+ T cells, these cells demonstrated an upward trend in the frequency of cells singly expressing 2B4 (Figure 46. Panel A) and doubly expressing TIGIT+VISTA+, VISTA+LAG-3+ and LAG-3+2B4+ (Figure 46. Panel B). In terms of the septic non-PA_{224}-specific CD8+ T cells, these cells did not demonstrate any trend in the frequency of cells singly expressing the other exhaustion markers (Figure 46. Panel C); however, these cells did demonstrate a downward trend in the frequency of cells doubly expressing VISTA+2B4+ (Figure 46. Panel D).

Taken together, this information may suggest that septic non-NP_{366}-specific CD8+ T cells, but not septic non-PA_{224}-specific CD8+ T cells, are phenotypically more exhausted within the septic liver compared to the sham liver, which also potentially indicates that these cells were more activated compared to sham.

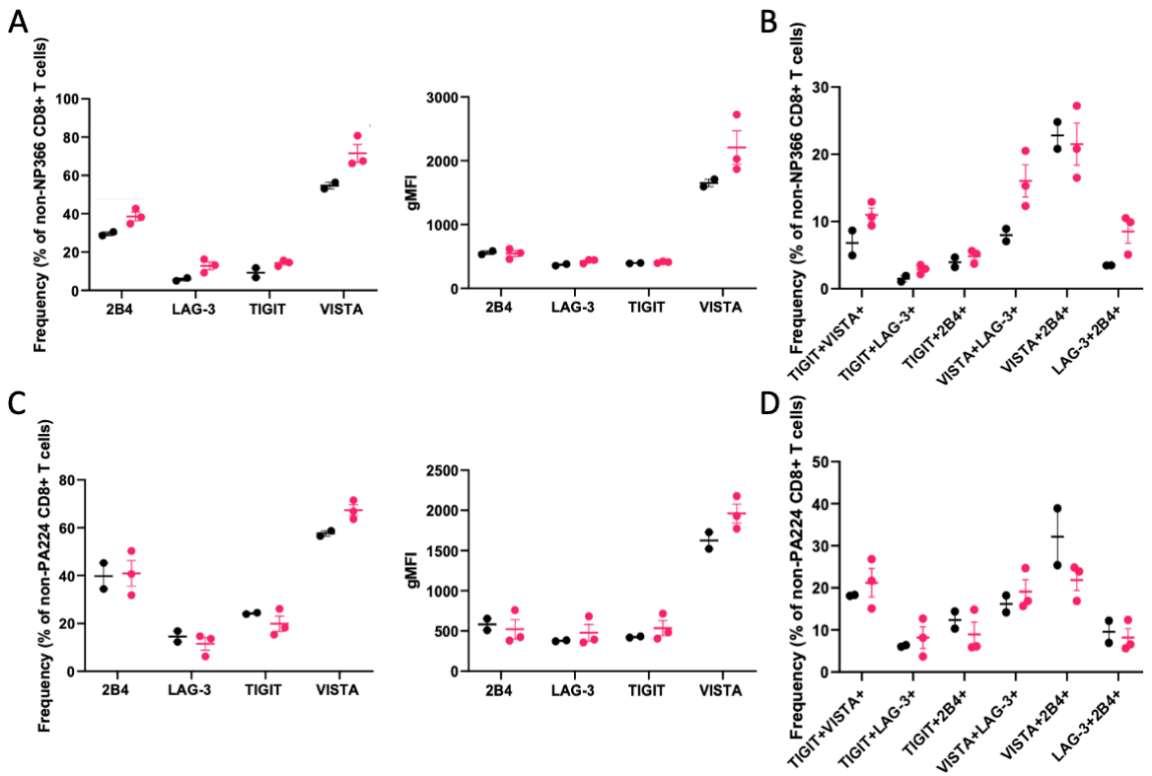

Figure 48. Non-IAV-specific CD8+ T cells within the septic liver differ in their exhaustion phenotype. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and gMFI (right) of non-NP_{366}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (B) Summary plot for the frequency of non-NP_{366}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (C) Summary plots for the frequency (left) and gMFI (right) of non-PA_{224}-specific CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (D) Summary plot for the frequency of non-PA_{224}-specific CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A-D) No statistics were performed due to the low replicate number.

3.2.17 Activated CD8+ T cells within the septic liver may be similar in frequency compared to sham, but they may be numerically more plentiful due to the increased presence of central and effector memory CD8+ T cells; memory CD8+ T cell populations may be less phenotypically exhausted within the septic liver

Next, I had decided to examine the different memory CD8+ T cell subsets within the septic liver to provide a better understanding on the IAV-specific CD8+ T cells of interest and of the whole CD8+ T cell pool.

Within the septic liver, it was discovered that the frequency of activated CD8+ T cells as measured by the expression of CD44 were comparable to the levels within the sham condition (Figure 47. Panel A); however, there is an upward trend in the absolute numbers of activated CD8+ T cells (Figure 47. Panel A). This information may suggest that there is a higher presence of activated CD8+ T cells within the septic liver, and this could be significant as these cells are capable in causing tissue pathology[381–385]. To further interrogate this trend, I had decided to examine the frequencies and absolute numbers of the different memory CD8+ T cell subsets. I discovered within the septic liver that there were no differences in the frequency of central and effector memory CD8+ T cells compared to sham (Figure 47. Panel B); however, there was an upward trend in the absolute numbers of both central and effector memory CD8+ T cells (Figure 47. Panel B). From this information, it may be suggested that activated CD8+ T cells within the septic liver are higher in presence and this is due to the increasing presence of central and effector memory CD8+ T cells; however, due to the low number of replicates, more experiments are needed to confirm this observation.

Thereafter, I had wanted to the examine the exhaustion profile of the different memory CD8+ T cell subsets at the peak primary CD8+ T cell response during protracted sepsis. This information would provide a more robust understanding on how sepsis effects the IAV-specific CD8+ T cells of interest and also shed some light on the effects sepsis has on the whole CD8+ T cell compartment. Of important note, the IAV-specific CD8+ T cells would all be characterized as effector memory CD8+ T cells.

In terms of septic central memory CD8+ T cells, these cells demonstrated a downward trend in the frequency of cells singly expressing 2B4, while displaying an upward trend in the frequency of cells singly expressing VISTA (Figure 47. Panel C). Furthermore, these cells also demonstrated a downward trend in the frequency of cells doubly expressing VISTA+2B4+ (Figure 47. Panel D). In terms of effector memory CD8+ T cells, these cells displayed a downward trend in the frequency of cells singly expressing 2B4 (Figure 47. Panel E) and doubly expressing VISTA+2B4+ (Figure 47. Panel F). Taken together, this information suggests that both central and effector memory CD8+ T cells may be phenotypically less exhausted within the septic liver compared to the sham liver, which may also imply that these cells were less activated compared to sham. Furthermore, comparing these phenotypes to the exhaustion profile of IAV-specific CD8+ T cells, it could be suggested that (1) IAV-specific CD8+ T cells may be more exhausted compared to the other CD8+ T cells present within the septic liver, which implies that they are more activated and (2) that 2B4 could be specifically upregulated on IAV-specific CD8+ T cells as within the effector memory CD8+ T cells, there was a downward trend in the expression of this marker; however, within IAV-specific CD8+ T cells, there was an upward trend in the expression of 2B4.

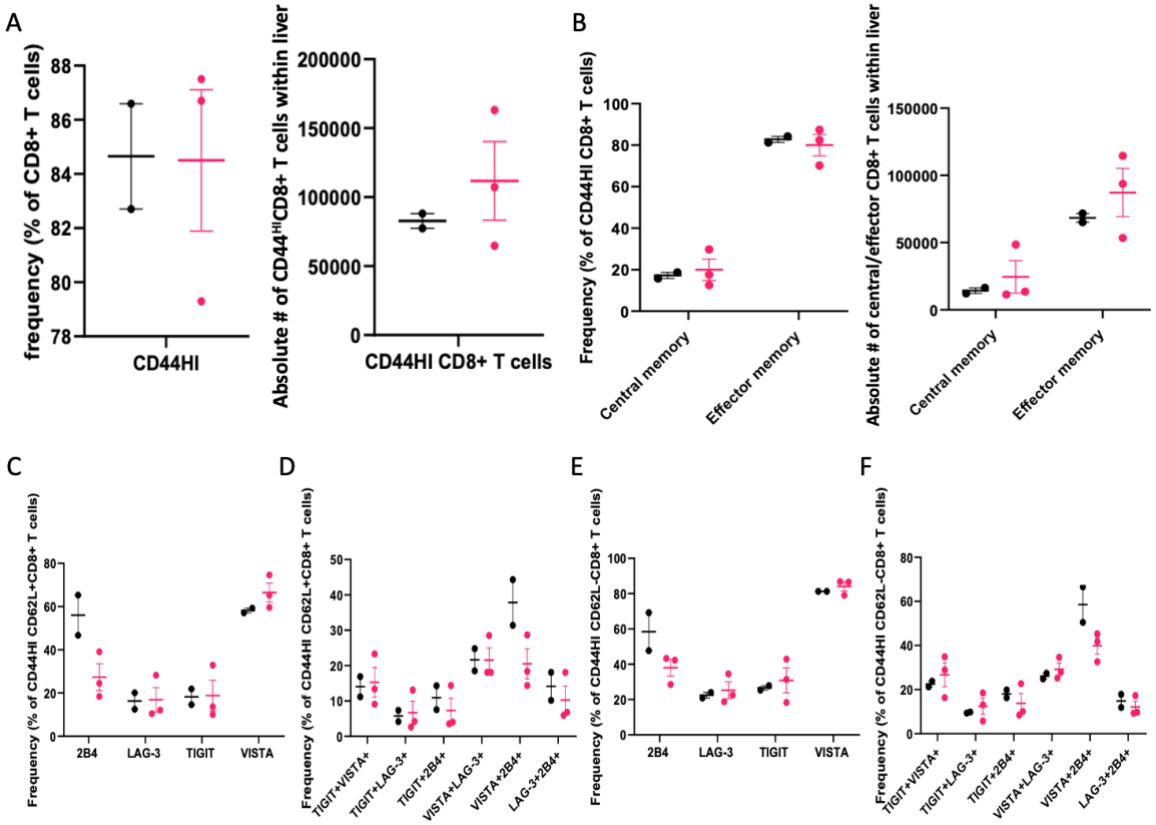

Figure 49. Within the septic liver, activated CD8+ T cells may be similar in frequency compared to sham, but they appear to be higher in absolute numbers due to the increased numbers of central and effector memory CD8+ T cells; additionally, memory CD8+ T cells may be less phenotypically exhausted. Sham is denoted in black dots, while CLP are red dots. (A) Summary plots for the frequency (left) and absolute numbers (right) of CD8+ T cells expressing high levels of CD44 within the liver of each mouse. (B) Summary plots for the frequency (left) and absolute numbers (right) of central and effector memory CD8+ T cells populations measured by the expression of CD62L gated on $CD44^{HIGH}$ CD8+ T cells within the liver of each mouse. (C) Summary plot for the frequency of central memory CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (D) Summary plot for the frequency of central memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse. (E) Summary plot for the frequency of effector memory CD8+ T cells singly expressing the different exhaustion markers within the liver of each mouse. (F) Summary plot for the frequency of effector memory CD8+ T cells doubly expressing the different exhaustion marker combinations within the liver of each mouse.

The data was pooled from 1 independent experiment: CLP = 3 mice; Sham = 2 mice. (A-F) No statistics were performed due to the low replicate number.

Chapter 4: Discussion and Conclusions

4.1 Discussion for the Recall Response

Table 5. Summary table for the findings of aim 1. Note: the blacked-out boxes signify that this data is not available. N/A = not available, (+ or –) indicate significant results with more of these symbols meaning higher significance (example: ++++ = ****p<0.0001), ~ = trend (either in the + or – direction). Important note: double-expressors for the different exhaustion marker combinations were not included.

				Exhaustion markers (Frequency/gMFI)									Other markers (Frequency/gMFI)					Functionality markers (ICS/gMFI)				
Tissue	Cell type	Frequency (Tetramer/ ICS)	Absolute numbers (Tetramer/ ICS)	PD-1	BTLA	TIM-3	CTLA-4	TIGIT	LAG-3	2B4	VISTA	KLRG1	CD25	CD69	CD95	CD127	CD44	IFN-γ (Frequency/ gMFI or MFI)	TNF-α	IL-2	Granzyme B	IFN-γ, TNF-α, IL-2
Spleen	NP_{366}	+++/++++	0/0	+/0	0/0	0/0	0/0	0/0	0/0	+/0	0/0	++/0	0/0	0/0	~+/0	++/0	N/A	++++/++	0/0	0/~-	+/0	0
	PA_{224}	0/~+	0/0	+++ +/0	0/0	0/0	0/0	0/0	0/0	+/0	0/0	+++/0	0/0	0/0	0/0	++++/0	N/A	~+/++	0/0	0/~-	++/0	0
	Non-NP_{366}			+/0	- - /~+	0/+	0/0	0/0	0/0	+/~-	0/0		0/0	0/-	0/0	0/0	~+/0					
	Non-PA_{224}			++++ /0	- - - - /~+	0/++	0/0	~+/0	0/0	+/0	0/0		0/0	0/-	0/0	0/0	~+/0					
	Central memory	-/N/A	-/N/A	0/0	++/0	0/0	0/0	0/0	0/0	+/+ +	0/0		0/0	0/0	0/0	0/0						
	Effector memory	+/N/A	0/N/A	++/0	0/~+	0/~+	0/0	0/0	0/0	0/0	~+/0		0/0	0/0	0/0	0/0						
PECs	NP_{366}	-/~-	~-/0	0/0	0/0	0/0	0/0											0/~ -				
	PA_{224}	0/~-	0/0	0/0	0/0	0/0	0/0											0/-				
	Non-NP_{366}			~-/0	~+/~ +	0/0	0/0															
	Non-PA_{224}			~-/0	~+/~ +	0/0	0/0															
	Central memory																					
	Effector memory																					
Lung	NP_{366}	~+/N/A	~+/N/A	0/0	0/~-	0/0	0/0															
	PA_{224}	0/N/A	0/N/A	0/0	0/~-	0/0	0/0															
	Non-NP_{366}			0/0	~-/~-	0/0	0/0															
	Non-PA_{224}			0/0	~-/~-	0/0	0/0															
	Central memory	~-/N/A	0/N/A	0/0	0/0	0/0	0/0															
	Effector memory	~+/N/A	~+/N/A	0/0	~-/~-	0/0	0/0															
Liver	NP_{366}	~+/N/A	~+/N/A	~+/0	0/0	0/0	0/0															
	PA_{224}	~+/N/A	~+/N/A	0/0	0/0	0/0	~-/0															
	Non-NP_{366}			0/0	~-/~-	0/0	0/0															
	Non-PA_{224}			0/0	~-/~-	0/0	0/0															
	Central memory	~-/N/A	~-/N/A	0/0	0/0	0/0	0/0															
	Effector memory	~+/N/A	~+/N/A	0/0	0/0	0/0	0/0															

4.1.1 IAV-specific CD8+ T cell numbers and frequency within the spleen

It was discovered through my research that IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression were higher in frequency within the septic spleen; however, they are numerically similar compared to sham. This was an intriguing finding as I initially hypothesized that these cells would be numerically decreased within the septic environment. I wanted to further characterize this by examining the frequencies and absolute numbers of the previous populations, namely CD8+ T cells and lymphocytes. I found, in terms of the frequency, that there was a significant decrease within the lymphocyte population; however, the frequency of CD8+ T cells were similar between sham and CLP. Moreover, looking at the absolute numbers, I discovered that there was a two-fold reduction in the numbers of lymphocytes and CD8+ T cells. One interesting point of observation is that despite there being a significant decrease in the absolute numbers of CD8+ T cells, I found a similar number of IAV-specific CD8+ T cells between sham and CLP. This might suggest that these cells have either (1) a higher proliferative capacity, (2) are recruited from different anatomical sites or (3) are more resistant to sepsis-induced apoptosis.

There is evidence within the literature that suggests that these cells do in fact have a higher capacity to proliferate. In a recent paper by Jensen et al.[194], the authors discovered that within human septic blood specimens, collected within 24 hours of admission, there was a higher frequency of CD8+ T cells expressing the proliferation marker Ki-67, which is indicative of a higher proliferative potential. They further demonstrated that central memory CD8+ T cells have the highest capacity to proliferate among any other CD8+ T cell subset, which is consistent with a previous study[284]. Furthermore, the researchers had also examined pre-existing memory CD8+ T cell proliferative capacity within a CLP mouse model of polymicrobial sepsis. In order to establish immunological memory and study pre-existing memory CD8+ T cells, this group adoptively transferred naïve Thy1.1^{+} TCR-transgenic P14 CD8+ T cells (specific for GP_{33} of LCMV) into C57BL/6 mice and infected them with LMCV a month prior to surgery. It was discovered that central memory P14 CD8+ T cells within septic mice had a higher frequency of cells expressing

Ki-67 compared to both sham mice central memory P14 CD8+ T cells and septic mice effector memory P14 CD8+ T cells, which corroborates the findings within the human septic blood specimens. Despite Ki-67 being a good marker to assess for proliferative potential, it is not sufficient to conclude that these cells possess a higher proliferative capacity as it does not measure proliferation directly. To address this issue, the researchers performed proliferation assays involving the incorporation of BrdU into cells; higher BrdU incorporation indicates more proliferation. Just as what was previously shown, it was discovered that septic central memory antigen specific and bulk CD8+ T cells had more proliferation compared to both septic effector memory antigen specific and bulk CD8+ T cells and sham central memory antigen specific and bulk CD8+ T cells. From this information, and taking into consideration that my IAV-specific CD8+ T cells of interest would have a greater proportion classified as central memory CD8+ T cells prior to the induction of the recall response[282,284,444], it could be suggested that my IAV-specific CD8+ T cells within the septic spleen have a higher proliferative capacity after sepsis induction.

Despite these findings, there are differences between their study and mine. For instance, the authors had assessed pre-existing memory CD8+ T cell recovery from the septic insult, and I have examined their secondary expansion due to antigen re-encounter. From their experiments, the memory CD8+ T cells would be presumably recovering through homeostatic proliferation, and they have eloquently shown that after sepsis, central memory antigen specific and bulk CD8+ T cells have the highest capacity to proliferate among any other CD8+ T cell subset leading to their ultimate overrepresentation within the CD8+ T cell compartment. This information is still applicable to my research as they have shown that memory CD8+ T cells after the septic insult have a higher proliferate capacity; however, in terms of my study, their findings may underestimate the amount of proliferation that I have seen. In a study by Cheung et al.[443], it was discovered that true-memory CD8+ T cells outcompeted homeostatic proliferated memory CD8+ T cells specific for the same antigen; furthermore, it was also demonstrated that compared to homeostatic proliferated memory CD8+ T cells, antigenic proliferated memory CD8+ T cells, seven days post-infection had a significantly higher number within the spleen. Moreover, within a study by Geginat et al.[433], it was illustrated that compared to

homeostatic proliferation, antigenic proliferation had significantly greater absolute numbers of proliferated central memory CD8+ T cells within the spleen; furthermore, the authors had also discovered that in response to cognate antigen, central memory CD8+ T cells had significantly higher proliferation compared to effector memory CD8+ T cells. From this information, in terms of my data, I can conclude that after sepsis induction, my IAV-specific memory CD8+ T cells will begin to proliferate through homeostatic proliferate mechanisms; however, when the antigen is re-encountered, antigenic proliferation will take over as it can outcompetes homeostatic mechanisms leading to their overrepresentation within the CD8+ T cell compartment. Moreover, compared to the study by Jensen et al.[194], it would make sense that I would see an enhanced proliferation from my IAV-specific memory CD8+ T cells as there are more driving forces in enhancing proliferation: (1) there would be an enhanced IAV-specific central memory CD8+ T cell proliferative potential from the sepsis-induced lymphopenia; (2) antigenic proliferation would take over and lead to a higher magnitude of IAV-specific central memory CD8+ T cells proliferating within the spleen.

Continuing, within the study by Jensen et al.[194], it was also demonstrated that the higher proliferative capacity of central memory CD8+ T cells after sepsis led to the remodelling of the different memory CD8+ T cell subsets, ultimately leading to the higher proportion of central memory CD8+ T cells within the septic host. In my data, I described a shift from central memory CD8+ T cells to effector memory CD8+ T cells within the septic spleen, which might seem contradictory to their findings; however, it is not. The IAV-specific memory CD8+ T cells prior to the induction of the recall response would be mostly classified as central memory CD8+ T cells[282,284,444], with a smaller proportion consisting of effector memory CD8+ T cells; however, upon recall response, central memory CD8+ T cells can switch to an effector memory CD8+ T cell phenotype[282,432,433]. My data fits well in the literature for multiple reasons: Firstly, it has been shown that during sepsis-induced lymphopenia, antigen specific memory CD8+ T cells have a higher proliferative capacity compared to sham, which would lead to their predominance within the CD8+ T cell compartment; secondly, antigen specific central memory CD8+ T cells have been shown to have a higher proliferative capacity compared to antigen specific effector memory CD8+

T cells, among other T cell subsets[194,284]; thirdly, prior to the recall response, there would be a greater presence of central memory CD8+ T cells compared to effector memory CD8+ T cells; fourthly, it has been demonstrated by a couple of groups that central memory CD8+ T cells are the predominant contributors to recall responses[284,445]; Fifthly, central memory CD8+ T cells have the ability to switch to an effector memory CD8+ T cell phenotype during a recall response[282,432,433]. Hence, I may conclude that after sepsis, prior to the induction of a recall response, IAV-specific central memory CD8+ T cells will expand more readily leading to their predominance, then subsequently upon recall response, IAV-specific central memory CD8+ T cells will expand due to antigenic proliferation and will switch to an effector memory CD8+ T cell phenotype. Despite all this information, further studies would have to be performed to confirm that the IAV-specific CD8+ T cells of interest prior to recall response are in fact central memory CD8+ T cells.

4.1.2 IAV-specific CD8+ T cell phenotypes within the spleen

Next, I wanted to assess IAV-specific CD8+ T cell phenotypes of exhaustion at the peak memory CD8+ T cell response during sepsis-induced immunosuppression. I revealed that IAV-specific CD8+ T cells within the spleen of septic mice were more exhausted compared to sham as demonstrated by the increased frequencies of cells singly expressing PD-1 and 2B4. Furthermore, I had also wanted to assess their co-expression of the different exhaustion markers, which would provide stronger evidence of an exhaustive phenotype. Single-expression of different exhaustion markers may be indicative of CD8+ T cell exhaustion; however, it is not conclusive. CD8+ T cell exhaustion is dependent on the simultaneous co-expression of different inhibitory markers[424,425]. I discovered that IAV-specific CD8+ T cells within the septic spleen have a higher frequency of cells doubly expressing the different exhaustion markers, and this finding was illustrated across the majority of the double-expressors examined. In terms of septic NP_{366}-specific CD8+ T cells, they possessed higher frequencies of cells doubly expressing PD-1+BTLA+, TIGIT+2B4+ and VISTA+2B4+, while septic PA_{224}-specific CD8+ T cells had higher frequencies of double-expressors for PD-1+BTLA+ and VISTA+2B4+. Furthermore, within the ICS data, I demonstrated that septic IAV-specific CD8+ T cells had a higher frequency of cells expressing KLRG1. KLRG1 is a co-inhibitory marker that gets upregulated in highly proliferative and cytotoxic effector CD8+ T cells, which were stimulated by strong TCR and inflammatory signalling[413,414]. From the results, it is evident that at the peak memory CD8+ T cell response in the context of sepsis-induced immunosuppression, IAV-specific CD8+ T cells within the septic spleen are more phenotypically exhausted compared to sham.

These results made me question whether there are exhaustion markers that are unique to IAV-specific CD8+ T cells during the recall response in the context of protracted sepsis. To address this question, I turned my attention to the non-IAV-specific and bulk memory CD8+ T cell compartments. I discovered that 2B4 could potentially be unique for these IAV-specific CD8+ T cells. In terms of the non-IAV-specific CD8+ T cells, there was a higher frequency of cells expressing 2B4 within the CLP condition; however, there is some evidence to show that 2B4 is unique for IAV-specific CD8+ T cells. Studying the non-IAV-specific CD8+ T cell populations is limited as there will be contamination within the populations with other IAV-specific CD8+ T cells, for example, non-NP_{366}-specific CD8+ T cells would have contamination from PA_{224}-specific CD8+ T cells and vice versa. Despite this limitation, I can use this contamination to my advantage as there is a higher frequency of cells specific for NP_{366}, while PA_{224}-specific CD8+ T cells are less frequent during recall response[429]. Within the non-PA_{224}-specific CD8+ T cell compartment, the frequency of cells expressing 2B4 is higher compared to the non-NP_{366}-specific CD8+ T cell compartment, which makes sense as there is more contamination from NP_{366}-specific CD8+ T cells within the non-PA_{224}-specific CD8+ T cell compartment. Further evidence comes from the gMFI data, there is a trend towards reduced 2B4 expression on a per-cell basis within the non-NP_{366}-specific CD8+ T cell compartment and this trend is dampened within the non-PA_{224}-specific CD8+ T cell compartment, which suggests that more contamination from IAV-specific CD8+ T cells leads to a higher 2B4 signal. Stronger evidence, however, comes from examining the bulk memory CD8+ T cell compartment. Within the effector memory CD8+ T cell compartment, which would consist of the majority of the IAV-specific CD8+ T cells of interest, there is no statistical difference in the expression of 2B4 between sham and CLP. This information suggests that 2B4 could potentially be unique to IAV-specific CD8+ T cells during the recall response as the signal of 2B4 on IAV-specific CD8+ T cells is being dampened by the presence of many other effector memory CD8+ T cells. From all this information, it might be suggested that during the recall response, 2B4 is preferentially upregulated on IAV-specific CD8+ T cells in the context of sepsis-induced immunosuppression.

To my knowledge, this is the first study to suggest that 2B4 upregulation is unique to antigen specific memory CD8+ T cells during recall responses to their cognate antigen in the context of sepsis-induced immunosuppression. Previous studies have focused on the expression of 2B4 in memory CD8+ T cells during the acute phase of sepsis[274,285]. For instance, in a study by Xie et al.[274], the authors discovered that 2B4 was preferentially upregulated on memory CD8+ T cells 24 hours post-CLP in memory mice. Within another study by Xie et al.[285], it was discovered that three days post-CLP, both antigen experienced and antigen specific memory CD8+ T cells possessed higher frequencies of cells expressing 2B4 compared to sham; furthermore, the authors demonstrated that the expression of 2B4 was unique to antigen specific memory CD8+ T cells as non-tetramer-specific memory CD8+ T cells did not show an increased expression of 2B4 compared to sham. This information suggests that during the acute phase of sepsis, memory CD8+ T cells, specifically antigen specific memory CD8+ T cells have a unique upregulation of 2B4. Despite these findings being attributed to the acute phase of sepsis, this research is still translatable to my findings as these authors have demonstrated that 2B4 upregulation in the context of sepsis is specific for antigen specific memory CD8+ T cells. Hence, these findings strengthen my hypothesis that 2B4 upregulation is unique to IAV-specific memory CD8+ T cells during the recall response to IAV in the context of sepsis-induced immunosuppression.

Furthermore, the increased expression of 2B4 may be detrimental to the survival of the cells that express this marker as it could lead to their depletion. Within the study by Xie et al.[274], the researchers demonstrated that the preferential upregulation of 2B4 in memory CD8+ T cells was associated with increased apoptotic factors such as caspase-3/7, which would indicate that these cells are more susceptible to apoptosis. Moreover, within human blood samples of septic individuals, the researchers discovered that CD8+ T cells expressing 2B4 had a higher caspase-3/7 activation, which is indicative of a higher apoptotic potential. This information suggests that during the acute phase of sepsis, memory CD8+ T cells may have an enhanced susceptibility to sepsis-induced apoptosis leading to a greater degree of lymphopenia due to in part by the upregulation of 2B4. This finding was reflected within their experiments, comparing naïve and memory mice, the

authors discovered that memory mice had a more profound loss of CD8+ T cells within the septic spleen compared to naïve mice. To show further proof of concept that 2B4 upregulation on memory CD8+ T cells during sepsis enhances lymphopenia, both studies by Xie et al.[274,285] performed 2B4 knockout experiments. Within the first study[274], knocking out 2B4 in the CLP memory mouse model led to increased mouse survival from 20% to 60% with an associated increase in CD8+ T cell numbers and decreased caspase-3/7 activation. Furthermore, within the other study by Xie et al.[285], knocking out 2B4 in CLP memory mice led to increased frequencies and numbers of antigen experienced and antigen specific memory CD8+ T cells, which were similar to the levels of sham mice. All this information suggests that memory CD8+ T cells are preferentially depleted during acute sepsis, with 2B4 expression being a major contributor to this depletion.

Taken together, despite their studies examining pre-existing memory CD8+ T cells during acute sepsis, this information is still important in demonstrating how 2B4 upregulation is unique to antigen specific memory CD8+ T cells. Incorporating their findings into my study, it could be suggested that during the initial phase of sepsis, memory CD8+ T cell loss is enhanced by in part the preferential upregulation of 2B4 on pre-existing antigen specific memory CD8+ T cells, which disproves the notion that my memory IAV-specific CD8+ T cells possess a resistance to sepsis-induced apoptosis. Furthermore, the pre-existing IAV-specific memory CD8+ T cells that survive the initial phase of sepsis, upon cognate antigen exposure may specifically upregulate 2B4 leading to higher apoptotic activity and their subsequent exacerbated cell loss after the peak memory CD8+ T cell response.

In addition to examining exhaustion markers within the IAV-specific CD8+ T cells at the peak memory CD8+ T cell response in the context of sepsis-induced immunosuppression, I had also examined the expression of different activation, death, and survival/maintenance markers. I demonstrated that IAV-specific CD8+ T cells within the septic spleen possessed lower frequencies of cells expressing CD127, while having higher frequencies of cells expressing KLRG1 and showing a trend towards higher expression of CD95.

To further discuss CD127 expression and why it is important, first, I must discuss how memory CD8+ T cells are generated *in vivo*. A typical CD8+ T cell response goes through three phases: effector cell expansion and differentiation, contraction, and generation of a memory pool, which is stable for months and years. It has been demonstrated that memory CD8+ T cell precursors exist at the peak CD8+ T cell response; however, these antigen specific CD8+ T cells do not show functional characteristics of memory CD8+ T cells until one-to-two months after infection[439]. Furthermore, the typical characteristics of long-lived memory CD8+ T cells are the ability to rapidly expand during recall responses, express increased levels of Bcl-2, CD62L, and CCR7, and are able to persist for long-term due to homeostatic proliferation under the influence of IL-15 and IL-7 [439]. In terms of CD127, it was discovered by Kaech et al.[439] that CD127 was required to establish a memory CD8+ T cell pool capable of responding to subsequent pathogen re-encounter. This finding was demonstrated in their experiments, where the authors had adoptively transferred either $IL7R^{HIGH}$ or $IL7R^{LOW}$ P14 Thy-1.1+ CD8+ T cells specific for LCMV-GP_{33} into naïve mice, and then challenged these mice with *Listeria monocytogenes in vivo* at a later time-point. They discovered that the $IL7R^{HIGH}$ population expanded greatly and comprised ~20-30% of the CD8+ T cell pool; however, the $IL7R^{LOW}$ population only comprised ~0.2-0.5% of total CD8+ T cells. From this information, it is appropriate to use the frequency of CD127 as a marker for their capability to produce a robust memory CD8+ T cell pool. Within my data, I found a reduced frequency of cells expressing CD127 at the peak memory CD8+ T cell response within the septic spleen, which would indicate that these cells are compromised in their ability to produce a memory CD8+ T cell pool.

In another train of thought, lower expression of CD127 could potentially be a marker for higher CD8+ T cell activation. In a study by Paiardini et al.[426], the authors measured *ex vivo* cytokine production by stimulation with PMA and ionomycin and demonstrated that CD8+CD127- T cells from both HIV-infected and control patients had a higher capacity to produce IFN-γ, while almost totally depleted of IL-2 production. Moreover, it was also revealed that CD8+CD127+ T cells had a limited ability to produce IFN-γ, while having higher capabilities to produce IL-2. This information suggests that CD8+ T cells lacking CD127 are more functionally active compared to CD8+ T cells expressing CD127; however, they may possess a dampened ability to proliferate. Furthermore, the researchers had also assessed spontaneous and activation-induced apoptosis by looking at the expression of annexin V after a 48-hour incubation period with either media or phytohaemagglutinin (PHA). It was discovered that CD8+CD127- T cells had a 2- to 5-fold increase in both the level of spontaneous and activation-induced apoptosis, which would indicate that they are more short-lived compared to their CD127-expressing counterpart. To further show prove of concept, in another study by Mouillaux et al.[427], the authors demonstrated that septic shock patients compared to healthy volunteers had an increased proportion of $CD127^{low}PD\text{-}1^{high}$ CD8+ T cells after septic shock; additionally, these cells also co-expressed the late activation marker HLA-DR, which would indicate that CD127 gets down-regulated upon T cell activation. Using all this information, I may conclude that the IAV-specific CD8+ T cells within the spleen of septic mice are consistent with a phenotype of higher activation and a reduced ability to produce a good quality CD8+ T cell memory pool.

Next, I will discuss the implications of CD95 in my research. CD95 was found to show a trend in upregulation in the septic spleen; however, NP_{366}-specific CD8+ T cells showed a higher increase compared to PA_{224}-specific CD8+ T cells. CD95 has been well characterised as a death mediator for activated T cells by guiding these cells into the apoptotic pathway[428], which would indicate that our cells of interest could potentially be more sensitive to CD95-induced apoptosis. Taking another perspective, during CD8+ T cell responses, CD95 and CD95L are upregulated to control effector CD8+ T cell homeostasis, this would imply that higher T cell activation and proliferation would lead to higher signalling through CD95-CD95L pathway to maintain homeostasis[428]. CD95 could be looked at as an activation marker due to this nature, so this is further evidence that my IAV-specific CD8+ T cells are more activated within the septic spleen compared to sham.

To provide further evidence that my IAV-specific CD8+ T cells are more activated within the septic spleen, I will discuss KLRG1. Within the literature, the phenotype $CD127^{LOW}KLRG1^{HIGH}$ is commonly referred to as terminal effector CD8+ T cells[408,441,446–448], which would imply that after they respond, they die off. Hence, within my data, it could be suggested that there is a higher frequency of IAV-specific CD8+ T cells that are short-lived, which would mean that there is a more pronounced contraction phase. This ties in well with the 2B4 data, as it has been shown that 2B4 expression leads to significant apoptosis of this population[274,285]. Furthermore, KLRG1 expression may affect the ability of effector CD8+ T cells to produce a robust memory CD8+ T cell pool. Within a study by Hand et al.[449], it was shown that the ectopic overexpression of CD127 was not sufficient to induce the formation of memory CD8+ T cells. It was discovered that the expression of KLRG1 was a heavy determinant on the formation of memory CD8+ T cells as CD8+ T cells expressing high levels of KLRG1 were not able to produce a memory CD8+ T cell pool, despite these cells expressing CD127. Furthermore, the authors had also demonstrated that CD8+ T cells expressing high CD127 and KLRG1 were unable to proliferate under the influence of IL-7, which has been corroborated by another group[446]. From this information, I may state that the IAV-specific CD8+ T cells of interest within the septic spleen have a reduced capability to produce a good memory CD8+ T cell pool as they express lower frequencies of CD127, while expressing higher frequencies of

KLRG1. Moreover, the expression of KLRG1 may be indicative of a higher functionality of these IAV-specific CD8+ T cells at the peak memory CD8+ T cell response, which will be further be touched upon later[444,450–453]. This enhanced functionality suggests that these cells may be more activated in the context of sepsis. Next, to provide a better understanding on why KLRG1 may be used to assess CD8+ T cell activation, I will discuss why and how KLRG1 gets expressed. It has been demonstrated by multiple groups that KLRG1 is expressed due to chronic antigenic exposure, repeated antigenic exposure, age, multiple rounds of cell division and inflammation[413,444,446–448,450]. In the context of sepsis, three of these conditions are met: chronic antigen exposure as there is typically a failure of memory T cells to clear the infection after sepsis; multiple rounds of cell division, which I have previously demonstrated that IAV-specific memory CD8+ T cells will have an increased ability to proliferate; inflammation due to the increased presence of inflammatory stimuli after CLP due to the continuous leakage from the septic focus[454]. In terms of inflammation, in a study by Joshi et al.[413], it was discovered that higher levels of inflammatory stimuli within the presence of cognate antigen led to significantly more KLRG1+CD127- effector CD8+ T cells, which typically possess a greater ability to produce cytokines and cytotoxic effects[431,455]. Hence, considering that my IAV-specific CD8+ T cells possessed higher frequencies of cells expressing KLRG1, while having reduced CD127, it could be suggested that my IAV-specific CD8+ T cells within the septic spleen are more activated and functionally active.

Taking another perspective, I may be able to further suggest that IAV-specific CD8+ T cells at the peak memory CD8+ T cell response within the septic spleen are more activated compared to sham due to their enhanced exhaustion phenotype. Currently, within the literature, there is not a good consensus on what defines exhaustion and activation when these markers are expressed. Many studies have demonstrated that exhaustion markers are associated with decreased responses[419–423,456], while others have demonstrated increased responses[415–418,431,435,436,457]. For example, in a study published by Blazkova et al.[418], it was discovered that TIGIT expression on CD8+ T cells positively correlated with the production of different cytokines and cytolytic effector molecules within human blood samples. Moreover, the authors had also examined genetic expression profiles in TIGIT+CD8+ and TIGIT-CD8+ T cells; the researchers discovered that CD8+ T cells expressing TIGIT had an upregulation in the genes of IFN-γ, TNF-α, granzyme (A, H, K). However, in a different study by Sun et al.[419], it was demonstrated in septic patients compared to healthy controls that CD8+ T cells expressing TIGIT had a significant reduction in their ability to produce IFN-γ, TNF-α and IL-2, which points to these cells being exhausted rather than active. Furthermore, it was discovered in a study by Pombo et al.[457] that individuals capable of controlling HIV infections had a distinct HIV-specific CD8+ T cell population that co-expressed 2B4 and CD160, which was correlated with a higher cytotoxic ability. However, 2B4 has also been shown to suppress CD8+ T cell responses[421]. From this information, it is not clear what constitutes exhaustion as these exhaustion markers have been shown to be associated with both enhanced and dampened responses. Although, considering the nature of expression of exhaustion markers (i.e. induced upon T cell activation to control excessive responses), it is reasonable to suggest that CD8+ T cells expressing the different exhaustion markers would infer that they have been more activated and functional. Recently, it has been suggested that true exhaustion consists of the expression of multiple exhaustion markers simultaneously[424,425]. Furthermore, CD8+ T cell exhaustion is a progressive phenomenon and occurs during multiple situations, such as persistent antigen exposure and/or inflammation[458,459]. In a study by Angelosanto et al.[458], the authors wanted to assess the effect of CD8+ T cell exhaustion on their cytokine productive capabilities and their ability to form memory CD8+ T cells. To accomplish this, the authors infected mice with LCMV clone-13, which

is a model for chronic viral infection, and at different time points (eight, 15, 30 days post-infection) isolated GP_{33}-specific CD8+ T cells, then subsequently adoptively transferred them into mice that have been infected with either LCMV clone-13 or LCMV-Arm (acute infection) for the same amount of time and analyzed their responses at multiple time-points after transfer (eight, 15, 30 and 60 days). It was demonstrated that transferring GP_{33}-specific CD8+ T cells eight days post-infection into the acute infection model led to these cells having reduced PD-1 and increased CD127 expression 60 days post-transfer, similar to the expression within endogenous GP_{33}-specific CD8+ T cells; however, when they were transferred into the chronic infection model, there was an increased PD-1 and decreased CD127 expression, which was comparable to the expression of endogenous GP_{33}-specific CD8+ T cells of these markers. Furthermore, it was also shown that transferring GP_{33}-specific CD8+ T cells 15 days post-infection into the acute infection model led to these cells having higher PD-1 and similar CD127 expression 60 days post-transfer compared to endogenous GP_{33}-specific CD8+ T cells expression levels. Although, when they were transferred into the chronic infection model, there was a similar expression of PD-1 and CD127 compared to the endogenous GP_{33}-specific CD8+ T cells. Moreover, within the both sets of experiments, the ability to produce IFN-γ was preserved, however, transferring GP_{33}-specific CD8+ T cells 15 days post-infection into either the acute or chronic model had reduced polyfunctionality. Although this study is very powerful, it examined the exhaustion onset during a primary CD8+ T cell response. To address the exhaustion during a recall memory CD8+ T cell response, I will turn my attention to a study that was published by Nolz et al.[460]. In their experiments, the authors had examined the response from naïve, primary memory and secondary memory P14 CD8+ T cells during the chronic viral infection with LCMV-clone 13. Compared to both naïve and secondary memory P14 CD8+ T cells, primary P14 CD8+ T cells demonstrated the ability to clear the infection by day 10 post-infection. Furthermore, it was also discovered that primary memory P14 CD8+ T cells had a significantly higher frequency of cells capable of producing IFN-γ and TNF-α seven days post-infection, while on day 15 post-infection had a reduced expression of PD-1, 2B4 and LAG-3 compared to naïve and secondary memory P14 CD8+ T cells, although, this decrease in exhaustion markers may be due to the absence of antigen as it has been shown that upon viral clearance, exhaustion markers are downregulated[438].

Moreover, in a different set of experiments that is more comparable to our study, the authors had administered LCMV-clone 13 and ensured that there would be a consistent viral load, which would mimic what I see during the course sepsis. It was discovered that seven days post-infection, primary memory CD8+ T cells had a similar expression of IFN-γ and TNF-α as the previous experiment; however, 13 days post-infection, there was a reduction in the production of these cytokines, with an associated increase in the expression of different exhaustion markers. From these experiments, I may be able to conclude that 13 days post-infection, there will be the onset of exhaustion and considering that within my experiments, our timeline was 11 days, the onset of exhaustion may not have set in yet. Hence, the expression of the exhaustion markers may be utilized in concluding that the IAV-specific memory CD8+ T cells of interest are indicative of both higher activation and exhaustion, although, the exhaustion pathway has taken full effect.

Taken together, there is strong evidence to suggest that the IAV-specific CD8+ T cells of interest, at the peak memory CD8+ T cell response in the context of sepsis-induced immunosuppression are phenotypically more activated and exhausted compared to sham as illustrated by their enhanced expression of CD95 and the different exhaustion markers, and the reduced expression of CD127. Furthermore, these IAV-specific CD8+ T cells within the septic spleen may also possess a dampened ability to produce a robust memory CD8+ T cell pool as these cells are more short-lived effector CD8+ T cells.

4.1.3 IAV-specific CD8+ T cell functionality within the spleen

Next, I had also wanted to examine the functionality of IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression, which was determined by their capacity to produce different cytokines and cytolytic effectors molecules. What I found was contrary to my initial hypothesis, I initially thought that the IAV-specific CD8+ T cells of interest would be functionally dampened during sepsis-induced immunosuppression; however, I discovered that our IAV-specific CD8+ T cells demonstrated increased functionality. In my research, I demonstrated that IAV-specific CD8+ T cells within the septic spleen had higher frequencies, albeit similar numbers, and higher per-cell expression of IFN-γ. Moreover, I also showed that the polyfunctionality of these cells are retained within the septic spleen as the frequencies of triple-expressors for IFN-γ, TNF-α and IL-2 were similar compared to sham. I also measured the expression of the cytolytic effector molecule granzyme B and discovered that there were higher frequencies of cells expressing granzyme B within the septic spleen, despite their being a similar expression on a per-cell basis. From these results, it is reasonable to conclude that IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during sepsis-induced immunosuppression within the spleen are more functionally active compared to their sham counterpart.

In the next paragraphs, I will briefly discuss the research that has been done on memory CD8+ T cell responses during sepsis (extensively discussed in section 1.2.3), the problems with these studies, then further expand on my findings.

Commonly, it has been widely accepted that memory T cell responses were dampened during sepsis. The first piece of evidence that suggests memory T cell dysfunction during sepsis-induced immunosuppression was the reported loss of delayed-type hypersensitivity reactions within septic patients[461–463], which was associated with diminished T cell proliferation and cytokine production including IL-2 and IFN-γ. Proceeding along, I will further discuss the aberrations seen within memory CD8+ T cell responses during early and late sepsis, with a heavy focus on antigen specific memory CD8+ T cells.

In terms of early memory CD8+ T cell responses, within a study by Duong et al.[275], it was discovered that early in sepsis (two days post-CLP), splenic pre-existing antigen specific memory CD8+ T cells have a dampened ability to produce IFN-γ under the simulation with cognate peptide. Furthermore, within a study by Dahany et al.[272], it was demonstrated that splenic pre-existing antigen specific memory CD8+ T cells had a reduced absolute number, frequency and per-cell expression of cells capable of producing IFN-γ under the stimulation with cognate peptide two days post-CLP; however, tissue resident antigen specific memory CD8+ T cells within the ear did not demonstrate this defect in IFN-γ production. Moreover, within a paper by Choi et al.[271], the authors had examined early memory CD8+ T cell responses in human septic blood specimens that either had CMV reactivation or did not have reactivation within three days of admission to the ICU for severe sepsis. The researchers discovered that memory CD8+ T cells between CMV-reactivated and non-reactivated did not differ in their frequency and absolute number of single-expression of the different cytokines examined (IFN-γ, TNF-α, CD107a, MIP-1β), although, there was a reduced per-cell expression of TNF-α and CD107a. However, it was found that the septic patients with reactivation of CMV had a reduced frequency and absolute number of CMV-specific memory CD8+ T cells quadruple- and triple-expressing the various cytokines, which was correlated with the exhaustion marker PD-1. Hence, this information suggests that single-expression of cytokines may not be affected during the early septic event; however, these antigen specific memory CD8+ T cells are more exhausted in patients with reactivation, which might contribute to their dampened polyfunctionality leading to reduced control of CMV. Furthermore, within the study by Duong et al. [275], the authors had also examined the ability of antigen specific memory CD8+ T cells and bulk memory

CD8+ T cells in their innate ability to respond to heterologous infection, which assessed their ability for bystander activation and functionality. Five days post-CLP, the researchers had administered the heterologous LCMV strain and examined the responses 20 hours post-infection; it was discovered that bulk and P14 memory CD8+ T cells had a reduced frequency and absolute number of cells capable of producing IFN-γ, while P14 memory CD8+ T cells had a reduced expression of granzyme B, CD25 and CD69, which indicates that P14 memory CD8+ T cells had a lower activation and functionality at the five day time-point after sepsis induction. So far, it is clear from the results presented for systemic (blood and spleen) memory CD8+ T cell functionality (antigen-dependent and -independent) during early sepsis is dampened, while tissue resident memory CD8+ T cell functionality is persevered, however, the degree of severity of sepsis can influence their functionality[276].

Next, I will further expand on late sepsis functionality of antigen specific memory CD8+ T cells, in which there has only been one study to address this. Within a paper by Jensen et al.[194], it has been demonstrated that splenic pre-existing antigen specific memory CD8+ T cells 30 days post-CLP had an increased functionality as measured by the expression of IFN-γ and IL-2; it was shown that IFN-γ production was similar compared to sham, however, they possessed higher frequencies of cells expressing IL-2 and doubly-expressing IFN-γ+IL-2+, which would indicate (1) there may be an enhanced ability to proliferate as IL-2 is a canonical cytokine involved in CD8+ T cell expansion[369] and (2) there quality of response may be enhanced during late sepsis. From this one study, it is evident that late septic memory CD8+ T cell responses may not be affected, but it may actually be enhanced, however, this may not be true. Within the same study by Jensen et al.[194], the authors had adoptively transferred these splenic antigen specific memory CD8+ T cells from either sham or CLP mice into naïve mice, then infected them one day later and examined the viral titers within the spleen and liver. The researchers discovered that compared to the sham antigen specific memory CD8+ T cells, the septic antigen specific memory CD8+ T cells had a reduced capability to control the infection as there was a higher viral titer five days post-infection. From this one study, it is evident that splenic septic memory CD8+ T cells had an enhanced capability to produce cytokines, while possessing an increased

polyfunctionality, however, they still may be dysfunctional as they may not be able to control re-infection.

Thereafter, I will now discuss some of the limitations and issues with these aforementioned studies to provide a better understanding on why and how my study is novel and is significant. Firstly, within all the functionality experiments examined, including early and late, the researchers had focused on the early immune responses from pre-existing antigen specific memory CD8+ T cell without the induction of an *in vivo* recall response. However, some of the groups have examined the response from an *in vivo* stimulation with their cognate peptide, although, it was only a few hours of stimulation. These experiments were good in assessing the early memory CD8+ T cell responses to cognate antigen; however, it omits their responses at the peak memory CD8+ T cell response during an *in vivo* infection. It is crucial to examine the memory CD8+ T cell responses at the peak as failing to do so would compromise their results because they are not capturing the entire breadth of response. Memory CD8+ T cells are quick in responding to cognate antigens by rapidly producing different cytokines and controlling infections sooner than naïve CD8+ T cells[282,284,460,464–467]; however, they are slower in their proliferative capacity. Within the spleen, memory CD8+ T cells begin to expand and proliferate as early as three days post-infection and their numbers peak around day seven-to-eight post-infection[284,460,465–467]. Furthermore, it has also been demonstrated that memory CD8+ T cell productive capabilities of different cytokines peaks at the height of their numbers[284,460,465,467]. From this information, I can state that it is appropriate to examine the early responses in memory CD8+ T cells to cognate antigen as they are potent in controlling the initial infection; however, due to the slower onset of proliferation, examining the early responses would not capture the entirety of the response and this could severely compromise their findings. Secondly, the majority of the experiments that have focused on the antigen specific memory CD8+ T cell responses were performed two days post-CLP, which has been shown to be the peak CD8+ T cell loss[273,395]. Furthermore, it has been shown by multiple groups that memory CD8+ T cells were significantly more susceptible to sepsis-induced apoptosis compared to any other T cell subset as they have a more pronounced cell loss[273,274,395]. Hence, it would make sense that the researchers reported reduced frequencies and absolute

numbers of functionally active memory CD8+ T cells as there is a reduced absolute number and frequency of memory CD8+ T cells within the septic condition at these early time-points. Taken together, considering that the majority of the studies examining memory CD8+ T cells responses during sepsis have (1) examined early functionality of antigen specific memory CD8+ T cells, without examining the functionality at the peak memory CD8+ T cell response during an *in vivo* recall response and (2) examined the early functionality of antigen specific memory CD8+ T cells at peak CD8+ T cell loss, there is a clear lack of knowledge on how sepsis effects memory CD8+ T cell responses during a recall response at the peak memory CD8+ T cell response.

Now, I will further discuss my findings, regarding the functionality of antigen specific memory CD8+ T cells at the peak memory CD8+ T cell response within sepsis-induced immunosuppression. My research is extremely important as no one has previously investigated the functionality of antigen specific memory CD8+ T cells during an *in vivo* recall response in the context of sepsis-induced immunosuppression at the peak memory CD8+ T cell response. In terms of granzyme B production, it has been reported by multiple groups that after the onset of sepsis, there is an increased expression of granzyme B within CD8+ T cells[468,469]. For example, within human septic blood samples, it has been discovered that CD8+ T cells had an increased intracellular expression of granzyme A, B and M[468]. Furthermore, the authors had also demonstrated that CD8+ T cells tended to co-express the three granzymes together. Moreover, within a paper by Napoli et al.[469], it was determined within septic patients that there was a higher expression of granzyme B in CD8+ T cells compared to healthy and acutely ill non-septic patients. Furthermore, the researchers had also demonstrated that the expression of granzyme B within CD8+ T cells correlated strongly with the acute physiology and chronic health evaluation II (APACHE II) score, which suggests that higher intracellular expression of granzyme B within CD8+ T cells may lead to a higher disease severity from sepsis. These two studies have addressed the intracellular expression of the different granzymes within CD8+ T cells during the acute phase of sepsis; currently, to my knowledge, there is only one study that has examined the expression of granzyme B in the context of sepsis-induced immunosuppression. Within a study by Duong et al.[275], the authors had examined the innate functionality of antigen

specific memory CD8+ T cells in response to a heterologous infection. The infectious agent was administered five days post-CLP, which is a time-point where immunosuppression has set in, then the researchers examined the intracellular expression of granzyme B within antigen specific memory CD8+ T cells 20 hours post-infection. It was discovered that septic splenic antigen specific memory CD8+ T cells had a reduced frequency of cells expressing granzyme B, which is contrary to our findings; however, there are a few differences between my study and theirs. For instance, they had examined early innate antigen specific memory CD8+ T cell responses to a heterologous infection, while I have examined homologous antigen specific memory CD8+ T cell responses at the peak of the memory CD8+ T cell response, which are immutable differences between our studies. In terms of IFN-γ expression, like the granzyme B study, the majority of the studies have examined early memory antigen specific CD8+ T cell responses, which are significantly different compared to my study. To my knowledge, currently, there are no studies that confirm my findings, however, I can utilize the phenotyping experiments to provide stronger evidence of increased activity. Firstly, I described that there was an increased frequency of effector memory CD8+ T cells at the peak memory recall response and this was most likely due to the conversion of central memory CD8+ T cells to effector memory CD8+ T cells after their expansion within the post-septic environment. Furthermore, it has been demonstrated by multiple groups that effector memory CD8+ T cells are functionally more active compared to central memory CD8+ T cells as they possess a higher capacity to produce different cytokines and cytolytic effector molecules, while possessing a greater cytotoxic ability[431,455,470,471]. Hence, it would be reasonable to suggest that within the septic spleen, there are more functionally active memory CD8+ T cells; furthermore, IAV-specific CD8+ T cells of interest at the peak memory CD8+ T cells are mostly classified as effector memory CD8+ T cells, therefore, these cells are more functionally active. Secondly, within my data, I discovered that there was a higher frequency of cells expressing KLRG1, with an associated lower expression of CD127. This phenotype has been shown by many groups to be more functionally active compared to cells highly expressing CD127, but low KLRG1[414,425,444,450,452,471–473]. For instance, within a study by Brandstetter et al.[472], it was discovered that effector memory CD8+ T cells expressing KLRG1 and lacking CD127 had an increased frequency of cells capable of producing granzyme B and IFN-γ

compared to effector memory CD8+ T cells possessing low KLRG1 and high CD127. Furthermore, within a study by Masopust et al.[444], it was demonstrated that CD8+ T cells expressing KLRG1 have a higher capability to produce TNF-α and granzyme B. moreover, within a study by Ouyang et al.[450], it has been reported that KLRG1+ CD8+ T cells tended to possess higher frequencies of cells capable of producing IFN-γ. Therefore, it would be reasonable to conclude that the higher expression of KLRG1 and lower CD127 may be indicative of a higher functionality within the IAV-specific CD8+ T cells. Thirdly, the exhausted phenotype is indicative of higher activation and function of the IAV-specific memory CD8+ T cells. As previously discussed, exhaustion markers could be used as a marker for higher activation and functionality within CD8+ T cells. One of the first signs of exhaustion is the downregulation of IL-2[371], which would be reflected within the single-expression and co-expression of IL-2. Within my data, there was no differences in the expression of IL-2, in terms of co- and single-expression, which might indicate that exhaustion hasn't set in yet. Taken together, it can be strongly suggested that IAV-specific CD8+ T cells at the peak memory CD8+ T cell response during protracted sepsis are more functionally active compared to sham.

4.1.4 IAV-specific CD8+ T cell responses within the PECs

Up to now, the discussion has been focused on my data from the spleen; I will next discuss the other tissues that I have examined. Compared to all other tissues, the PECs differed the most in their IAV-specific memory CD8+ T cell responses. In my data, I demonstrated that IAV-specific CD8+ T cells within the septic PECs may be hampered in their responses upon re-encountering the cognate antigen during sepsis-induced immunosuppression.

In terms of their frequencies and numbers, I discovered that there was a reduced frequency of NP_{366}-specific CD8+ T cells as measured by tetramer reagent, with an associated trend towards lower absolute numbers, albeit there was a high variation within the data, which may suggest that their absolute numbers are in fact similar compared to sham. Furthermore, from the ICS data, IAV-specific CD8+ T cells demonstrated a trend towards reduced frequencies of cells capable of producing IFN-γ under the stimulation with cognate peptides. This information may suggest that within the septic peritoneal cavity, IAV-specific CD8+ T cells have a dampened ability to expand in the context of sepsis-induced immunosuppression during a recall response.

Currently, within the literature, there are no studies that address antigen specific memory CD8+ T cell responses during a recall response within the PECs in the context of protracted sepsis. However, there has been one study by Duong et al.[275], which examined the numbers of pre-existing antigen specific memory CD8+ T cells within the PECs five days post-CLP. It was discovered that there was a trend towards higher absolute numbers of antigen specific memory CD8+ T cells within the PECs, although not significant. This information suggests that during sepsis, there is an enhanced proliferation or migration from antigen specific memory CD8+ T cells within the PECs. Furthermore, within a study by Ding et al.[253], the authors assessed *ex vivo* proliferation of antigen specific T cells (clone D10.G4.1 – CD4+ T cell) in the presence of septic peritoneal cavity dendritic cells. To perform this experiment, the researchers performed CLP and sham surgery, then 24 hours post-surgery isolated dendritic cells from the PECs, in which the authors had then co-cultured dendritic cells with antigen specific T cells in the presence or absence of antigen. Interestingly, the

authors discovered that the septic dendritic cells lead to higher amounts of T cell proliferation compared to sham dendritic cells, regardless of antigen presence. This information suggests that the increased proliferation seen in the septic condition is due mainly to antigen-independent mechanisms. Furthermore, within the septic condition, antigenic proliferation may be dampened as this group has also shown that immature dendritic cells within the PECs were severely depleted 24 hours post-CLP. This information suggests that during the acute phase of sepsis, antigen specific memory CD8+ T cells may enhance their numbers through antigen-independent mechanisms; however, during recall response reactions in the immunosuppressive environment, they may be dampened in their ability to participate in antigenic proliferation as there is a severe depletion of immature dendritic cells[131,253].

Another potential reason for the reduced proliferative capacity of antigen specific memory CD8+ T cells within the PECs during immunosuppression is the presence of different immunosuppressive cell types. For instance, it has been demonstrated by Molinaro et al.[474], within the PECs 24 hours post-CLP, there was a three- and four-fold increase in the frequency and absolute numbers of CD4+ Treg cells, respectively. Furthermore, it has been demonstrated by Haeryfar et al.[469] that CD4+ Treg cells have the capability to dampen antigenic proliferation of antigen specific CD8+ T cells. This information together suggests that CD4+ Treg cells may have a severe impact on the IAV-specific CD8+ T cell expansion within the septic PECs.

Furthermore, another potential reason for reduced proliferative capacity of septic IAV-specific CD8+ T cells within the PECs is the consistent release of microbial species. It has been shown by Xiao et al.[454] that within the peritoneal cavity during the immunosuppressive phase of sepsis, there is a failure to clear bacterial species. The authors determined that the main source of bacteria within the peritoneal cavity is due to the leakage of microbial species from the abscess. During the CLP procedure, the immune system will form an abscess at the location of the puncture, which allows the local control of the septic focus[400,475]. Furthermore, Xiao et al.[454] demonstrated that cecal resection performed four days post-CLP lead to reduced abscess formation, mortality and virtually no bacterial

presence within the peritoneal cavity. This information strongly suggests that the abscess formation contributes heavily to late mortality within the CLP model of polymicrobial sepsis. In terms of memory CD8+ T cell responses, this information is extremely important. During the course of sepsis, there will be continuous leakage of antigen from the abscess, which has the capability to induce IAV-specific CD8+ T cell exhaustion and loss[476–479]. This could explain the reduced secondary expansion; however, this is speculative.

Next, I wanted to phenotype and functionally assess the IAV-specific memory CD8+ T cells within the PECs at the peak memory CD8+ T cell response during sepsis-induced immunosuppression. In terms of the phenotype, it was demonstrated that there were no differences in the expression patterns of the different exhaustion markers examined compared to sham. This may suggest that IAV-specific CD8+ T cells within the septic may not be different in their exhaustion and activation status; however, these conclusions are not definitive as the number of replicates is too low to state anything with certainty. In terms of functionality, I discovered that there was a reduced frequency, absolute number, and per-cell expression of cells capable of producing IFN-γ, which would indicate that these cells are less capable of producing IFN-γ under the stimulation with their cognate peptides. Within the previous paragraph, I discussed that the PECs after sepsis would be severely immunosuppressive, and this could have an effect on IAV-specific CD8+ T cell functionality. For instance, the increased presence of CD4+ Treg cells within the PECs could negatively regulate their cytokine productive capabilities as well as their cytolytic effector functions[474,480]. Furthermore, the continuous leakage of antigen and inflammatory stimuli may lead to IAV-specific CD8+ T cell exhaustion and loss, which would affect their functionality as they would be unable to produce a response[424,476–479,481,482].

In addition, I had also wanted to examine the non-IAV-specific CD8+ T cell populations within the PECs at the peak memory CD8+ T cell response during protracted sepsis to obtain a better understanding on the effect that sepsis has on the whole CD8+ T cell compartment. Herein, I demonstrated that non-IAV-specific CD8+ T cells show an upward trend in BTLA expression in terms of frequency and per-cell expression in the CLP condition. Furthermore, non-IAV-specific CD8+ T cells within the septic PECs had reduced frequencies of cells expressing PD-1, while displaying reduced co-expression of the different exhaustion markers examined. Reflecting on these results, it could be suggested that non-IAV-specific CD8+ T cells are less phenotypically exhausted and activated compared to sham. As previously discussed, the presence of exhaustion markers could indicate activation[418,435,436,457,483,484]; however, in terms of BTLA expression, increased BTLA expression on non-IAV-specific CD8+ T cells may indicate reduced ability to respond and decreased activation. In a study Otsuki et al.[434], it was discovered that BTLA is constitutively expressed on memory CD8+ T cells, which subsequently gets downregulated upon T cell activation. Furthermore, it was also demonstrated that decreasing BTLA expression paralleled increasing cell division and IFN-γ production, which is indicative of T cell activation. Taken together, I may suggest that during recall responses within the septic PECs, non-IAV-specific CD8+ T cells appear to be less activated and able to respond to pathogenic encounter. However, I would need to increase the number of replicates as two replicates is too low to make any firm conclusions; additionally, I would need to perform more extensive immunophenotyping experiments and functional assays to be able to conclude that they are less activated and functional.

4.1.5 IAV-specific CD8+ T cell responses within the lungs

Next, I will further discuss the antigen specific memory CD8+ T cells responses within the lungs at the peak memory CD8+ T cell response during protracted sepsis. Within the septic lungs, I discovered that IAV-specific CD8+ T cells demonstrated a trend towards higher frequencies and absolute numbers. There are a few potential reasons for their increased presence within the lungs: firstly, there could be an increased migration of IAV-specific CD8+ T cells into the lungs; secondly, there could potentially be an increased *in situ* proliferation of these cells. The first reason is the most rational explanation as it has been demonstrated by multiple studies[485–487] that memory CD8+ T cells present within the lungs do not proliferate *in situ*; however, these cells still maintain their functionality in response to cognate antigen as they are robust producers of different cytokines and cytolytic effector molecules, and maintain their cytolytic activity. Despite their lack of *in situ* proliferation within the lungs, this property is not due to a T cell intrinsic defect[485,486]. It has been displayed that isolating antigen specific memory CD8+ T cells from the lungs and culturing them *ex vivo* with their cognate peptides and IL-2 led to significant proliferation. This suggests that antigen specific memory CD8+ T cells within the lungs maintain their proliferative capacity; however, within this environment, it renders them unable to proliferate. Now, I will further expand on the increased migratory abilities of the IAV-specific CD8+ T cells, but I first must discuss some key pieces of information. Within the literature, multiples researchers have demonstrated that both naïve and central memory CD8+ T cells continuously circulate through the blood and secondary lymphoid tissues, while effector and effector memory CD8+ T cells preferentially migrate to peripheral tissues[352,431,488,489]. Within my data, I discovered that there was a higher frequency of effector memory CD8+ T cells within the septic spleen, which are heavily composed of the IAV-specific CD8+ T cells of interest. Moreover, the spleen is considered to be the site of systemic immune response. From all this information, I may state that due to the higher presence of effector memory CD8+ T cells within the septic spleen, there will be a higher migrative potential of both effector memory and IAV-specific CD8+ T cells to the lungs. Despite these findings and hypothesis, I would need to increase the number of replicates to make firm conclusions within this tissue.

In addition to examining IAV-specific CD8+ T cell frequencies and numbers, I had also performed immunophenotyping of these cells. IAV-specific CD8+ T cells displayed a trend towards reduced per-cell expression of BTLA. As previously discussed, BTLA downregulation occurs during T cell activation[434]; therefore, the IAV-specific CD8+ T cells of interest may be more phenotypically activated compared to sham. However, this is not conclusive as I would need to increase the number of replicates and perform more extensive immunophenotyping to be able to provide a firm conclusion.

In terms of IAV-specific CD8+ T cell functionality, I had not performed any functional assays; however, I can speculate that these cells would have an enhanced functionality. Within a study by Harris et al.[486], it was discovered that antigen specific effector CD8+ T cells within the lungs, which originated from the circulation, had a similar functionality in terms of IFN-γ production compared to circulatory and splenic antigen specific effector CD8+ T cells. Moreover, in a paper by Marshall et al.[490], it was demonstrated that during a recall response to an IAV respiratory infection, IAV-specific CD8+ T cells within the lungs had a higher capacity to produce inflammatory cytokines and possessed a greater cytolytic ability compared to splenic IAV-specific CD8+ T cells. Similar to the previous study, it was discovered by Masopust et al.[488] that lung antigen specific memory CD8+ T cells, which had migrated from the secondary lymphoid tissues, had an increased cytolytic ability *ex vivo* compared to splenic antigen specific memory CD8+ T cells. Despite the aforementioned studies not examining antigen specific memory CD8+ T cell responses in the context of sepsis-induced immunosuppression, the information is still applicable to my research. Within my research, I discovered that septic splenic IAV-specific CD8+ T cells had an increased functionality as demonstrated by the increased expression of both IFN-γ and granzyme B. Taken together, it would be reasonable to hypothesis that lung IAV-specific CD8+ T cells would have an enhanced functionality compared to their sham counterpart; however, these cells may also be more functionally capable compared to septic splenic IAV-specific CD8+ T cells. This hypothesis is speculative, and I would need to perform functionally assays within the lungs to confirm or deny this thought.

Within the lungs, I had also wanted to examine the different memory CD8+ T cell populations. I discovered in the septic lungs that there is a higher frequency and absolute number of activated CD8+ T cells as measured by the expression of CD44. Furthermore, I also demonstrated that there was a phenotypic shift in frequency from central memory to effector memory CD8+ T cells, which was due to the numerical increase of effector memory CD8+ T cells. These findings further support the notion that there is an increased recruitment of effector memory CD8+ T cells within the septic lungs, which would be majorly composed by the IAV-specific CD8+ T cells of interest. Furthermore, it also provides further evidence that there is a higher activation and functionality within the septic lungs as there is a higher presence of effector memory CD8+ T cells, which are highly functional cells[431]. In terms of their phenotype, I demonstrated that effector memory CD8+ T cells within the septic lungs had a reduced frequency of cells expressing BTLA, which has been previously discussed to be downregulated during T cell activation[434]. Hence, it would be appropriate to suggest that effector memory CD8+ T cells, which are mostly composed of IAV-specific CD8+ T cells, are higher in presence within the septic lungs, while demonstrated an increased activation and functionality. However, despite the evidence pointing towards higher activation and function, I would need to perform more extensive immunophenotyping and functional assays to confirm this hypothesis.

4.1.6 IAV-specific CD8+ T cell responses within the liver

In addition to the lungs, I have also decided to examine both septic liver IAV-specific CD8+ T cell and memory CD8+ T cell numbers and frequencies at the peak memory CD8+ T cell response during protracted sepsis. I discovered that septic liver IAV-specific CD8+ T cells were higher in frequency and absolute numbers compared to sham. Furthermore, within the septic liver, I had also demonstrated that there is a shift in frequency from central memory to effector memory CD8+ T cells due to the numerical loss of central memory CD8+ T cells and the numerical gain of effector memory CD8+ T cells. Compared to the lungs, the potential reasons for the increased presence of both IAV-specific and effector memory CD8+ T cells within the septic liver is fuzzier due to liver biology. Firstly, both cell types may have an increased migration to the liver. Secondly, the increase in presence could be attributed to a higher *in situ* proliferation.

In order to discuss the potential reasons for the increased presence of IAV-specific and effector memory CD8+ T cell within the septic liver, I will provide necessary background information in a step-wise approach, beginning from the initial memory CD8+ T cell generation to the final assessment of memory CD8+ T cell responses at the peak memory CD8+ T cell response.

To begin, I will first discuss the ability of primary activated CD8+ T cells to migrate to and take residence within the liver. In a study by Masopust et al.[491], it was discovered that primary activated antigen specific CD8+ T cells migrated to multiple different nonlymphoid tissues, such as the lungs and liver, amongst others, which occurred irrespective of the location of antigen encounter. In the context of my research, this information demonstrates that *in vivo* immunization/infection leads to primary activated antigen specific CD8+ T cells that have the ability to home to the liver, which may or may not become tissue resident antigen specific memory CD8+ T cells[492]. Furthermore, within a paper by Su et al.[493], it was discovered that adoptively transferring activated antigen specific CD8+ T cells into naïve mice led to a high proportion of different antigen specific memory CD8+ T cell subsets among all CD8+ T cells within the liver. Moreover, it was

also demonstrated that these different antigen specific memory CD8+ T cell subsets were maintained within the liver for 150 days post-transfer; notably, the liver had the highest proportion of these cells compared to every other tissue examined (bone-marrow, spleen, lymph nodes). Additionally, Within a study by Holz et al.[492], it was discovered that adoptively transferring activated antigen specific CD8+ T cells led to an increased memory CD8+ T cell population within the liver, such as central, effector and tissue resident memory CD8+ T cells. In terms of absolute numbers, there were similar numbers of both central and tissue resident memory CD8+ T cells; however, effector memory CD8+ T cells were 2-fold less compared to the other two memory CD8+ T cell populations. Furthermore, the authors had also revealed that TCR stimulation, which requires antigen, was required for the formation of liver tissue resident memory CD8+ T cells during the activation of naïve CD8+ T cells. From this information, I may state that within the liver of the mice under investigation, there will be a prominent presence of different IAV-specific memory CD8+ T cell subsets, which are maintained during the 2-month period after PR8 priming. Furthermore, considering that both tissue resident and effector memory CD8+ T cells are phenotypically characterized as $CD44^{HIGH}$ CD62L- (tissue resident memory CD8+ T cells are $CD44^{HI}$ CD62L- CD69+), there will be a mixture of these two cell types within the liver of pre-sepsis mice; however, tissue resident IAV-specific memory CD8+ T cells will approximately account for two thirds of the $CD44^{HIGH}$ CD62L-CD8+ T cells, while the rest will be effector memory CD8+ T cells.

Thereafter, I will briefly touch upon *in situ* proliferation of these different liver memory CD8+ T cell subsets to maintain and increase their numbers within the liver. Within a study by Su et al.[493], the authors had separately activated *in vitro* Vβ5+ and Vβ8+ CD8+ T cells, then subsequently mixed them in equal numbers and adoptively transferred them into naïve mice and examined their numbers within the liver seven and 60 days post-transfer. It was discovered at day seven post-transfer, there were 13 clusters of tissue resident memory CD8+ T cells, with six of them being exclusively constituted by Vβ5+ CD8+ T cells, while six other clusters were exclusively constituted by Vβ8+ CD8+ T cells. Furthermore, 60 days post-transfer demonstrated 20 clusters of tissue resident memory CD8+ T cells, with half of them being exclusively Vβ5+ CD8+ T cells and the other half being exclusively

Vβ8+ CD8+ T cells. This information suggests that specific CD8+ T cell clonotypes form clusters within the liver, which do not intermix. Furthermore, this information also suggests that the liver enhances the numbers of tissue resident memory CD8+ T cells through *in situ* clonal expansion of single cells.

Taken together, during the initial PR8 priming, different IAV-specific memory CD8+ T cell populations will arise and migrate to the liver, which will either take residence or not. The cells that take residence within the liver will clonally expand during the two-month period leading to their enhanced numbers.

Next, I will further discuss how sepsis affects the different IAV-specific memory CD8+ T cell subsets generated during PR8 priming. Within a study by Sharma et al.[255], it was discovered that 20 hours post-CLP, hepatic CD8+ T cells significantly increased in terms of frequency and absolute numbers compared to sham, 1.8- and 1.9-fold increase, respectively. Furthermore, within a different study by Soldato et al.[494], the authors had demonstrated that there was a 2-fold increase in the frequency and absolute numbers of CD8+ T cells 24 hours post-CLP compared to sham, which is consistent with the previous study. Despite these important findings, currently within the literature, there are not many studies in which examine the frequencies and absolute numbers of the different CD8+ T cell subsets during the course of sepsis within the liver. Presently, to my knowledge, there is only one study that examines hepatic memory CD8+ T cell numbers at multiple time points after sepsis. In a study by Taylor et al.[395], it was discovered that septic hepatic memory CD8+ T cells were numerically reduced during a 72 hour period post-CLP; the most pronounced loss occurs at 48 hours post-CLP, while at 72 hours post-CLP, the numbers started to recover. At first glance, this study appears to be contradictory to the aforementioned studies; however, these studies are not comparable as Taylor et al.[395] had not used an appropriate control, namely a sham procedure. In determining memory CD8+ T cell numbers, Taylor et al.[395] had statistical compared septic mice to mice that had not undergone any surgery, which is problematic as mice undergoing sham surgery experience a reduction in CD8+ T cell numbers, albeit not to the same degree as sepsis[273]. Taken together, from the information presented, it would be reasonable to suggest that during the

early phase of sepsis, there will be an increased presence of CD8+ T cells, which may consist of the different IAV-specific memory CD8+ T cell subsets. To further characterize and explain this increased frequency and number of CD8+ T cells during early septic events, I must turn the attention to the biology of the liver. The liver is a special organ as it anatomical has two distinct networks of blood supply: hepatic artery, which provides oxygenated blood to the organ; hepatic portal vein, which is nutrient-rich as it directly comes from the blood network of the intestines. This special feature of the liver makes it extremely important to study during abdominal sepsis because there will a constant migration of pathogens and inflammatory stimuli from the gut lumen into the bloodstream of the intestines ultimately ending up in the liver[495]. Of important note, possessing a higher pathogen load within the liver will lead to liver injury and excessive systemic inflammation, which could progress to cirrhosis[495,496]. These pieces of information are tremendously important in providing a possible mechanism for the increased accumulation of CD8+ T cells within the septic liver. Within a study by Holz et al.[492], it was discovered that co-administering activated antigen specific CD8+ T cells with different inflammatory stimuli led to the increased formation of tissue resident antigen specific memory CD8+ T cells within the liver. Despite this study examining the initial generation of tissue resident memory CD8+ T cells, their findings are still applicable as many research groups have demonstrated that memory CD8+ T cells, unlike naïve CD8+ T cells, have the capability to readily get activated under the stimulation with many different combinations of cytokines[497–500]. Moreover, within the study by Holz et al.[492], it was concluded that activated CD8+ T cells can constitutively form tissue resident memory CD8+ T cells within the liver as antigen recognition within the liver is not necessary. Hence, it would be reasonable to suggest that due to sepsis, IAV-specific memory CD8+ T cells will become re-activated, which will then lead to their increased numbers within the liver as tissue resident IAV-specific memory CD8+ T cells. To show further proof of this hypothesis, I will turn the attention to a recently published paper by Lefebvre et al.[501]. The authors had discovered that liver infection caused by multiple different methods (malaria infection, CLP, and *Listeria monocytogenes* infection) led to the rapid infiltration of effector memory CD8+ T cells within the liver, which was antigen- and tissue resident memory T-independent. Taken together, there is strong evidence to suggest that during the early stages

of sepsis, IAV-specific memory CD8+ T cells will become reactivated due to the inflammatory milieu of the host leading to their quick infiltration into the liver and their subsequent enhanced formation of tissue resident IAV-specific memory CD8+ T cells; however, despite all this evidence, I would need to perform more experiments to elucidate whether IAV-specific memory CD8+ T cells after the septic insult are in fact higher in numbers ad frequencies within the liver.

Lastly, I will now discuss the effect of inducing a recall response on the frequencies and numbers of IAV-specific CD8+ T cells within the septic liver at the peak memory CD8+ T cell response. Currently, to my knowledge, there are no studies that have examined peak memory antigen specific CD8+ T cell responses within the septic liver. Hence, our discussion is limited, and the conclusions provided herein are not absolute.

In terms of migration, within a study by Masopust et al.[491], the authors demonstrated that reactivation of central and effector memory CD8+ T cells led to their widespread migration, regardless of the site of infection or tissue origin. This finding in conjunction with the fact that the spleen is responsible for systemic immune responses allows me to make suggestive conclusions utilizing the data I have acquired for the spleen. Within the septic spleen, I discovered that effector memory and IAV-specific CD8+ T cells (mostly classified as effector memory CD8+ T cells) were higher in frequency compared to sham, which may suggest that they have an enhanced capacity to migrate to the liver as effector memory CD8+ T cells preferential migrate to non-lymphoid organs[352,431,488,489]. Furthermore, IAV-specific CD8+ T cells may or may not have an increased capacity to produce tissue resident memory CD8+ T cells. As previously alluded to, activated CD8+ T cells constitutively form tissue resident memory CD8+ T cells as there is no requirement for antigen to be present within liver. From this information, it could be suggested, while taking into consideration that I discovered that septic splenic IAV-specific CD8+ T cells were more functional and phenotypically activated, that septic splenic IAV-specific CD8+ T cells may have an enhanced ability to form tissue resident memory CD8+ T cells as they are more activated and plentiful. However, septic splenic IAV-specific CD8+ T cells may have a reduced capacity to form tissue resident memory CD8+ T cells as they possessed reduced

expression of CD127 with an increased expression of KLRG1, which is a phenotype described as terminal effector memory T cells with a reduced memory formation potential[408,441,446–448]. To provide further evidence of increased migration to the liver, I must discuss the significance of increased microbial presence within the liver. In my experimental design, I induced a recall response by intraperitoneally injecting septic and sham mice with X31 four days post-CLP. It has been shown by Zheng et al.[502] that intraperitoneal injection of murine cytomegalovirus (MCMV) led to viral presence and replication within the liver 5 days post-infection. From this information, it could be suggested that after inducing a recall response, there would be the presence of IAV within the liver. Furthermore, within the septic condition, there may be more microbial presence compared to sham. Within a study by Jensen et al.[194], the authors performed adoptive transfer of P14 memory CD8+ T cells (specific for GP_{33}), originating from either septic or sham mice 30 days post-surgery, into naïve mice, which were then subsequently infected with virulent *Listeria monocytogenes* (*L.m.*) expressing GP_{33}. The researchers discovered that the mice receiving CLP-derived P14 memory CD8+ T cells had a higher bacterial presence within the liver 5 days post-infection. From this information, it could suggested that at the peak recall response within septic mice, there will be a higher viral presence of IAV within the liver, which would potentially lead to effector memory and IAV-specific CD8+ T cells having a higher potential to migrate to the liver[501].

In terms of *in situ* proliferation, there is evidence to suggest that upon migration to the liver, there will be an enhanced ability to proliferate. Within a study by Isogawa et al.[503], the authors had assessed antigen specific memory CD8+ T cell expansion within the liver with a focus on whether their expansion is due to migration or *in situ* proliferation. The authors had utilized a transgenic model of mice that constitutively expressed hepatitis B virus (HBV) within the liver and a non-transgenic model as a control. The researchers had isolated splenic HBV-specific memory CD8+ T cells from a primed mouse, then subsequently labelled them with CFSE and adoptively transferred them into the two different mouse strains, which the authors then assessed their proliferation within the liver at two and a half and five days post-transfer. It was discovered that at two and a half days, there was a small fraction of HBV-specific CD8+ T cells that divided up to five times;

however, at day five post transfer, the vast majority of the cells divided six times, which suggests that there is a strong capability to proliferate *in situ*. Furthermore, the non-transgenic model demonstrated no proliferation, which suggests that proliferation occurred due to the presence of antigen within the liver. As previously discussed, it would be expected within the septic liver, that there would be a higher presence of IAV during recall response, which could potentially lead to higher proliferation of IAV-specific CD8+ T cells within the septic liver. Moreover, within a different study by Su et al.[493], the authors had adoptively transferred activated antigen specific memory CD8+ T cells labelled with CFSE and subsequently examined their proliferation 28 days post-transfer. The researchers demonstrated that compared to the spleen, liver antigen specific memory CD8+ T cells had significantly proliferated more, which suggests that upon entry and residency within the liver, these cells can expand massively in numbers compared to any other tissue. In terms of my research, this information may suggest that upon activation and migration to the liver, IAV-specific CD8+ T cells will have an enhanced proliferation leading to high numbers within the liver.

Another important point to address is the tissue residency capability of the liver to maintain the numbers of memory CD8+ T cells. In the study by Holz et al.[492], it was demonstrated within the liver that there is no competition for the niche and successive immunizations/infections led to the addition of these cells to the liver niche. Hence, it may be suggested that during multiple rounds of infection/immunization, the formation of new liver tissue resident memory CD8+ T cells will simply just add to the pre-existing.

Next, I will briefly discuss the phenotypic differences within the IAV-specific memory CD8+ T cell population, non-IAV-specific CD8+ T cell populations, and the different memory CD8+ T cell subsets within the septic liver during the peak memory CD8+ T cell response. In terms of IAV-specific CD8+ T cells, NP_{366}-specific CD8+ T cells displayed an upward trend for the expression of PD-1, which may indicate that these cells are more activated and exhausted. PA_{224}-specific CD8+ T cells demonstrated a phenotype that was associated with reduced exhaustion and activation. In terms of non-IAV-specific CD8+ T cells, it was demonstrated that these cells have reduced frequencies and per-cell expression of BTLA, which may indicate that these cells are more activated[434,504,505]. In terms of the different memory CD8+ T cell populations, both central and effector memory CD8+ T cells demonstrated a reduced BTLA expression, which might suggest that they are more activated compared to their sham counterpart. Despite these findings, due to the small number of replicates of the immunophenotyping experiments and no functional experiments performed, it is difficult to make any discernable conclusions regarding their activation and function. Further experiments will need to be performed to make any definite conclusions.

4.1.7 Potential contributions of memory CD8+ T cell responses to organ dysfunction during secondary infection after the septic insult

Another interesting point that needs to be discussed, in my data, I discovered that there was a higher presence of effector and effector memory CD8+ T cells within the septic lungs and liver of memory mice, which was not present within the naïve mice. This could be very significant in explaining the mortality discrepancies between memory and naïve mice. It has been demonstrated within the literature that compared to naïve mice, mice possessing a good memory population are more susceptible to sepsis-induced mortality[274,395,396]. For example, in a paper by Taylor et al.[395], the authors demonstrated that memory mice compared to their naive counterpart had a worse five-day survival after CLP. Furthermore, the researchers had also assessed liver organ dysfunction by standard measure of liver injury (transaminases, bilirubin), and examined the expression of genes encoding the sodium/bile acid co-transporter (*SLC10a1a*) and the organic anion transporter protein 1 (*SCLO1a1*). It was discovered that memory mice had higher serum levels of both alanine aminotransferase and bilirubin, and had decreases in both SLC1Oa1a and SCLO1a1, which indicates a higher level of organ dysfunction. Corroborating these findings, Huggins et al.[396] demonstrated that mice occupied with increased microbial species leading to a prominent memory T cell pool led to increased mortality within a CLP mouse model of polymicrobial sepsis compared to pathogen free mice. Furthermore, the authors had also discovered that four days post-CLP, when immunosuppression has set in, microbial exposed mice had increased mortality after four days post-CLP compared to pathogen free mice that did not experience any mortality after four days. Moreover, in a paper by Xie et al.[274], the researchers discovered that memory mice after four days post-CLP had a stronger trend towards mortality compared to naïve septic mice, although not significant. All this information suggests that memory CD8+ T cells during sepsis may exacerbate septic mortality and organ injury during both the initial and later stages of sepsis. In terms of my research, there was a greater presence of IAV-specific CD8+ T cells within the septic lungs and liver, which was not present in naïve mice. This finding could potentially explain how memory CD8+ T cells could be contributing to mortality and organ damage during septic secondary infections. However, currently, it is not well known how memory CD8+ T cell responses during a secondary infection contribute to mortality and organ dysfunction

during sepsis induced immunosuppression. This is the first report suggesting that antigen specific memory CD8+ T cell responses may be pathogenic in the context of sepsis-induced immunosuppression during a secondary infection. Further evidence for this comes from studies demonstrating that activated antigen specific CD8+ T cells have the capability to cause tissue pathology[376]. Hence, it would be reasonable to suggest that IAV-specific memory CD8+ T cells during a secondary infection in the context of sepsis-induced immunosuppression may be responsible for enhanced tissue pathology and mortality; however, more experiments are necessary to further interrogate this hypothesis.

In the next paragraphs, I will discuss potential new avenues for sepsis treatment, which is focused on (1) secondary infections occurring after sepsis (2) utilizing the unique marker that I potentially elucidated (2B4) and (3) memory antigen specific CD8+ T cell responses. I propose herein that 2B4 could be a very important therapeutic target for treating sepsis-associated secondary infections. Now, I will further expand and provide my rationales for this proposal.

Firstly, 2B4 has been found to be more upregulated within septic patients and mouse models of sepsis, which has been described by me and others[273,274]. Secondly, 2B4 is preferentially upregulated on memory CD8+ T cells, which leads to a higher activation of caspase-3/7 and a more profound CD8+ T cell loss during early sepsis[274]. Thirdly, it has been shown that 2B4 is preferentially upregulated on antigen experienced and antigen specific memory CD8+ T cells during acute sepsis[285]. Although these studies have focused on the initial phase of sepsis, they are still important in demonstrating that 2B4 is preferentially upregulated on antigen experienced and antigen specific memory CD8+ T cells during sepsis. Fourthly, within my own data, I demonstrated that 2B4 upregulation may be unique to antigen specific memory CD8+ T cells during recall responses in the context of sepsis induced immunosuppression. From these four points of information, there is strong evidence to suggest that 2B4 expression could be unique to antigen specific memory CD8+ T cells during a secondary infection after sepsis.

Now, I will further expand on why 2B4 could potentially be an important therapeutic target during secondary infections in the context of sepsis-induced immunosuppression by focusing on the increased presence of antigen specific CD8+ T cells within the different tissues. Firstly, educated mice in three studies demonstrated increased mortality compared to uneducated mice[274,395,396], which points to memory CD8+ T cells contributing to sepsis-induced mortality. Furthermore, two of the three studies have demonstrated that memory septic mice had an increased mortality rate after the four-day time-point post-CLP compared to naïve mice , which suggests that memory CD8+ T cells are potential contributors to mortality during immunosuppression. Secondly, educated mice show higher levels of organ dysfunction compared to uneducated mice, which strongly suggests that memory CD8+ T cells contributes to tissue pathology during sepsis. Thirdly, in my own research, I demonstrated that upon recall response during the immunosuppressive phase of sepsis, there is a higher presence of antigen specific memory CD8+ T cells within the lungs and liver, which constitutes the majority of the activated CD8+ T cells seen in these tissues. Despite the previous studies describing memory CD8+ T cells contributing to mortality and tissue injury without antigen re-encounter, and my work focusing on their responses during recall responses, the information is still translatable because memory CD8+ T cells may be pathogenic and contribute to tissue pathology and mortality during infection, which has been shown by multiple groups examining IAV infections[381–385]. From this information, it is reasonable to suggest that during a recall response to a secondary infection in the context of sepsis-induced immunosuppression, antigen specific memory CD8+ T cells may be contributing heavily to mortality and organ damage. Fourthly, comparing my results from the primary and recall response experiments, I noticed within the primary response, there was no notable differences in the frequency and absolute numbers of either activated and antigen specific CD8+ T cells within the septic lungs and liver. This finding signifies that within the primary response, there are less activated antigen specific CD8+ T cells within the tissues to cause damage, which could potentially explain the differences in mortality and organ injury in educated and uneducated mice. Fifthly, upon recall response, I described a shift from central memory CD8+ T cells to effector memory CD8+ T cells leading to more activated memory CD8+ T cells present within different tissues. These cells would be capable of mediating organ damage during the response to secondary

infections. Furthermore, within the spleen, it was demonstrated that memory CD8+ T cells were more functionally active as measured by their increased intracellular expression of IFN-γ and granzyme B. Despite the lack of functionality experiments within the lungs and liver, it is reasonable to suggest that antigen specific memory CD8+ T cells are more active in their cytokine productive capabilities as the spleen is a site for systemic responses, so they could migrate from the spleen and circulatory system into these different tissues. In terms of 2B4, specifically targeting 2B4 during secondary infections after sepsis could dampen the excessive responses from antigen specific memory CD8+ T cells, which could aid in preventing mortality and organ dysfunction.

From all these observations and rationales, I hypothesis that during a secondary infection in the context of sepsis-induced immunosuppression, antigen specific memory CD8+ T cells will respond in greater quantity and magnitude leading to a more pronounced organ dysfunction and mortality within septic subjects. Moreover, 2B4 could potentially be an immunotherapeutic target to specifically target antigen specific memory CD8+ T cells responding to the secondary infection as I have reported in my research that 2B4 may be uniquely upregulated on these antigen specific memory CD8+ T cells during recall response. This proposed hypothesis, currently contrasts what is commonly thought within the literature. The current knowledge proposes that mortality and morbidity from secondary infections within the immunosuppressive environment of sepsis is due to the host not being able to respond to these infections; however, I believe that the organ damage and mortality associated with secondary infections may be due to an increased response within host. This is speculative, more experiments are required to either confirm or deny this hypothesis.

4.2 Discussion for the Primary Response

Table 6. Summary table for the findings of aim 2. Note: the blacked-out boxes signify that this data is not available. N/A = not available, (+ or –) indicate significant results with more of these symbols meaning higher significance (example: ++++ = ****p<0.0001), ~ = trend (either in the + or – direction). Important note: double-expressors for the different exhaustion marker combinations was not included.

				Exhaustion markers (Frequency/gMFI)									Other markers (Frequency/gMFI)					Functionality markers (Frequency/gMFI or MFI)				
Tissue	Cell type	Frequency (Tetramer/ ICS)	Absolute numbers (Tetramer/ ICS)	PD-1	BTLA	TIM-3	CTLA-4	TIGIT	LAG-3	2B4	VISTA	KLRG1	CD25	CD69	CD95	CD127	CD44	IFN-γ	TNF-α	IL-2	Granzyme B	IFN-γ, TNF-α, IL-2
Spleen	NP_{366}	+++/++++	0/+++	0/0	0/0	0/0	0/0	+/0	0/0	++/0	0/+	++++/0	0/0	0/0	0/0	~-/0	-/~-	++++/++	0/~+	0/+	++++/0	~-
	PA_{224}	~+/+	0/+	~-/0	0/0	0/0	0/0	~+/0	0/0	+++/0	0/0	++++/0	0/0	0/0	0/0	- - - -/0	-/~-	+/+	0/~+	0/+	++++/0	~-
	Non-NP_{366}			~+/0	0/0	~+/0	~+/0	~+/0	0/0	~+/~-	0/~+		0/0	0/-	0/0	- -/0	+++/0					
	Non-PA_{224}			~+/0	0/0	~+/0	0/0	~+/0	0/0	~+/~-	0/0		0/0	0/-	0/0	- - -/0	+++/0					
	Central memory	- - -/N/A	- - - -/N/A	0/0	+++/0	0/0	0/0	0/0	0/0	~+/0	-/0		0/0	0/0	0/0	0/0						
	Effector memory	+++/N/A	0/N/A	~+/0	0/0	0/0	0/0	~+/0	0/0	+/0	0/0		0/0	~-/0	0/0	- - -/0						
PECs	NP_{366}	0/~+	~-/~+	- -/~-	-/+++	-/~+	0/~+											~+/-				
	PA_{224}	0/0	~-/0	-/0	- - /+	~-/~+	0/0											0/- -				
	Non-NP_{366}			~-/~+	0/0	0/+	0/+															
	Non-PA_{224}			~-/0	0/0	0/0	0/+															
	Central memory																					
	Effector memory																					
Lung	NP_{366}	0/0	0/0					0/0	0/0	~+/0	0/~+	~+/0						0/~+	0/~+	0/0	~+/0	~-
	PA_{224}	0/0	0/0					~+/0	0/0	~+/0	0/~+	~+/0						0/~+	0/~+	0/0	~+/0	~+
	Non-NP_{366}							0/0	0/0	~+/0	~+/~+											
	Non-PA_{224}							0/0	0/0	~+/0	~+/~+											
	Central memory	0/N/A	0/N/A					0/0	0/0	0/0	0/0											
	Effector memory	0/N/A	0/N/A					0/0	0/0	~+/~+	0/~+											
Liver	NP_{366}	0/0	~+/0					~+/0	~+/0	~+/0	~+/~+	+/0						0/~+	0/0	0/0	0/0	~-
	PA_{224}	0/~+	0/0					0/0	0/0	0/0	0/~+	+/0						~+/~+	0/0	0/0	0/0	-
	Non-NP_{366}							0/0	~+/0	~+/-	~+/~+											
	Non-PA_{224}							0/0	0/0	0/0	~+/~+											
	Central memory	0/N/A	~+/N/A					0/0	0/0	~- / ~+	~+/~+											
	Effector memory	0/N/A	~+/N/A					0/0	0/0	~- /~+	0/~+											

4.2.1 IAV-specific CD8+ T cell numbers and frequency within the spleen

To begin, I will discuss the presence of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response during sepsis-induced immunosuppression within the spleen. Through my tetramer reagent staining experiments, I discovered that IAV-specific CD8+ T cells within the septic spleen were higher in frequency; however, they were numerically similar compared to sham. Furthermore, I had also assessed the frequency and absolute numbers of IAV-specific CD8+ T cells capable of producing IFN-γ under the stimulation with their cognate peptides and discovered that these cells were higher in frequency and absolute numbers within the spleen of septic mice. In addition, I complemented these experiments by examining the different memory CD8+ T cell subsets, which would enhance our understanding of this difference but would also provide a more complete understanding of the effects sepsis has on the whole CD8+ T cell compartment. Even though IAV-specific CD8+ T cells during the primary response are not memory CD8+ T cells, I can still make comparisons with the effector memory CD8+ T cell subset as my definition (CD44+ CD62L-) encompasses effector CD8+ T cells, which consists of the IAV-specific CD8+ T cells of interest. It was discovered that there is a higher frequency of activated CD8+ T cells within the septic spleen as measured by the expression of CD44; however, they are numerical decreased within the septic condition. Moreover, there was a frequency shift from central to effector memory CD8+ T cells due to the decreased absolute numbers of central memory CD8+ T cells, which has also been described within the recall response experiments. Taken together, in terms of IAV-specific CD8+ T cells, their increased presence may be due to (1) an increased proliferative capacity or (2) a resistance to sepsis-induced apoptosis. Furthermore, in terms of memory CD8+ T cells, there may be (1) an increased phenotype switch to effector memory CD8+ T cells, (2) a resistance to sepsis-induced apoptosis of effector memory CD8+ T cells, (3) an increased susceptibility to sepsis-induced apoptosis within central memory CD8+ T cells (4) an increased proliferative capacity.

I will first touch upon the potential reasons for the increased presence of IAV-specific CD8+ T cells within the septic spleen after sepsis insult, but prior to inducing a primary response. I will begin by discussing the potential for increased resistance to sepsis-induced apoptosis. Within a study by Jensen et al.[194], the authors utilized a CLP mouse model of polymicrobial sepsis and assessed naïve CD8+ T cell numbers within the blood. It was discovered that naïve CD8+ T cells two days post-CLP had significantly reduced numbers within the septic blood, which has been corroborated within the spleen[274]. Moreover, in a study by Condotta et al.[180], it was demonstrated that two and 30 days post-CLP, naïve antigen specific CD8+ T cells were numerically reduced within the septic spleen. From this information, it is reasonable to assume that naïve IAV-specific CD8+ T cells after the septic insult would not possess an enhanced resistance to sepsis-induced apoptosis, which indicates that the most likely reason for their increased presence at the peak primary CD8+ T cell response within the septic spleen is due to their increased proliferative capacity. In a paper by Jensen et al.[194], the authors discovered that naïve CD8+ T cells had an increased proliferative capacity within the septic condition. Within human septic blood specimens at 24 hours after admission to the ICU, naïve CD8+ T cells had a higher intracellular expression of Ki-67 compared to control patients, which is indicative of a higher proliferative potential. Furthermore, within a study by Serbanescu et al.[273], it was demonstrated within a CLP model, two days post-CLP, splenic naïve CD8+ T cells had a more profound numerical reduction compared to sham; however, three and five days post-CLP displayed an enhanced number of splenic naïve CD8+ T cells compared to sham. These pieces of information suggest that naïve CD8+ T cells after the septic insult, after the most pronounced loss of CD8+ T cells, have an enhanced proliferative capacity, which enables them to recover their numbers.

Next, I will further expand on the increased presence of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response within the septic spleen. Prior to discussing the IAV-specific CD8+ T cell expansion in response to cognate antigen, I must first expand on one of the important factors that influences the magnitude of the primary CD8+ T cell. It has been demonstrated by a few studies that the magnitude of the primary CD8+ T cell response depends heavily on the size of the naïve antigen specific CD8+ T cell precursor pool at the

time of antigen encounter[397,398]. Furthermore, as previously mentioned, in a study by Serbanescu et al.[273], it was discovered that between three and five days post-CLP, there was a higher number of splenic naïve CD8+ T cells within the septic condition. Hence, it would be reasonable to suggest that my naïve IAV-specific CD8+ T cells at the time of pathogen administration may be numerically higher compared to sham, which would indicate that they might have a higher magnitude of response.

Now, I will touch more upon the expansion of naïve IAV-specific CD8+ T cells at the peak primary CD8+ T cell response; however, I will first briefly discuss what has been shown within the literature. For the focus of the discussion, I will only provide highlights of the research as I have extensively reviewed them within the introduction of this thesis (section 1.2.3); additionally, I will only focus on the studies that have examined antigen specific CD8+ T cell numbers and frequencies through tetramer reagent staining as examining IFN-γ productive capability relies on functionality of these cells. In a study by Condotta et al.[180], the researchers induced a primary response 30 days post-CLP and examined antigen specific CD8+ T cell expansion at the peak primary CD8+ T cell response. It was demonstrated within the septic blood, that there was a reduced frequency and absolute number of antigen specific CD8+ T cells. In a different study by Condotta et al.[267], the authors examined peak primary antigen specific CD8+ T cell responses at multiple time-points after sepsis induction within the spleen (two, nine and 29 days post-CLP) and discovered that at each time-point, there were similar frequencies of antigen specific CD8+ T cells compared to sham. From this information, it appears that naïve antigen specific CD8+ T cells may not be affected in their ability to expand upon antigen encounter; however, this is at odds with my research as I have demonstrated that they have an enhanced capability to expand. One potential reason for this difference could be attributed to the route of administration. Within their experiments, they had infected these mice by intravenous administration, while I have by intraperitoneal administration. It has been demonstrated that different routes of infection/immunization affect the magnitude of the primary CD8+ T cell response[506]. During intravenous administration, naïve CD8+ T cells will be primed within the spleen; however, for intraperitoneal administration, naïve CD8+ T cells will be primed within the draining lymph nodes of the gut. This could potentially

be explaining the differences in the magnitude as the gut is the location of the initial septic event. It would be expected that microbial antigens will leak into the peritoneal cavity, then move into the draining lymph nodes of the gut, which would induce higher local inflammation compared to the spleen as the spleen will receive blood that has already been processed by the liver. Furthermore, it has been shown that inflammation has the potential to enhance the magnitude of CD8+ T cell responses[507]. Taken together, IAV-specific CD8+ T cells are higher in frequency within the spleen, which could potentially be due to their higher proliferative capabilities after sepsis and the method of administration of IAV.

Next, I will further discuss the potential reasons for higher frequencies of activated and effector memory CD8+ T cells within the septic spleen during the peak primary CD8+ T cell response. As previously mentioned, during primary response, IAV-specific CD8+ T cells will not be considered memory cells as they are effector cells that have been differentiated from naïve CD8+ T cells; however, they will be within the effector memory CD8+ T cell population as effector CD8+ T cells are part of this population based on the phenotypic characteristics utilized during my study. Furthermore, the mice under investigation are housed in pathogen free conditions; hence, they typically will not possess memory CD8+ T cells that have been generated by antigenic exposure. Despite this, it has been shown that age changes the composition of the different CD8+ T cell subsets with increasing age shifting towards possessing more memory CD8+ T cells[508,509]. In a study by Chiu et al.[508], it has been demonstrated that central memory CD8+ T cells develop over the age of C57BL/6 mice; however, this process occurs in the absence of antigenic stimulation as they lack the expression of CD49d, which is a marker that gets upregulated during antigenic stimulation . These 'central memory' CD8+ T cells have been termed by multiple groups as virtual memory CD8+ T cells[510,511]. Throughout this discussion, I will refer to these virtual memory CD8+ T cells as central memory CD8+ T cells; however, a special consideration must be made as they are not conventional central memory CD8+ T cells. Within my data, there was a reduced absolute number of central memory CD8+ T cells. To explain this, it has been shown by Serbaescu et al.[273] that compared to naïve CD8+ T cells, memory CD8+ T cells (virtual memory CD8+ T cells included), defined as $CD44^{HIGH}CD11a^{HIGH}$, are preferentially depleted compared to sham. From this

information, I may be able to conclude that there is an increased susceptibility of these memory CD8+ T cell phenotypes to undergo sepsis-induced apoptosis leading to the loss of these 'central memory' CD8+ T cells.

Now, I will expand more on the potential reasons for the increased frequencies of activated and effector memory CD8+ T cells. Firstly, IAV-specific CD8+ T cells at the peak primary CD8+ T cell response would be classified as effector memory CD8+ T cells; hence, the increased frequency of effector memory CD8+ T cells can be majorly explained by the exuberant expansion of naïve IAV-specific CD8+ T cells. Secondly, there could be an enhanced presence of effector memory CD8+ T cells due to the phenotypic alterations seen within naïve CD8+ T cells under a lymphopenic environment. It has been reported by multiple research groups that under lymphopenic conditions, which entails sepsis, homeostatic proliferation of naïve CD8+ T cells will lead to their expansion and alter their phenotype to appear more memory-like[180,185–187,215–218]. For instance, these naïve CD8+ T cells acquire the expression of CD44, CD122, CD11a and acquire effector function such as expressing IFN-γ and cytolytic ability; however, these cells fail to upregulate some markers of activation, such as CD69 and CD25, while they fail to downregulate CD62L[180,187,216–218]. One important aspect to consider to determine the degree of homeostatic proliferation within my study is the timeline. Within a study by Krishna et al.[216], the authors compared the kinetics of homeostatic proliferation versus antigenic proliferation on naïve P14 transgenic CD8+ T cells. 72 hours post-lymphopenia, both homeostatic and antigen-driven proliferation had significant proliferation within a CFSE assay; however, antigen-driven proliferation was greater. Furthermore, in another study by Goldrath et al.[218], the researchers adoptively transferred naïve OT-I transgenic CD8+ T cells (recognize ovalbumin (OVA) peptide) into irradiated mice and discovered that five days after the onset of lymphopenia, there was a significant proportion of OT-I CD8+ T cells that had adapted a memory-like phenotype. From 12 to 20 days after lymphopenia, the majority of OT-I cells possessed the memory-like phenotype; however, between 20 to 30 days, these cells began to downregulate these memory/activation markers. Ultimately, between 40 to 50 days after lymphopenia, the majority of OT-I CD8+ T cells lose the expression of these markers. Thus, homeostatic proliferation is a transient mechanism in

restoring T cell loss. From this information, and applying it to my research, I may conclude that the increased frequency of effector memory CD8+ T cells is mainly due to the increased ability of naïve IAV-specific CD8+ T cells to proliferate in response to cognate antigen within the septic environment; however, there will be a minor contribution from homeostatic proliferation. Within my data, there was an increased presence of effector memory CD8+ T cells, which do not possess CD62L, but during homeostatic proliferation of naïve CD8+ T cells, CD62L expression will be maintained. Despite this, it would not be far-fetched to consider the ability of homeostatic proliferated naïve CD8+ T cells to downregulate CD62L during an infection as it has been shown that bystander activation can induce downregulation of CD62L[512]. Furthermore, one research group noted that homeostatic proliferation started as early as 30 hours post-lymphopenia, while showing significant proliferation 72 hours post-lymphopenia. This timeline is important to consider within my study, as lymphopenia occurs two days post-CLP, which has been shown to be the peak of CD8+ T cell loss[273,395,396]. Therefore, homeostatic proliferation within my experiments, hypothetically should begin to occur 78 hours post-CLP, with significant proliferation at 120 hours post-CLP. I had induced a primary response at 96 hours post-CLP, which is between this timeframe. During antigen encounter, naïve CD8+ T cells that have not expanded due to homeostatic proliferation will expand through antigen-dependent mechanisms.

4.2.2 IAV-specific CD8+ T cell phenotypes within the spleen

Next, I wanted to phenotypically characterise the IAV-specific CD8+ T cells within the septic spleen at the peak primary CD8+ T cell response during protracted sepsis. It was discovered that IAV-specific CD8+ T cells possess higher frequencies of cells expressing 2B4, while NP_{366}-specific CD8+ T cells have an increased frequency of TIGIT and PA_{224}-specific CD8+ T cells only showing a trend. Furthermore, IAV-specific CD8+ T cells also possessed higher frequencies of cells expressing KLRG1. In terms of co-expression of the different exhaustion markers, NP_{366}-specific CD8+ T cells possessed higher frequencies of cells doubly expressing TIGIT+VISTA+ and TIGIT+2B4+, while PA_{224}-specific CD8+ T cells had reduced frequencies of PD-1+TIM-3+. From this information, it may be concluded that IAV-specific CD8+ T cells are phenotypically more exhausted within the septic condition compared to sham. Furthermore, as previously discussed (section 4.1.2), exhaustion markers may also be an indicator of higher activation of these cells. Considering the study by Angelosanto et al.[458], it evident that exhaustion starts to set in at around day 15 post-infection, which works well for my timeline as I sacrificed mice 11 days post-CLP (seven days post-infection); hence, the exhaustion phenotype experiments will most likely provide a good indication of activation and subsequent exhaustion, although exhaustion will set it past the experimental timeline. Taken together, I may conclude from my data that IAV-specific CD8+ T cells, at the peak primary CD8+ T cell response, are more activated and exhausted compared to sham, however, T cell exhaustion has not yet set in.

In addition to examining exhaustion markers, I had also examined the expression of different activation, death, and survival/maintenance markers. I discovered that IAV-specific CD8+ T cells had reduced frequencies of cells expressing CD44, while NP_{366}-specific CD8+ T cells demonstrated a trend towards reduced frequencies of cells expressing CD127 and PA_{224}-specific CD8+ T cells had a significant reduction in CD127. In terms of the expression of CD44, despite the statistics determining significance, it only differed by a few percent compared to sham, which is most likely not biologically relevant. In terms of the expression of CD127 and KLRG1, in which I had previously discussed extensively within the discussion of the recall response (section 4.1.2), the reduced

expression of CD127 and increased expression of KLRG1 within IAV-specific CD8+ T cells may suggest that these cells (1) have an impaired ability to produce a good quality memory CD8+ T cell pool and (2) that these cells are more activated and functionally active within the septic spleen.

Subsequently, I had also wanted to phenotypically characterize the non-IAV-specific CD8+ T cells within the spleen at the peak primary CD8+ T cell response to acquire a better understanding on the IAV-specific CD8+ T cells of interest and the whole CD8+ T cell compartment. I discovered that there was a trend towards increased frequencies of cells singly expressing PD-1, 2B4 and TIGIT compared to sham. Furthermore, non-IAV-specific CD8+ T cells demonstrated a strong upregulation of co-expression of exhaustion markers as they doubly-expressed multiple different combinations of exhaustion markers. In addition, I had also examined the expression of different activation, death, and survival/maintenance markers on these cells populations. I discovered that non-IAV-specific CD8+ T cells possess a higher frequency of cells expressing CD44 and a lower frequency of CD127. From this information, it may be suggested that non-IAV-specific CD8+ T cells within the septic spleen at the peak primary CD8+ T cell response are more activated and exhausted compared to sham. Furthermore, these cells may be compromised in their ability to produce a good quality memory CD8+ T cell pool as they express less CD127; however, it may also suggest higher activation. Curiously, I also noticed a reduction on a per-cell basis of CD69. Reflecting further on the differences for CD44, CD127 and CD69 compared to sham, these differences may be due to homeostatic proliferation. As previously discussed, homeostatic proliferation leads to the higher expression of CD44, while not inducing the expression of CD69. Furthermore, CD127 downregulation is also a characteristic of homeostatic proliferation[513]. Taken together, non-IAV-specific CD8+ T cells are more phenotypically activated and exhausted in part due to homeostatic proliferation.

Also, I had wanted to phenotypically assess the different memory CD8+ T cell populations at the peak primary CD8+ T cell response in the context of sepsis-induced immunosuppression. Within the central memory CD8+ T cell population (virtual memory CD8+ T cells), there were increased frequencies of cells expressing BTLA, while there were lower frequencies of VISTA; however, VISTA on a per-cell basis expression showed an increasing trend. Furthermore, in terms of co-expression, central memory CD8+ T cells had an increased frequency of cells doubly expressing TIGIT+VISTA+, TIGIT+2B4+ and VISTA+2B4+. From this information, I may conclude that these cells are more exhausted within the septic spleen compared to sham. Furthermore, central memory CD8+ T cells may be less responsive to either their cognate antigen or inflammatory signals as both BTLA and VISTA are expressed at higher levels. BTLA and VISTA are negative regulators of T cell activation[434,514–517], and have been shown to downregulate their expression during T cell activation[434,517]. In terms of effector memory CD8+ T cells, there were a higher frequency of cells expressing 2B4 and a trend towards higher frequencies of PD-1 and TIGIT. Moreover, they also had higher frequencies of cells doubly expressing PD-1+TIM-3+, TIGIT+2B4+ and VISTA+2B4+, which would indicate that these cells are more phenotypically exhausted and activated compared to their sham counterparts. In addition, I also had examined the activation of these cells. Within the effector memory CD8+ T cell compartment, there were reduced frequencies of cells expressing CD127, and a trend towards reduced frequencies of CD69. From this information, it may be suggested that effector memory CD8+ T cells at the peak primary CD8+ T cell response have a compromised ability to form a memory CD8+ T cell pool, are more activated and exhausted.

4.2.3 IAV-specific CD8+ T cell functionality within the spleen

Thereafter, I wanted to examine the functionality of IAV-specific CD8+ T cells at the peak primary CD8+ T cell response in the context of sepsis-induced immunosuppression. To address this, I stimulated these cells with their cognate peptides *ex vivo* then performed an intracellular stain, which assessed for different cytokines and cytolytic effector molecules. Through my research, I discovered that there was a higher frequency and absolute number of IAV-specific CD8+ T cells capable of producing IFN-γ within the septic spleen, which had an associated increase in per-cell expression of IFN-γ. Furthermore, I also discovered that these cells may have a higher cytotoxic ability as demonstrated by the increased intracellular expression of granzyme B compared to sham. Despite these increases, their polyfunctionality as measured by triple expressors of IFN-γ, TNF-α and IL-2 may be dampened during sepsis. Within the NP_{366}-specific CD8+ T cell compartment, there was a trend towards reduced frequencies of triple-expressors; however, this same trend was not apparent within the PA_{224}-specific CD8+ T cell compartment. In discussing these results, I will first touch upon studies that support my findings, then will proceed to discuss contradictory research and potential reasons for these discrepancies. In a study by Markwart et al.[266], the authors had performed CLP surgery on naïve mice, then 10 days later induced a primary response and subsequently examined the *in vivo* and *ex vivo* antigen specific CD8+ T cell functionality by assessing expression of IFN-γ and TNF-α within the spleen. It was discovered that challenging antigen specific CD8+ T cells with their cognate peptide *in vivo* led to a slight increase in frequency of cells expressing IFN-γ and TNF-α within the septic condition. Furthermore, upon *ex vivo* challenge, it was demonstrated that per-cell expression of IFN-γ was increased within the septic condition compared to sham; however, per-cell expression of TNF-α was similar compared to sham. These findings corroborate the results within my study as I have shown that the per-cell expression of IFN-γ was enhanced within the septic condition. Furthermore, to provide additional evidence that my IAV-specific CD8+ T cells are increased in functionality within the septic spleen, I can turn to the immunophenotyping experiments, specifically the expression of KLRG1 and CD127, which has been previously discussed (section 4.1.2 and 4.1.3). It has been shown in the literature that persistent antigen exposure and/or persistent inflammation,

which encompasses sepsis, leads to the upregulation of KLRG1 and downregulation of CD127[447,518–520]. Furthermore, the KLRG1+CD127- phenotype is associated with higher functionality as it has been shown to possess higher production of IFN-γ, TNF-α, granzyme B, and CD107a, while having a greater cytolytic ability[444,520,521]. Considering that the IAV-specific CD8+ T cells under investigation displayed reduced frequencies of CD127 and increased frequencies of KLRG1, I may suggest from these phenotyping experiments that they are more functionally active. In another train of thought, taking into consideration that the CLP model entails a state of persistent inflammation and antigen exposure[522], this might have had an effect on the priming of IAV-specific CD8+ T cell response. In a study by Obar et al.[520], it was demonstrated that co-administering the pathogen and an inflammatory stimuli led to an increased frequency and per-cell expression of IFN-γ at the peak primary CD8+ T cell response. Hence, during IAV priming, IAV-specific CD8+ T cells may have an increased response due to the inflammatory state after CLP.

Next, I will discuss the research within the literature that has shown results contradictory to my study. Within a study by Condotta et al.[267], it was demonstrated that inducing a primary response two and nine days post-CLP and examining the peak response led to a reduced frequency of antigen specific CD8+ T cells capable of producing IFN-γ and TNF-α within the septic spleen compared to sham; although, they did possess similar frequencies of antigen specific CD8+ T cells as measured by tetramer reagent. Furthermore, within a study by Gurung et al.[523], it was discovered that inducing a primary response two days post-CLP and examining the peak response led to a significant reduction in absolute numbers of antigen specific CD8+ T cells expressing IFN-γ within the septic spleen. Moreover, within another study by Condotta et al.[180], the authors demonstrated that inducing a primary response two and 30 days post-CLP led to decreased frequencies and absolute numbers of antigen specific CD8+ T cells capable of producing IFN-γ at the peak primary CD8+ T cell response within the septic spleen. The authors had also shown at the late septic time point (30 days post-CLP) that there were reduced absolute numbers of antigen specific CD8+ T cells expressing TNF-α, IL-2, granzyme B and CD107a; although, the frequency of granzyme B and CD107a were similar compared to sham. Firstly, I will discuss the potential problems with inducing a primary response two days post-CLP. Two days post-

CLP is the peak of CD8+ T cell loss, with both naïve and memory CD8+ T cells being lost; although, memory CD8+ T cells appear to be more numerically depleted compared to naïve[180,273,395,396]. Furthermore, within a study by Serbanescu et al.[273], it was reported that at two days post-CLP, there were reduced absolute numbers of naïve CD8+ T cells compared to sham; however, three days post-CLP, naïve CD8+ T cells demonstrated a trend towards higher absolute numbers compared to sham. Moreover, within the study by Condotta et al.[180], it was discovered that antigen specific CD8+ T cells were significantly reduced two days post-CLP compared to sham. From this information, I may confidently state that two days post-CLP, there will be a reduction in the naïve CD8+ T cell compartment. It has been shown within the literature that the precursor naïve CD8+ T cell pool size correlates strongly with the magnitude of the primary response[397,398]. Taken all this information together, it can be concluded that these studies observed a reduced primary antigen specific CD8+ T cell response as there were reduced numbers of naïve CD8+ T cells at the time of antigen encounter. This is problematic as it might not address the inherent problems with the septic naïve CD8+ T cells as they have induced a primary CD8+ T cell response at the peak CD8+ T cell loss; it is more appropriate to study naive antigen-specific CD8+ T cell responses when the naïve CD8+ T cell pool recovers from the lymphopenia. Now, I will further expand on the discrepancies seen between the two studies that have induced a primary response at nine and 10 days post-CLP. Within the study that demonstrated an enhanced response, the authors had administered the infection by intraperitoneal injection, while the study that had demonstrated a decreased response had administered the infectious agent through intravenous injection. As previously discussed, this could severely affect the responses seen at the peak primary CD8+ T cell response as injecting through the intraperitoneal route will most likely encounter higher levels of inflammatory stimuli within the draining lymph nodes as CLP causes leakage of different microbial species into peritoneal cavity. Additionally, it has been shown by multiples groups that inducing a primary response with inflammatory stimuli led to their increased magnitude in response[507,520].

4.2.4 IAV-specific CD8+ T cell responses within the PECs

In this next section, I will discuss the results that I acquired for the PECs, in terms of IAV-specific CD8+ T cell responses at the peak primary CD8+ T cell response during sepsis-induced immunosuppression. I decided to study the PECs as it would give me information on the local immune responses within the peritoneal cavity, which is the site of sepsis induction. Of important note, to my knowledge, this is the first study to date examining CD8+ T cell primary responses within the PECs. I discovered that IAV-specific CD8+ T cells within the septic PECs are similar in frequency compared to sham; however, there is downward trend in absolute numbers. In terms of their phenotype, IAV-specific CD8+ T cells display a reduced exhausted phenotype as shown by the decreased frequencies of cells singly expressing BTLA, PD-1 and TIM-3. IAV-specific CD8+ T cells also have reduced frequencies of cells doubly expressing PD-1+BTLA+, PD-1+TIM-3+ and BTLA+TIM-3+. From these findings, it may be suggested that within the peritoneal cavity, IAV-specific CD8+ T cells are less exhausted compared to sham. Examining this data in a different lens, IAV-specific CD8+ T cells within the PECs may be less activated compared to sham. Within my data, I discovered that per-cell expression of BTLA was increased within the septic condition. As previously discussed, BTLA is a negative regulator of T cell activation, in which upon activation, BTLA will downregulate its expression[434,504,505]. Despite the differences between the frequency and per-cell expression of BTLA, it could be suggested that IAV-specific CD8+ T cells are responding; although, not to the same degree as sham because they possess a reduced single- and double-expression of exhaustion markers. Furthermore, the cells that are expressing BTLA, express BTLA at higher levels suggesting that they are less responsive to cognate antigen stimulation. This hypothesis is further corroborated within my ICS data. I discovered that there was a trend towards higher frequencies and absolute numbers of NP_{366}-specific CD8+ T cells producing IFN-γ, while PA_{224}-specific CD8+ T cells were similar to sham. However, this might seem contradictory, but it is not as the PECs from the septic condition contains more cells compared to sham. Strong evidence for this hypothesis comes from the per-cell expression of IFN-γ as IAV-specific CD8+ T cells demonstrate a significant reduction in the expression of IFN-γ.

Within the septic PECs, I had also wanted to examine the phenotypic differences in the non-IAV-specific CD8+ T cell populations. I observed that there was an overall trend towards decreased frequencies of cells singly expressing BTLA, PD-1 and TIM-3. Furthermore, in terms of co-expression, it was demonstrated that the non-IAV-specific CD8+ T cells tended to have a reduced double-expression of the different exhaustion marker combinations. Despite these findings, the per-cell expression of these markers had a different pattern of expression. There was an increase in the per-cell expression of CTLA-4 and TIM-3, while the other markers examined had higher averages, although not significant but a very weak trend. In terms of CTLA-4, it is not biologically relevant as the frequencies of cells that express this marker are very low. From this information, it could suggest that some of the non-IAV-specific CD8+ T cells are responding, and the cells that are responding are more phenotypically exhausted as they express these markers on a higher per-cell basis expression.

4.2.5 IAV-specific CD8+ T cell immune responses within the lungs

Now, I will further the discuss the results for the lungs; however, for these experiments, the sample number was too low to make any definite conclusions, but I can still note interesting observations.

Within the septic lungs, there were similar frequencies and absolute numbers of IAV-specific CD8+ T cells during the peak primary CD8+ T cell response. This information suggests that sepsis does not affect the ability of IAV-specific CD8+ T cells to migrate to nonlymphoid tissues, which has been corroborated within a study by Dahany et al.[272]. In terms of their phenotypic differences, within the septic lungs, there were higher frequencies of cells singly expressing 2B4 and KLRG1. In terms of 2B4, this finding may suggest that after the peak primary CD8+ T cell response, during the contraction phase, these cells will be more likely to die off compared to sham as they would express higher caspase-3/7 activation[274]. In terms of KLRG1, there increased expression within IAV-specific CD8+ T cells may indicate that these cells are (1) more phenotypically exhausted (2) more functionally capable in producing different cytokines and cytolytic effector molecules (3) less capable of producing a memory CD8+ T cell pool and (4) more phenotypically activated due to chronic inflammation, chronic antigenic stimulation and increased rounds of proliferation[446,447,449,458,473]. Furthermore, across the board, there were higher frequencies of cells double-expressing different exhaustion marker combinations. From this information, it may suggest that within the septic lungs, IAV-specific CD8+ T cells appear to be more phenotypically exhausted and activated compared to sham. To compliment the phenotyping experiments, I had also performed functional assays to determine IAV-specific CD8+ T cell functionality. Compared to the sham condition, IAV-specific CD8+ T cells within the septic lungs displayed similar frequencies and absolute numbers of cells capable of producing IFN-γ; however, per-cell expression of IFN-γ demonstrated an increasing trend, which would indicate that these cells are more functionally capable in producing IFN-γ. Moreover, IAV-specific CD8+ T cells possessed higher frequencies of cells expressing granzyme B within the septic lungs. From this information, it could be suggested that IAV-specific CD8+ T cells within the septic lungs

during the peak primary CD8+ T cell response in the context of sepsis-induced immunosuppression are more functionally active compared to sham, which corroborates the phenotyping experiments. Despite these apparent trends for increased functionality within IAV-specific CD8+ T cells, there was a differential effect on their quality of response. For instance, NP_{366}-specific CD8+ T cells demonstrated a downwards frequency trend of triple-expressors for IFN-γ, TNF-α and IL-2, which indicates that their quality of response might be dampened within the septic lungs. Contrary, PA_{224}-specific CD8+ T cells possessed an upwards trend in the frequency of cells triple-expressing IFN-γ, TNF-α and IL-2, which might suggest that these cells have a better quality of response. One curious observation is that PA_{224}-specific CD8+ T cells had a higher functionality compared to NP_{366}-specific CD8+ T cells, which might suggest that the immunodominant response have been altered. To expand on this, in a study by Wherry et al.[524], the authors had examined the functional responses from different dominant and subdominant antigen specific CD8+ T cells during chronic infection, which is applicable to my research as sepsis can be classified as a chronic infection. It was discovered, eight days post-infection, that dominant antigen specific CD8+ T cells had a slight reduction in the expression of IFN-γ, while TNF-α and IL-2 expression was basically gone; however, subdominant antigen specific CD8+ T cells retained their expression of IFN-γ, while TNF-α and IL-2 expression was reduced, albeit not to the same degree as the dominant antigen specific CD8+ T cells. From this information and taking into consideration that NP_{366}-specific CD8+ T cells are the most dominant in responding to PR8 (within B57BL/6 mice), while PA_{224}-specific CD8+ T cells are still considered dominant, however, they are less dominant compared to NP_{366}-specific CD8+ T cells; it may suggest that within the septic lungs, there is a skew in the immunodominance hierarchy.

Thereafter, I had also examined the phenotypic differences for the non-IAV-specific CD8+ T cell compartment and the different memory CD8+ T cells compartment within the septic lungs. In terms of the non-IAV-specific CD8+ T cell compartment, it was demonstrated that these cells possessed higher frequencies of cells expressing 2B4, while having higher double-expression of VISTA+LAG-3+ and VISTA+2B4+. In terms of the memory CD8+ T cell populations, it was discovered that there was a similar frequency and absolute number of activated CD8+ T cells as measured by the expression of CD44 within the septic lungs compared to sham. Furthermore, there were no differences in the frequency and absolute numbers of both central and effector memory CD8+ T cells, which is quite different than the results from the recall response experiments. Moreover, central memory CD8+ T cells demonstrated increased frequencies of cells doubly expressing VISTA+LAG-3+ and VISTA+2B4+, while effector memory CD8+ T cells displayed increased frequencies of cells singly expressing 2B4, and doubly expressing higher levels of VISTA+2B4+. Taken together, within the septic lungs, both non-IAV-specific CD8+ T cells and memory CD8+ T cells had a more exhausted and activated phenotype compared to sham at the peak primary CD8+ T cell response.

4.2.6 IAV-specific CD8+ T cell immune responses within the liver

In addition to examining the lungs, I had also examined the IAV-specific CD8+ T cell presence and responses within the septic liver. I had wanted to examine this tissue because it is closely connected with the septic insult as the biology of the liver permits this. However, just as the lungs, the number of replicates is too low to form any definitive conclusions, but I am able to note curious observations.

Within the septic liver, I discovered that IAV-specific CD8+ T cells were similar in frequency compared to sham; however, NP_{366}-specific CD8+ T cells may be numerically increased, while PA_{224}-specific CD8+ T cells were numerically similar compared to sham. As previously stated, this finding suggests that the migration to the liver is unaffected after the septic insult. In terms of phenotypic differences, NP_{366}-specific CD8+ T cells demonstrate a trend towards higher frequencies of cells singly expressing 2B4, LAG-3, TIGIT and VISTA, while PA_{224}-specific CD8+ T cells do not demonstrate this same trend. Furthermore, these IAV-specific CD8+ T cells had a higher frequency of cells expressing KLRG1. In terms of co-expression, IAV-specific CD8+ T cells displayed elevated frequencies of cells doubly expressing the different exhaustion marker combinations. From this information, it may be suggested that IAV-specific CD8+ T cells within the septic liver are more phenotypically exhausted and activated compared to sham.

Next, I determined the functionality of IAV-specific CD8+ T cells by stimulating them *ex vivo* with cognate peptides and performed an intracellular stain for different cytokines and cytolytic effector molecules. Within the septic liver, IAV-specific CD8+ T cells may have an increased frequency, absolute number, and per-cell expression of IFN-γ; however, there were no differences in the expression of the other markers examined. In terms of polyfunctionality, IAV-specific CD8+ T cells within the septic liver demonstrate a dampened quality of response as they possess lower frequencies of cells triple-expressing IFN-γ, TNF-α and IL-2. This dampened quality of response could be attributed to the chronic inflammation within the septic liver as they would encounter many inflammatory stimuli from the bloodstream of the intestines, which come into contact first with the

liver[495,496]. Chronic infection, eight days after infection, has been demonstrated to downregulate the expression of TNF-α and IL-2, while preserving IFN-γ expression[524]. Taken together, I may suggest that IAV-specific CD8+ T cells within the septic liver at the peak primary CD8+ T cell response are phenotypically more exhausted and activated compared to sham. Furthermore, they may be less functionally active compared to sham as they demonstrated reduced polyfunctionality, which is most likely due from the chronic inflammation within the septic liver and the increased expression of KLRG1 supports this notion.

Next, just as I had done previously for the lungs, I examined the phenotypic differences within the non-IAV-specific CD8+ T cell populations and the different memory CD8+ T cells populations for the septic liver. In terms of the non-IAV-specific CD8+ T cell populations, non-NP_{366}-specific CD8+ T cells possessed higher frequencies of cells singly expressing 2B4 and VISTA, while non-PA_{224}-specific CD8+ T cells did not display this trend. Furthermore, non-IAV-specific CD8+ T cells had increased per-cell expression of VISTA. Considering that VISTA is a negative regulator of T cell activation[514,515,517], as previously discussed, it could be suggested that the non-IAV-specific CD8+ T cells are less likely to respond upon either cognate antigen or inflammatory stimuli due to the chronic inflammatory state of the liver, which renders these cells unable to respond. In terms co-expressing, non-NP_{366}-specific CD8+ T cells had higher frequencies of cells doubly-expressing VISTA+LAG-3+, TIGIT+VISTA+ and LAG-3+2B4+, while non-PA_{224}-specific CD8+ T cells demonstrated a reduced frequency of double-expressors of VISTA+2B4+. From this information, it may be suggested that non-NP_{366}-specific CD8+ T cells are more phenotypically activated and exhausted compared to sham; however, non-PA_{224}-specific CD8+ T cells may be less phenotypically activated and exhausted compared to both sham and their non-NP_{366}-specific CD8+ T cell counterpart. In terms of the different memory CD8+ T cell subsets, I examined their phenotypic differences. I discovered that within the septic liver, there is a similar frequency of activated CD8+ T cells as measured by the expression of CD44; however, there was an upward trend in their absolute numbers due to the higher presence of central and effector memory CD8+ T cells. The increased presence of activated CD8+ T cells may be due to their increased migratory ability to the

liver and the potential for expansion. It has been demonstrated that activated CD8+ T cells preferentially migrate to the liver compared to any other tissue[493]. Furthermore, migration to the liver, during liver infection and inflammation, results in a quick migration from activated CD8+ T cells[501], which will then clonally expand due to the higher inflammatory state of the liver [493,507,520]. Hence, during both early sepsis and the induction of the primary response, activated CD8+ T cells will migrate quickly to the liver and clonally expand leading to their higher presence within the liver. In terms of phenotypic differences, central and effector memory CD8+ T cells had a reduced frequency of cells expressing 2B4 and VISTA+2B4+, which may indicate that these cells are less activated and exhausted compared to sham. However, this phenotype is consistent with the literature as it has been shown that activated CD8+ T cells that migrate to the liver acquire a dampened effector function as demonstrated by reduced *in vitro* cytotoxicity and IFN-γ production[493].

4.3 Limitations and future directions

4.3.1 Limitations to my study

In this next section, I will discuss the limitations of my study with a focus on the reasons for these limitations.

First, one of the major limitations to this study was the sample size for some of the tissues examined (i.e. PECs, lungs, liver). Some of the tissues examined had a replicate number as low as two, which is problematic as the minimum number of replicates needed to perform statistical analysis is three. In examining these tissues, I was not able to perform statistical analysis due to the low replicate number. Hence, lacking the statistical analysis severely affects the conclusions that I can provide and the observations that I have noted may or not be true. Furthermore, to add to this problem, sepsis is a very heterogenous syndrome[30–32,59]. For instance, within human septic patients, there are many patients that become immunosuppressed after the hyperinflammatory phase; however, some patients do not go through the hyperinflammatory phase, instead, they present with immunosuppression from the onset of sepsis. Furthermore, within my own data, there were signs of heterogeneity. For example, in Figure 19 (section 3.2.2), the frequency of IAV-specific CD8+ T cells singly expressing VISTA was quite variable from experiment to experiment. Considering that sepsis is very heterogenous, this makes the observations noted from a few replicates difficult to interpret and lacks power in the study. Next, I will address why some of the tissues there were so few replicates. In terms of the PECs, when I first started my Master's, I was isolating this tissue and performing the analysis; however, the results when first analyzed were quite messy and uninteresting. Hence, I decided to discontinue examining the IAV-specific CD8+ T cell responses within this tissue; however, when I became more knowledgeable and proficient in data analysis, I re-analyzed this tissue and found some interesting findings, but due to the later time point, I was not able to perform more experiments as my Master's was coming to an end. In terms of the lungs and liver, I had only started performing these analyses later in my Master's, when I was more efficient in performing these long experimental days. Furthermore, I had also decided to examine the

IAV-specific CD8+ T cell responses after acquiring a good number of mice for the splenic compartment as I wanted to extend my findings; however, the major reason for not performing and adding more replicates to these tissues was due to the timeline. I had only started examining the lungs and liver late in my Master's degree, and after performing these analysis, I did not have more time to perform further experiments as my Master's was coming to a close. In terms of the spleen, within the immunophenotyping and functional assays, there was a differential number of replicates for the different markers examined. This is also due to the timeline of my Master's as when I first started, I had only examined a few markers, then when I became proficient and comfortable performing these experiments, I had included more markers and more tissues. For example, within the splenic data, there are more replicates for the markers BTLA, PD-1, TIM-3 and CTLA-4 compared to the markers 2B4, TIGIT, VISTA and LAG-3. Again, this was due to me adding these markers in later experiments when I was more comfortable and proficient in the staining protocols. Taken together, due to the timeline of my Master's and my proficiency in performing these experiments, there was a variable replicate number for the different tissues and markers examined.

Second, another limitation that I must discuss is that I had not included the IAV-specific CD8+ T cell responses within the circulatory system. I had decided to not the include the blood compartment within my experiments for multiple reasons. Firstly, when I was proficient and comfortable performing the analysis on multiple tissues at once, I decided to omit the blood compartment as the experiments were quite long. For example, when I isolated the spleen, lungs and liver and then perform all their subsequent analysis, I was in the lab for approximately 14-17 hours. I had first started by sacrificing the mice and isolating the tissues examined, then subsequently isolated the non-parenchymal mononuclear cells within these tissues and performed cell counting. After the cell counting, I would resuspend the cells in cRPMI and set up the 5-hour stimulation assays. During the incubation time for the stimulation assays, I would perform the immunophenotyping surface staining experiments on the freshly isolated cells then subsequently read all of the tubes on the flow cytometer. After the stimulation was done, I would perform the ICS protocol and then read the cells on the flow cytometer upon completion. All of the

experiments were performed and completed on the day that I sacrificed the mice. Hence, considering that I was performing long day experiments and I had just gotten comfortable to add other tissues, I had not wanted to add to this workload as it already was very heavy. Secondly, I had also decided to omit the blood compartment as the majority of the studies within the literature had examined this tissue. Despite this, I believe that it would have been beneficial in my study to examine the blood compartment to provide a better global understanding on the effects sepsis has on the IAV-specific CD8+ T cells under study. Furthermore, adding the blood compartment, could have provided further evidence of some of the conclusions I have provided. For example, if I had performed this analysis and discovered that there was a higher frequency of effector memory CD8+ T cells within the blood, then it would provide stronger evidence that these cells may have an increased migratory ability to the peripheral tissues. Taken together, I had not examined the blood IAV-specific CD8+ T cell responses because of the short time frame for my Master's studies, the long duration of the experiments, in which I had just became proficient and comfortable performing the analyses on multiple tissues, and the evidence that has already been shown within the literature.

Third, the CLP polymicrobial model of sepsis is great; however, there are some limitations to this model of sepsis. In this paragraph, I will only focus on the limitations of this model as I have discussed the advantages and the rationale for using this model elsewhere (section 1.4.1). Firstly, reproducibility is somewhat difficult in the CLP model. CLP requires a surgical insult, and this inherently causes problems in its reproducibility from lab to lab and from experiment to experiment. There are three factors that influence the severity of sepsis within this model[402]: puncture quantity (number of pokes), the size of the puncture and the length of the cecum which is ligated. These parameters rely on the skills of the surgeon, which is different between person to person. Hence, if an individual performs a surgery with set parameters, while another individual performs the surgery with the same parameters, there could be differences in the mortality and immune responses due to the differences in the skill of the surgeon. As further proof of concept, I will provide an anecdote. During my Master's, I have trained a few of my lab mates to perform the CLP surgery. One difference that I noticed was the time it took to complete a surgery. My lab

mates, who are not as experienced as I am, performed the surgery in 30 minutes, while at that time it took me 15 minutes. This 15-minute difference can severely affect the outcome of these septic mice. Furthermore, the surgery may be different between experiments with the same surgeon. Considering that the surgery requires precise measurements, there may be slight differences in the length of cecal ligation from experiment to experiment and from animal to animal; however, it easier to control for the number of pokes and the size of the needle. Despite this, the number of pokes may also cause variability as you will be poking the ligated cecum in different locations. Secondly, standardization is difficult in the CLP model. One important fact to consider is that the CLP model relies on stool present within the cecum to leak out into the peritoneal cavity to cause peritonitis. This is a problem for standardization for multiple reasons. Firstly, depending on the batch of mice used, they may possess very different microbiota. This difference in microbes may severely affect the immune responses during sepsis, which could explain some of the heterogeneity seen within this model. Secondly, there is no way to control the amount of stool that leaks out into the peritoneal cavity. Some mice may have a lot of stool within their cecum, but others may not and this would severely affect the amount of stool that extrudes out. Hence, from mouse to mouse, the amount of stool that leaks out into the peritoneal cavity is quite different and this makes it difficult to standardize the model. Thirdly, within the CLP model, an abscess will form around the septic foci; however, there will be a constant leakage of microbes from this abscess leading to chronic inflammatory stimuli present[454]. This is problematic in terms of clinical relevance as one of the treatments for sepsis is source control. Hypothetically, if an abdominal septic patient presents with an abscess that is causing the sepsis, surgeons will drain the abscess and remove any necrotic tissue that is present. Hence, these septic mice that possess both necrotic tissue and an abscess may affect the clinical relevance as clinicians typically will remove these from patients. Taken together, the CLP model of polymicrobial sepsis is not perfect due to the problems of reproducibility, standardization, and clinical relevance; however, it is still considered the gold standard of sepsis models because the pros outweigh the cons.

Fourth, I had not performed these analyses within either (1) a different model of sepsis or (2) another mouse strain. This is a major limitation within my study as it makes my findings difficult to generalize as these findings could be specific to the model of sepsis and/or the strain of mice used. In terms of the sepsis model, I have outlined the limitations of CLP within the previous paragraph. To circumvent these limitations, it would have been ideal to examine the IAV-specific CD8+ T cell responses within a different model of sepsis. For example, I could have induced sepsis by the fecal-induced peritonitis (FIP) model. This model involves intraperitoneally injecting a defined concentration of fecal slurry into a mouse. Compared to CLP, the FIP model is generalizable as it does not rely on surgical skill, and it is also standardizable because a known concentration of fecal slurry is administered and typically it will contain the same microbiota as it is from one donor. In terms of using another mouse strain, this would be important to generalize the findings within this thesis. It has been demonstrated by multiple groups that different inbred laboratory mouse strains have different immune responses to different infections[525–530]. For example, BALB/c mice are more susceptible to sepsis-induced mortality compared to C57BL/6 mice[529]. Furthermore, in terms of CD8+ T cell responses, genetically diverse mice possess a wide variation in the size of effector and memory T cell responses following infection[526]. Hence, to make my findings more generalizable, it would have been ideal to perform these experiments within different mice strains and a different model of sepsis.

Fifth, another important limitation that needs to be discussed is the presence of blood within the lungs and liver. During the non-parenchymal mononuclear cell isolation (liver and lungs), I had not flushed these tissues to extrude excess blood left within the tissue. This could serve as a potential confounding factor when analysing the data from these tissues as there will be the presence of both IAV-specific and non-IAV-specific CD8+ T cells originating from the blood.

Sixth, there are some limitations associated with my definition of central and effector memory CD8+ T cells. For instance, within the effector memory CD8+ T cell population, in addition to true effector memory CD8+ T cells, there will also be the presence of activated effector CD8+ T cells. During the activation of naïve CD8+ T cells, CD62L expression is downregulated[531], while the expression of CD44 quickly upregulates and is maintained within the memory CD8+ T cells[532]. Hence, within my effector memory CD8+ T cells, there may be some contamination from activated effector CD8+ T cells that originated from naïve CD8+ T cells. Furthermore, just the expression of CD62L is not sufficient to define central memory CD8+ T cells because (1) it does not imply that they are in fact homing to the lymph nodes and (2) there is heterogeneity seen within the memory CD8+ T cell population. To define central memory CD8+ T cells, the chemokine receptor 7 is needed (CCR7), in addition to the expression of CD62L[282]. CCR7 is a G-protein coupled receptor that binds to chemokine ligand 19 and 21 (CCL19 and CCL21), which are highly abundant within the lymph nodes and spleen[533]. The dual expression of CCR7 and CD62L are needed to conclude that these cells are central memory CD8+ T cells[282]. Furthermore, it has been shown that there is heterogeneity seen within the memory CD8+ T cell pool. For instance, it was demonstrated that there is a prominent memory CD8+ T cell population that lacks CD62L, but expresses CCR7, which have been coined 'intermediate memory T cells'[534]. Hence, these intermediate memory CD8+ T cells may also contaminate the effector memory CD8+ T cell population.

Seventh, the co-expression of the exhaustion marker combinations was limited due to the restriction of eight-color flow cytometry. During my experiments, I had examined the co-expression of the different exhaustion marker combinations; however, I was not able to make certain combinations due to the restriction of the flow cytometer. The flow cytometer within my lab was restricted to eight-color panels, which prevented me from examining all of the possible combinations of double-expressors. This is a limitation as it would have been ideal to examine the co-expression of all of the exhaustion markers that demonstrated differences between the sham. For instance, within the septic spleen in the recall response, I demonstrated an increased expression of PD-1 and 2B4; however, these two markers were on different panels, therefore, I could not examine the co-expression of these markers.

4.3.2 Future studies

Now, I will discuss future studies that are necessary to (1) prove or disprove my hypotheses throughout this thesis and (2) provide direction to what comes next.

Firstly, it is necessary to increase the replicate number for the different tissues and markers examined. For example, the lungs and liver have sparce data and the noted observations from these tissues may or may not be significant upon increasing the replicate number. Furthermore, it would also be ideal to include the blood compartment in future studies to acquire a better global understanding on the effects sepsis has on the IAV-specific CD8+ T cell responses. Additionally, it would be best to perform these experiments within another model of sepsis and another strain of mice, which would render these findings more generalizable.

Secondly, it is necessary to include more functional assays to acquire a better understanding on the IAV-specific CD8+ T cell functionality. Within this thesis, I had examined IAV-specific CD8+ T cell functionality by assessing their productive capabilities of different cytokines and cytolytic effector molecules; however, this measurement omits their direct cytolytic ability, which is one of their main mechanisms of killing. It is extremely important to measure their cytolytic ability as this would measure their direct killing of target cells. One method that would accurately assess their ability for cytotoxicity is the *in vivo* killing assay[535]. I will provide a brief run down on this method and how it should be implemented within future studies. The *in vivo* killing assay is a flow cytometry-based method for quantification of CD8+ T cell killing; it relies on *in vivo* antigen-specific CD8+ T cells killing a target cell that has been labelled with CFSE and possesses the specific antigen in which is targeted. In terms of implementation, it is ideal to perform this method at either the peak memory CD8+ T cell response or the peak primary CD8+ T cell response. Hence, the methodology will be mostly similar (Figure 7 and 8); however, at the peak CD8+ T cell response (11 days post-CLP), target cells should be administered intravenously to the septic and sham mice, then two to four hours later, the mice will be sacrificed and splenocytes should be read for their CFSE levels.

Thirdly, within my thesis, I have hypothesized that IAV-specific CD8+ T cells have an increased proliferative capacity within the septic spleen. It would be extremely important to test out this hypothesis as it would provide more insight into antigen-specific CD8+ T cell responses during sepsis-induced immunosuppression. To test out this hypothesis, there are a few methods that would work; however, I will discuss a method that utilizes BrdU incorporation into proliferating CD8+ T cells. The experimental design would be relatively similar to what I have done (Figure 7 and 8); however, at the peak CD8+ T cell loss (two days post-CLP), BrdU would be administered intraperitoneally into the mice, then subsequently BrdU would be given in their drinking water[194]. Thereafter, at the peak IAV memory or primary CD8+ T cell response, mice will be sacrificed, and an ICS will be performed to detect BrdU incorporation in conjunction with tetramer reagent staining to specifically detect the BrdU incorporation into IAV-specific CD8+ T cells.

Fourthly, I have hypothesized that septic IAV-specific CD8+ T cells were more activated compared to their sham counterpart. This hypothesis is extremely important to study as it could provide more evidence to suggest that these cells are more functional or it could disprove my hypothesis; however, it is essential information to better understand the effect sepsis has on antigen specific CD8+ T cell responses. To accomplish this, a similar experimental design would be used (Figure 7 and 8); however, the only difference would be the time of immunophenotyping after the induction of the primary or recall response. The immunophenotyping for the different activation markers (CD25 and CD69) would need to be performed between the 24- and 120-hour timepoint after the administration of the infectious agent as this is when CD25 and CD69 are maximally expressed[536,537].

Fifthly, within my thesis, I had concluded that septic splenic IAV-specific CD8+ T cells possessed an increased functionality, while I speculated that septic liver and lung IAV-specific CD8+ T cells had an increased functionality. Considering these conclusions and speculations, the next reasonable step to take would be to assess their protection within a relevant secondary infection model. From my findings, it would be expected that they would provide enhanced protection from secondary infection as these cells appear to be more functional; however, rather than protection, they may be more detrimental as they

could damage these tissues more severely. To interrogate these hypotheses, the experimental design will be largely the same (Figure 7 and 8); however, there will be a few tweaks. For instance, rather than intraperitoneally injecting mice with different IAV strains at day four post-CLP, an intranasal administration should be given as it causes an active infection. Furthermore, the recall and priming steps within the recall response experiment would be different due to the infectivity of the different IAV strains. PR8 is more infectious and lethal compared to X31[538]. Hence, PR8 should be utilized for the active infection rather than the priming step because it is ideal to have the sham mice display some morbidity due to the infection; X31 may not be potent enough to induce morbidity within the sham mice. Despite this, there are some experimental problems with utilizing PR8 rather than X31 for an active infection within septic mice. For example, the weight change. During CLP, the weight of the mice decreases up to about 20% (experimental endpoint) and only starts to recover after day three or four post-CLP. This is problematic as PR8 intranasal infection also causes significant weight loss within healthy mice[539]. This double whammy of weight loss from CLP and PR8 intranasal would render these mice difficult to study as the vast majority would be sacrificed due to the excessive weight loss. Currently, the max weight loss that is acceptable within rodents is losing 20% of their weight[540]. However, there are a few changes that can be made to make this study more logistically possible. For example, the timeline of when to administer the active infection can be altered based on when the mice have decently recovered in their weight; however, this may also be problematic as it would no longer assess the IAV-specific CD8+ T cell responses at the four-day post-CLP time-point. Furthermore, another viable option would be to significantly reduce the inoculum of PR8 administered for the intranasal infection; however, in doing so, it would require an optimization step to determine the best inoculum concentration. The best inoculum concentration should display morbidity within sham mice, and it should not cause overt weight loss within septic mice.

Sixthly, it would be important to study the mechanism that I had proposed regarding the increased mortality and tissue damage due to memory CD8+ T cell responses during a secondary infection. To study this, there are a few necessary experiments that should be performed. The first experiment should utilize a similar experimental design (Figure 7 and 8); however, there will be an added treatment of anti-CD8 antibodies to deplete CD8+ T cells one day prior to the induction of the primary and recall response (with an isotype treatment). Furthermore, at the peak primary and memory CD8+ T cell response, it would be ideal to examine lung and liver damage by (1) assessing common serum markers of tissue damage for lung and liver and (2) histological examinations of these tissues. This experimental design would be the most optimal method to test my proposed mechanism because it would assess for lung and liver injury within both the primary and recall response with or without the contribution from CD8+ T cells; however, there is one foreseeable limitation. Depleting CD8+ T cells by anti-CD8 antibodies would result in the loss of all cell types expressing CD8. Hence, there may be some confounding factors. The next experiment that would be required to test out this hypothesis is performing these similar experiments; however, within a secondary infection model. Combining the methodology from this paragraph and the previous (intranasal IAV inoculation) would be a good means to test out this hypothesis within a secondary infection model.

5. Summary and significance

Our current understanding of how sepsis-induced immunosuppression impacts primary and recall CD8+ T cell responses is unclear. Herein, I report that both primary and recall CD8+ T cell responses are either enhanced or dampened during protracted sepsis, in a tissue context-dependent manner. Within the peritoneal cavity, both primary and recall CD8+ T cell responses are dampened with an associated reduction in their exhaustion phenotypes during sepsis-induced immunosuppression. Furthermore, I also discovered that primary CD8+ T cell responses within the spleen and lungs are enhanced during protracted sepsis, while the liver demonstrates a reduced response. In terms of recall CD8+ T cell responses within the spleen, there was an enhanced response during sepsis induced immunosuppression. Moreover, antigen specific CD8+ T cells during the primary and recall CD8+ T cell responses within the spleen, lungs and liver are associated with an enhanced exhausted phenotype.

These findings are extremely important to the field of sepsis. Firstly, this research has filled some of the gaps regarding how sepsis-induced immunosuppression effects the primary and recall CD8+ T cell responses. Secondly, the findings presented herein are contrary to the current notion that CD8+ T cell responses are dampened during sepsis-induced immunosuppression, which is significant as it raises the question whether antigen specific CD8+ T cell responses are enhanced or diminished during protracted sepsis. Thirdly, these novel findings may affect how sepsis-induced immunosuppression is viewed and treated within the clinic as the majority of the focus for treating protracted sepsis involves immunostimulants. This immunostimulatory treatment may exacerbate the CD8+ T cell response, which may in turn lead to significantly more tissue damage and mortality. Hence, this study is extremely relevant to the field of sepsis as it could potentially alter the commonly accepted notion that CD8+ T cell responses are dampened during protracted sepsis, which in turn would lead to new methods of treatment for the immunosuppression seen in sepsis.

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Appendices

Appendix 1

Cecal Ligation and Puncture Protocol:

List of ingredients needed:

1. Surgical pack (sterilized by autoclave)
    - Rat tooth forceps
    - Scissors (cutting tissue)
    - Scissors (cutting suture)
    - Adson forceps
    - Needle driver
    - Autoclave strip (place between drapes – necessary to ensure the inside of the pack is sterile)
2. Cleaning material
    - Bacteriostat soap (pink bottle – step 1)
    - Ethanol (step 2)
    - 2% chlorohexidine (blue bottle – step 3)
    - Gauze (cut up into quarters)
    - Clippers (with brush to clean between mice; we have two-sets of clippers, one is small and good for first timers, the other is big and should be used when surgeon is skilled because it can cause skin damage)
3. Preparation and recovery
    - Anal thermometer
    - Lubrification jelly (important for inserting thermometer in anus)
    - Eye ointment (very important – there eyes stay open during surgery so need this to prevent eye damage)
    - Analgesic (buprenorphine; 0.05mg/kg dosage)
    - 1mL needle (1 needle per mouse)
    - Saline
    - 4 different colored sharpies (black, blue, green, red; identifier)

- Ruler
- Scale

4. Surgery
    - 27.5G needle (depends on severity of sepsis you want to induce)
    - Silk suture (suture skin, but also used for ligation)
    - Vicryl suture (suture peritoneum)
    - Gauze (sterile)
    - Drape (with hole in it so you can just see abdomen)
    - Bead sterilizer
    - Sterilized PBS

Walkthrough of experimental procedure

1. Weigh the mouse and mark his tail with a sharpie (color, number of stripes)
2. Place mouse in induction chamber to anesthetize the mouse
    a. Oxygen flow rate is 1 L/min
    b. Put the dial for isoflurane at 4%
    c. Make sure the oxygen is on and it is at a pressure of about 55kPa
3. Wait until the mouse reaches a state of deep anesthesia (~3-5 minutes)
    a. There breathing is very exaggerated, and slower
    b. If you move the chamber they should fall on their side and not move
4. Once reached deep anesthesia, set the isoflurane to 0 to flush out the isoflurane prior to taking them out (~30 seconds)
5. Once out of the chamber, inject them subcutaneously with the analgesic (buprenorphine) at a dosage of 0.05mg/kg.
    a. For a 30g mouse, put 50ul of buprenorphine (we have) into 950ul of sterile PBS (makes 10 needles of 0.1mL)
6. After injection, put a drop of eye ointment onto their eyes (do not touch their eyes with the cap, make sure it is just the ointment touching the eyes).

Note: if they are waking up too soon, put them back into the chamber. If they are deeply anesthetized, you should be able to inject and apply eye ointment prior to them waking up

7. Place them on their back and put them into the cone.
    a. Make sure the hole in the cot is big enough to fit their nose in but not too big to allow for passage of gas into the air (harmful to the surgeon)
    b. Make sure that you change the passage of gas from the chamber to the cone
    c. Set the isoflurane to 2% (maintenance)
8. Check their anal temperature
    a. Put lubrification jelly on the thermometer reader and insert the tip into their anus and get a reading
9. Clip (generously) the hair on their abdomen
    a. For this, there are a few technique points that I can offer
    b. Use your index and middle finger of the hand not using the clippers and push down on their hind legs to help elongate the area you are going to clip and prevent any obstructions
    c. Depending on the clipper, press decently hard to make sure to cut the hair
    d. If there is a path of hair, use your other hand to pinch the skin up to make it easier to cut
10. Once a good portion of their hair is clipped, you need to disinfect and clean the area prior to surgery
    a. Use the bacteriostat soap first, put a drop or two on some gauze and rub it on the abdomen, in circular motion
    b. Spray the abdomen with ethanol (get rid of soap) and grab a different gauze and rub up (while applying pressure) towards their head (disinfect the surrounding area and area of abdomen
    c. Put a few drops of the 2% chlorohexidine onto some gauze and rub up (just like alcohol)
11. After they are cleaned and prepared, transfer them to the surgical area.

12. Check their reflexes (make sure they won't feel the surgery). Do a toe pinch to check if they are in deep anesthesia, if so, you can press hard and their legs wont jerk.
13. Once they have no reflexes, place the drape over them and make sure that the hole in the drape is placed over the surgical area (abdomen)
14. Pick up the skin with the Adson's forceps and using the scissors cut a vertical slit in the middle of the abdomen
    a. They have a bone that will be protruding and higher up than anything else near their thorax area, use this high peak to know where to cut. Cut ~3cm down from this protrusion.
    b. Make sure that when you pinch the skin with the forceps that the skin is folding upwards horizontally, if it is not, when you cut the skin, the hole will be diagonal. The best outcome is when the cut you make is vertical as it is easier to suture and will heal the best
15. Once the skin is cut, grab the peritoneum with the rat tooth forceps and pull up to tent the peritoneum. Cut with the scissors, and try to make the hole vertical just as the skin
    a. The peritoneum is very sturdy, so you do not need to be gentle with it
    b. There is a portion is the middle of the peritoneum that is clearer and more translucent, try to cut through this part as there are no blood vessels present. If you cut through the thicker parts, there is a potential for the mouse to bleed, although not significant, it is best to minimize bleeding.
16. Once the peritoneum and skin are open, use the Adson's and rat tooth forceps to find the cecum of the mouse
    a. This is the hardest part, but I have some pointers that can help
    b. The cecum is large and is a different color than the small intestines, when you are looking into the abdominal cavity, it is easy to find the cecum based on the color, it is darker than the small intestine.
    c. The cecum is usually on the mouses left side, so your right when you are looking into their cavity, however, it can be found on the other side too (not set in stone)

    d. Fatter mice are much harder to deal with as they have a lot of adipose tissues in the cavity, which makes it hard to find especially if you made a small incision. However, you can take out some of the fat and place it on a gauze near the incision so you can see more clearly in the cavity (make sure to keep it moist)

17. Once the cecum is located, take it out onto the piece of gauze that you place near the opening of the incision. Make sure to use PBS to keep the tissue moist. After wetting the tissue, flatten it by pushing down with your forceps and pushing the contents of the cecum towards the apex of the cecum.
    a. Pushing the contents and making the cecum flat will help with standardization of the procedure as you would control for fecal matter at the apex (or at least try your best)
    b. Once you flatten out the cecum, make sure to put the rest back into the cavity as you only need a little bit of the cecum of to perform the procedure
18. Using the silk suture, make a loop and feed the cecum through the loop (from the apex) until you have reached the desired length of ligation. And tighten the loop so it clenches the cecum
    a. The length from the apex will vary depending on the severity of sepsis. In my mode, I use a ligation of 0.5cm from the apex.
    b. You can do a surgical knot on top of the other knot to make sure it will stay in place
19. Perforate the cecum while simultaneously holding it with the Adson's forceps. Once you pull out the needle, make sure to squeeze the forceps so you will get the excretion of the fecal matter from the pores formed.
20. Put the cecum back into the abdominal cavity
    a. Preferably put it back in the spot that you found it as you want minimal alterations to the locations of organs
    b. Make sure that the stool that is protruding the pore, does not get wiped away on the skin, by holding up the peritoneum high with the rat tooth forceps, you can easily place the cecum back in without it touching the skin, or incision site

21. Suture the peritoneum with the vicryl and suture the skin with the silk.
    a. Make sure there are no holes in the peritoneum, this is important if you need to collect PECs and, you do not want the organs to protrude out of the peritoneum.
    b. When stitching, make sure you are not stitching with the peritoneum (or skin) another tissue
    c. When putting in the needle for stitching, make sure to be close to the edge of the incision, as you want it to close beside each other and not a tissue being on top of the other
    d. Do surgical knots for each stitch and make sure to do in this way: 1 loop around needle driver and pull, then another time with 1 loop and pull, 2 loop around and then pull
    e. When stitching the skin, elongate the incision vertically, so you can see exactly where you need to put stitches to make sure the skin does ruffle, you want the skin stitches to be precise so it will heal properly and there will be no holes in the skin.
22. Once finished stitching them up, prepare a needle with warmed saline. Inject the mice with 1mL of warmed saline subcutaneously and put them in a recovery cage
    a. The saline should be heated using heating labs or hot water. Test the heat by pressing the saline against your inside forearm, if it's too hot, wait for it to cool, it should feel warm on your skin but not boiling not.
    b. The recovery cage should have paper towels on the bottom with none of the usual cage popcorn. This towel will show you if a mouse is bleeding a lot and when they wake up from anesthesia, they might try to eat the popcorn.
    c. Watch them as they recover and once, they are back to their normal self, place them into a new clean cage.

Appendix 2

Variable	Score and description
Appearance	0 – Coat is smooth
	1 – Patches of hair piloerected
	2 – Majority of back is piloerected
	3 – Piloerection may or may not be present, mouse appears "puffy"
	4 – Piloerection may or may not be present, mouse appears emaciated
Level of consciousness	0 – Mouse is active
	1 – Mouse is active but avoids standing up
	2 – Mouse activity is noticeably slowed. The mouse is still ambulant
	3 – Activity is impaired. Mouse only moves when provoked, with possible tremor
	4 – Activity is severely impaired. Mouse remains stationary when provoked, movements have tremor
Activity	0 – Normal amount of activity. Mouse is any of: eating, drinking, climbing, running, fighting
	1 – Slightly suppressed activity. Mouse is moving around bottom of cage
	2 – Suppressed activity. Mouse is stationary with occasional investigative movements
	3 – No activity. Mouse is stationary
	4 – No activity. Mouse experiencing tremors, particularly in the hind legs
Response to stimulus	0 – Mouse responds immediately to auditory stimulus or touch
	1 – Slow or no response to auditory stimulus; strong response to touch (moves to escape)
	2 – No response to auditory stimulus; moderate response to touch (moves a few steps)
	3 – No response to auditory stimulus; mild response to touch (no locomotion)
	4 – No response to auditory stimulus. Little or no response to touch. Cannot right itself if pushed over

Eyes	0 – Open
	1 – Eyes not fully open, possibly with secretions
	2 – Eyes at least half closed, possibly with secretions
	3 – Eyes half closed or more, possibly with secretions
	4 – Eyes closed or milky
Respiration rate	0 – Normal
	1 – Slightly decreased respiration (rate not quantifiable by eye)
	2 – Moderately reduced respiration (rate at the upper range of quantifying by eye)
	3 – Severely reduced respiration (rate easily countable by eye, 0.5 s between breaths)
	4 – Extremely reduced respiration (>1 s between breaths)
Respiration quality	0 – Normal
	1 – Brief periods of laboured breathing
	2 – Laboured, no gasping
	3 – Laboured with intermittent gasps
	4 – Gasping

Appendix 3

Western

2018-122:10

AUP Number: 2018-122
AUP Title: Sepsis Related Immune Responses
Yearly Renewal Date: 06/01/2023

The **annual renewal** to Animal Use Protocol (AUP) 2018-122 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
    - Western's Senate MAPP 7.12 [PDF]; and
    - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
    - Completed the appropriate institutional OH&S training;
    - Completed the appropriate facility-level training; and
    - Reviewed related (M)SDS Sheets.

Submitted by: Cristancho, Martha on behalf of the Animal Care Committee

Appendix 4

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Aug 15, 2022

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Curriculum Vitae

Mr. Alex Roland Michaud
Sex: Male
Date of Birth: 4/17
Canadian Residency Status: Canadian Citizen

Research Disciplines: Immunology
Areas of Research: Diseases of the Immune System
Fields of Application: Biomedical Aspects of Human Health
Research Specialization Keywords: sepsis; flow Cytometry; mice models

Degrees

2020/9 (2022/8) - Master's Thesis, Master's of science, immunology, University of Western Ontario

2016/9 - 2020/4 - Bachelor's Honours, Medical science honours, Double Major in microbiology and immunology and interdisciplinary medical science, University of Western Ontario

Recognitions

2021/9 – Dr. Robert George Everitt Murray Graduate Scholarship in Microbiology and Immunology – 10,000$, University of Western Ontario

2020/9 - Dr. Frederick W. Luney Graduate Entrance Scholarship in Microbiology and Immunology - 2,000$, University of Western Ontario

2020/6 - The University of Western Ontario Gold Medal University of Western Ontario

2016/9 - 2020/6 - Dean's Honor List

2016/9 - The Western scholarship of excellence - 2,000$, University of Western Ontario

Publications

2022/6 - Yao T, Rudak PT, Laumont CM, **Michaud AR**, Rashu R, Knier NN, Foster PJ, McWilliam HEG, Villadangos JA, Nelson BH, DiMattia GE, Shepherd TG, Haeryfar SMM. MAIT cells accumulate in ovarian cancer-elicited ascites where they retain their capacity to respond to MR1 ligands and cytokine cues. Cancer Immunol Immunother. 2022 May;71(5):1259-1273. doi: 10.1007/s00262-021-03118-9. Epub 2021 Dec 2. PMID: 34854949.

Employment

2020/9 - Masters' student, Microbiology and Immunology, Schulich school of medicine and dentistry, University of Western Ontario

Courses Taught

2020/09/09 - 2020/12/31 - Graduate student, Microbiology and immunology, University of Western Ontario

Teachers' assistant, University of Western Ontario

Course Title: Clinical Immunology
Course Topic: immunology
Course Level: Undergraduate

2021/09/09 - 2021/12/31 - Graduate student, Microbiology and immunology, University of Western Ontario

Teachers' assistant, University of Western Ontario

Course Title: Clinical Immunology
Course Topic: immunology
Course Level: Undergraduate

Community and Volunteer Activities

2013/2 - 2016/9 - Receptionist at wellness centre, YMCA Stoney Creek

Service at reception desk, providing assistance and resources to guests and visitors, communicating in a professional manner Assistance in the achievement of client's personal fitness goals, by providing guidance and direction during training sessions Maintenance of cleanliness through regular cleaning of the facility

2016/1 - 2016/7 - Recreation therapy, Mount hope centre for long-term care

Contribution to enhancing the quality of life of patients through the organization of daily events and activities such as card games, trivia days, outings into the community Assurance of happiness and satisfaction by interacting, and engaging in meaningful conversations with individuals living with mild to severe dementia Demonstrated compassion and initiative to follow-up with patients and assess how they are doing