5-14-2014 12:00 AM

Analysis of Invariant Natural Killer T Cells in Intra-Abdominal Sepsis

Ram Venkatesh Anantha
The University of Western Ontario

Supervisor
Dr. John K. McCormick
The University of Western Ontario Joint Supervisor
Dr. S.M. Mansour Haeryfar
The University of Western Ontario

Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

Analysis of Invariant Natural Killer T Cells in Intra-Abdominal Sepsis

(Thesis Format: Monograph)

by

Ram Venkatesh Anantha

Graduate Program

In

Microbiology and Immunology

Thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science

The School of Graduate and Postdoctoral Studies

Western University

London, Ontario, Canada

Abstract

Sepsis is characterized by a severe systemic inflammatory response to infection that is associated with high morbidity and mortality despite optimal care. Invariant natural killer T (iNKT) cells are potent regulatory lymphocytes that can produce pro- and/or anti-inflammatory cytokines, thus shaping the course and nature of immune responses; however, little is known about their role in sepsis. We demonstrate here that patients with sepsis/severe sepsis have significantly elevated proportions of circulating iNKT cells in their peripheral blood, as compared to non-septic patients. We therefore investigated iNKT cells in mice with intra-abdominal sepsis (IAS). Our data show that iNKT cells are pathogenic in IAS, and that T helper (Th)2-type polarization of iNKT cells using the synthetic glycolipid OCH significantly reduced mortality from IAS. This reduction in mortality is associated with the systemic elevation of the anti-inflammatory cytokine interleukin (IL)-13, and reduction of several pro-inflammatory cytokines within the spleen, notably IL-17. Finally, we show that administration of OCH in septic mice is associated with significantly reduced apoptosis of splenic T and B lymphocytes, as well as macrophages, but not natural killer cells. We propose that modulation of iNKT cell responses towards a Th2 phenotype may be an effective therapeutic strategy in sepsis.

Keywords

Sepsis, Intra-abdominal Sepsis, invariant Natural Killer T cells, Peritonitis, Animal Model

Acknowledgements

My desire to enrol in graduate studies was not simply a realization of what I found to be intellectually challenging, but a declaration of the role I wanted to play as a surgeon-scientist: a researcher who contributes to the science of surgery for the improvement of patient care and an educator who participates in the development of future generations of aspiring surgeon-scientists. I am grateful to the Division of General Surgery and my program directors, Dr. Ken Leslie and Dr. Michael Ott, for providing me with protected time to conduct my research within my residency program. I am also grateful to them and to Dr. Tina Mele for providing me with strong mentorship support and career advice.

I am grateful to the Department of Microbiology and Immunology at Western University for giving structure and discipline to my graduate training, and providing such a stimulating environment where researchers supported one another and were eager to share their knowledge and expertise with me. I would also like to thank Dr. Douglas Fraser and Dr. Claudio Martin for giving me access to the Critical Care and Trauma Centre as well as the Medical-Surgical Intensive Care Unit at the London Health Sciences Centre to obtain blood samples from critically-ill patients.

I would like to especially thank my supervisors, Dr. John McCormick and Dr. Mansour Haeryfar, for giving me the independence to pursue my research interests and set up my own experiments, and yet always being available to discuss results and provide novel interpretations of my data. I have learned so much through my discussions with them, and I am grateful for their positive encouragements and their sheer excitement at seeing new results. I would also like to thank the lab members of both the Haeryfar and McCormick laboratories: Delfina, who has been an invaluable source of information and technical tips; and Kelcey, Brent, Katie, Stacey, Kevin,

Joe, Arya, Peter, Maryam, Courtney, Ankur, Matthew, and Jin. All of them have been so helpful and supportive, and it has been a pleasure working with them.

Lastly, I would like to thank my wife and son, for always being there. This was a tough couple of years for all of us but we made it, thanks to you. I love you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

IAS	Intra-abdominal Sepsis
7-AAD	7-aminoactinomycin D
ACCP	American College of Chest Physicians
ACK	Ammonium-Chloride-Potassium (for lysis buffer)
ALI	Acute Lung Injury
ALT	Alanine transaminase
ANOVA	Analysis of Variance
APACHEII	Acute Physiology and Chronic Health Evaluation II
APC	Antigen Presenting Cell
APC	Allophycocyanin
aPTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
ATS	American Thoracic Society
B6	C57BL/6J mice
BCL-2	B-cell lymphoma 2
BHI	Bovine Heart Infusion
BTLA	B- and T-lymphocyte attenuator
CASP	Colon Ascendens Stent Peritonitis
CCTC	Critical Care and Trauma Centre
CD	Cluster of Differentiation
CDR3	Complementarity Determining Region
CFU	Colony Forming Unit

CIA	Collagen-induced Arthritis
CLI	Cecal Ligation and Incision
CLP	Cecal Ligation and Puncture
CMV	Cytomegalovirus
cRPMI	complete Roswell Park Memorial Institute (medium)
DC	Dendritic cells
EGDT	Early Goal Directed Therapy
ELISA	Enzyme-linked Immunosorbent Assay
ESICM	European Society of Intensive Care Medicine
FACS	Fluorescence-activated Cell Sorting
FasL	First Apoptosis Signal Ligand
FasR	First Apoptosis Signal Receptor
FiO2	Fraction of inspired oxygen
FIP	Fecal-induced peritonitis
FITC	Fluorescein isothiocyanate
FS	Fecal slurry
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GSL	Glycosphingolipid
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
i.p.	Intraperitoneal

i.v.	Intravenous
IAS	Intra-abdominal Sepsis
IFN-gamma	Interferon-gamma
IL	Interleukin
INR	International Normalized Ratio
ISDC	International Sepsis Definitions Conference
kg	Kilogram
KRN7000	Synthetic alpha-galactosylceramide
L	Litre
LHSC	London Health Sciences Centre
LOS	Length of Stay
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAP	Mean Arterial Pressure
MDSC	Myeloid Derived Suppressor Cells
mg	Milligrams
MHC	Major Histocompatibility Complex
mmHg	Millimeters of Mercury
mmol	Millimoles
MPI	Mannheim Peritonitis Score
MSC	Mesenchymal Stem Cells
MS-ICU	Medical/Surgical-Intensive Care Unit
MSS	Murine Sepsis Score

NK cell	Natural Killer cell
NK1.1	Natural Killer 1.1
NKT cell	Natural Killer T cell
NOD	Non-obese diabetic
OCH	Sphingosine truncated derivative of alpha-galactosylceramide
PAF	Platelet Activating Factor
PaO2	Partial pressure of oxygen
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PMN	Polymorphonuclear cells
pRBCs	packed Red Blood Cells
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute (medium)
RR	Respiratory rate
SBP	Systolic Blood Pressure
SCCM	Society for Critical Care Medicine
SD	Standard Deviation
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome

SIS	Surgical Infection Society
SSC	Surviving Sepsis Campaign
T	Temperature
TCR	T-cell Receptor
TGF-β	Transforming growth factor-beta
Th	T helper response
TNF	Tumour Necrosis Factor
Treg	Regulatory T cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U/L	Units/Litre
WBC	White Blood Cells
zVAD.fmk	Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone
α-GalCer	α-Galactosylceramide

Chapter 1: Introduction

1.1 Introduction to Sepsis

1.1.1 Epidemiology of Sepsis

Sepsis is defined as an overwhelming systemic inflammatory response to an infection [1]. It is one of the leading causes of death among patients in non-coronary intensive care units [2, 3], and the tenth leading cause of death overall in North America [4]. With a mortality rate of 20% to 50% in the acute setting [5], sepsis also substantially reduces the quality of life among survivors [6, 7]. The management of sepsis also presents a huge financial burden for the healthcare system: the care of septic patients costs as much as $50,000 per patient [8], resulting in an economic burden of nearly $17 billion annually in Canada and the United States [2]. It is more worrisome that a 75% increase in the number of patients diagnosed with severe sepsis has been observed over the past two decades. This may be explained partly by the improved care of the increasing number of individuals surviving into their 70s, 80s, and 90s and by the associated co-morbidities of the elderly, including cancer and diabetes [9]. Therefore, as the general population continues to age, the incidence of sepsis is projected to increase significantly in the forthcoming years, leading, for example, to over 1 million cases of severe sepsis in 2020 in the United States alone [2].

1.1.2 Diagnosis of Sepsis

Definitions of sepsis, severe sepsis, and septic shock were previously based on expert advice, using criteria that identified progression of the infection along with appropriate physiological responses [10]. In particular, the presence of the systemic inflammatory response syndrome (SIRS; **Table 1**) was suggested to be a precursor of severe sepsis [11].

Table 1: Clinical criteria for severe inflammatory response syndrome (SIRS).

SIRS Criteria
At least two or more of the following:
1. Temperature >38°C or < 36°C
2. Heart Rate > 90/min
3. Respiratory Rate > 20/min or $PaCO_2$ < 32 mmHg
4. White Blood Cell Count >12 × 10^9 / L or < 4 × 10^9 / L

However, studies illustrating the limited value of SIRS criteria in predicting the risk of developing organ dysfunction, shock, and death [12-14], prompted the development of new scoring systems and clinical criteria. Consequently, the 2001 International Sepsis Definitions Conference (ISDC), sponsored by the Society for Critical Care Medicine (SCCM), the European Society of Intensive Care Medicine (ESICM), the American College of Chest Physicians (ACCP), the American Thoracic Society (ATS), and the Surgical Infection Society (SIS), expanded the list of signs and symptoms of sepsis (Tables 2 and 3) to reflect clinical bedside experience [15]. These definitions of sepsis, severe sepsis, and septic shock were also based on consensus guidelines and expert opinion, and exhibit broad physician endorsement. Additionally, evidence-based recommendations from the Surviving Sepsis Campaign (SSC) Management Guidelines Committee [16] provided treatment algorithms to appropriately resuscitate and manage patients with sepsis.

Table 2: Diagnostic criteria for sepsis.

Infection, documented or suspected, and two or more of the following:
General variables
Fever (>38.3°C)
Hypothermia (core temperature <36°C)
Heart rate >90 min^{-1} or >2 SD above the normal value for age
Tachypnea
Altered mental status
Significant edema or positive fluid balance (>20 mL/kg over 24 hrs)
Hyperglycemia (plasma glucose >140 mg/dL or 7.7 mmol/L) in the absence of diabetes
Inflammatory variables
Leukocytosis (WBC count >12,000 μL^{-1})
Leukopenia (WBC count <4000 μL^{-1})
Normal WBC count with >10% immature forms
Plasma C-reactive protein >2 SD above the normal value
Plasma procalcitonin >2 SD above the normal value
Hemodynamic variables
Arterial hypotension (SBP <90 mm Hg; MAP <70 mm Hg; or an SBP decrease >40 mm Hg in adults or >2 SD below normal for age)
Organ dysfunction variables
Arterial hypoxemia (PaO_2/FIO_2 <300)
Acute oliguria (urine output <0.5 mL/Kg hr or 45 mmol/L for at least 2 hrs, despite adequate fluid resuscitation)
Creatinine increase >0.5 mg/dL or 44.2 µmol/L
Coagulation abnormalities (INR >1.5 or a PTT >60 secs)
Ileus (absent bowel sounds)
Thrombocytopenia (platelet count, <100,000/µL)
Hyperbilirubinemia (plasma total bilirubin >4 mg/dL or 70 µmol/L)
Tissue perfusion variables
Hyperlactatemia (> upper limit of lab normal)
Decreased capillary refill or mottling

Abbreviations: WBC: white blood cell; SBP: systolic blood pressure; MAP: mean arterial pressure; INR: international normalized ratio; aPTT: activated partial thromboplastin time.

Table 3: Diagnostic criteria for severe sepsis.

Severe sepsis - sepsis-induced tissue hypoperfusion or organ dysfunction (any of the following thought to be due to the infection):
Sepsis-induced hypotension
Lactate greater than the upper limits of normal laboratory results
Urine output <0.5 mL/kg.hr for >2 hrs, despite adequate fluid resuscitation
ALI with PaO2/FIO2 <250 in the absence of pneumonia as infection source
ALI with PaO2/FIO2 <200 in the presence of pneumonia as infection source
Creatinine >2.0 mg/dL (176.8 µmol/L)
Bilirubin >2 mg/dL (34.2 µmol/L)
Platelet count <100,000
Coagulopathy (INR > 1.5)

1.2 Management of Sepsis

The management and treatment of sepsis has evolved dramatically over the last forty years [17, 18]. While there is an abundance of treatment algorithms for managing patients with sepsis, I will briefly highlight the most critical therapeutic strategies, as recommended by the Surviving Sepsis Campaign [16, 19, 20].

1.2.1 Early Goal-Directed Therapy

The landmark study by Rivers *et al* [21] emphasized the concept of early goal-directed therapy (EGDT) in the treatment of sepsis: measures to improve physiological parameters, such as blood pressure and tissue oxygen delivery, *immediately* upon diagnosis of sepsis significantly reduced patient mortality, disease severity scores, and severity and duration of organ dysfunction [22]. These measures included the use of fluids, vasopressors, and packed red cells, and early initiation of mechanical ventilation to attain physiologically-normal hemodynamic parameters as rapidly as possible. The overwhelmingly positive results reported by Rivers *et al* prompted hospitals to deploy "sepsis teams" and "critical care outreach teams" to manage patients with severe infections in the wards [20, 23-26], resulting in improved clinical outcomes [27].

1.2.2 Antibiotic Therapy

Early parenteral broad-spectrum antimicrobial therapy significantly improves clinical outcomes in sepsis [28]. Antibiotic administration within four hours of diagnosing elderly patients (age over 65) with community-acquired pneumonia significantly reduced in-hospital mortality, 30-day mortality, and length of stay in hospital [28]. Every additional hour without antibiotics increased the risk for death in hypotensive septic patients by 7.6% during the first 6 hours [29]. However, treatment effectively targeting the responsible pathogen is critical [30], since the ineffectiveness of antimicrobial treatment against a micro-organism identified in blood

cultures is strongly associated with death [31]. Compliance with the SSC guidelines, however, remains low with respect to antibiotic administration: the mean delay to first infusion of antibiotics remained in excess of 3 hours [23], and as many as 68% of patients did not receive their first dose within this period [19].

1.2.3 Hemodynamic Resuscitation

Efficient restoration of circulating blood volume is the primary goal of resuscitation in septic patients [32], although modalities of treatment continue to evolve. The use of crystalloids rather than colloids is supported by current literature. While both result in similar ejection stroke volume and oxygen delivery [33], patients receiving colloids had greater renal impairment [34]. The use of albumin in sepsis also remains controversial. Although it was the first product to be broadly used for intravenous fluid loading, a meta-analysis comparing albumin with other fluid loading agents identified an increased risk for death among patients who received albumin for supportive treatment during shock [35]. In septic patients with hypoalbuminemia, however, the use of albumin improved fluid loading [36], although its cost-effectiveness has been questioned [37]. Since the publication of the EGDT results by Rivers *et al* [21], the transfusion of packed red cells has also been regarded as a valuable approach to improving tissue oxygenation. Liberal transfusion of blood, however, has been shown to be potentially ineffective [38, 39]. However, in the setting of severe sepsis and septic shock the theoretical risks appear balanced by the benefits in terms of tissue oxygenation [40].

1.2.4 Vasoactive Drugs

Vasopressors may allow therapies to be applied earlier and more aggressively in order to improve physiological parameters [41], although their influence on mortality is unclear [42]. Norepinephrine and dobutamine improve hepatic and splanchnic circulation [43], while

dopamine and epinephrine are vasoconstrictors that also increase cardiac output. However, the latter may cause harmful metabolic effects if used inappropriately [44]. Vasopressin (an analogue of anti-diuretic hormone) is recommended as a second-line vasopressor, although it may be used as a first-line agent in the treatment of septic shock in select cases [45, 46].

1.2.5 Adjunct Therapeutic Strategies

Low tidal volume mechanical ventilation and strict blood glucose control are crucial components of the care provided to critically-ill patients. Low tidal volume (≤ 6 ml/kg) improves survival in patients with acute respiratory distress syndrome [47, 48], compared to "standard" mechanical ventilation (12 ml/kg). Landmark studies by van den Berghe *et al* [49] suggested that aggressive insulin therapy improved 30-day survival in critically-ill patients, and dramatically reduced their morbidity and length of hospital stay.

1.2.6 Prognosis in Sepsis

Earlier identification of patients with sepsis (through guidelines and training of healthcare personnel) and improved treatment algorithms have significantly reduced the early mortality in sepsis [17]. Most patients survive the early hyper-inflammatory phase of sepsis and enter a more protracted phase [50, 51]: more than 70% of deaths in sepsis occur after the first 3 days of the disorder, with many deaths occurring weeks later. If the patient dies in the first few days, death will probably have been caused by cytokine-driven hyper-inflammation and multiple organ failure, especially cardiovascular collapse (**Figure 1**). In many situations of protracted sepsis, however, death is due to the family's decision to change from aggressive support measures to comfort measures because of the patient's many, severe pre-existing comorbidities and small probability of meaningful recovery.

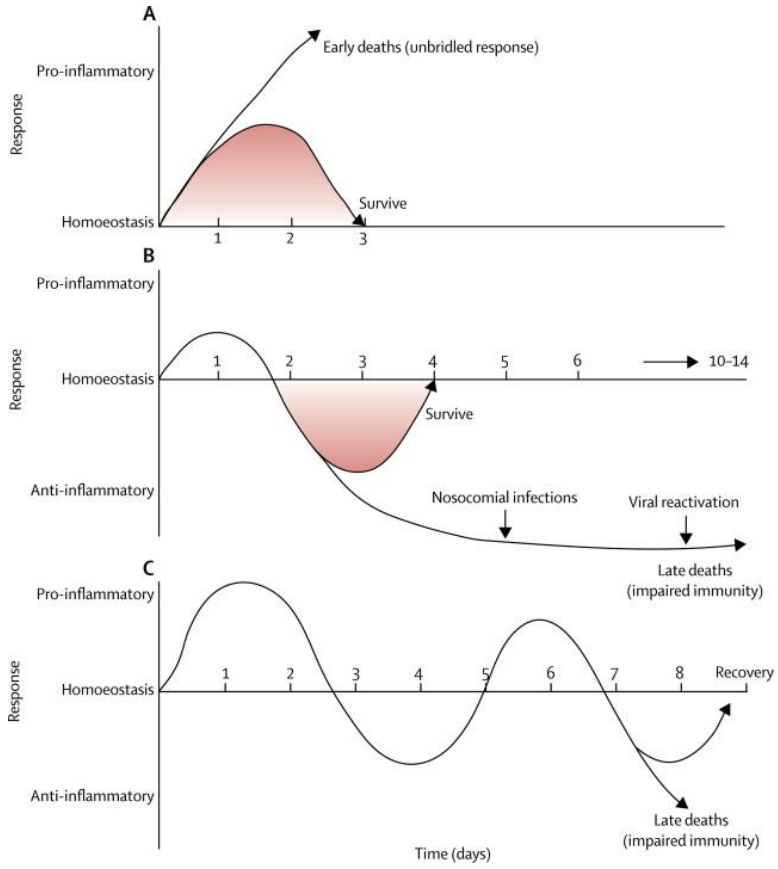

Figure 1: Inflammatory responses among septic patients.

This figure was adapted from Hotchkiss *et al* [17]. (A) The initial response in otherwise-healthy patients with severe sepsis is characterized by an overwhelming hyper-inflammatory phase with fever, hyperdynamic circulation, and shock. Deaths during this phase are generally due to cardiovascular collapse, metabolic and physiologic disruption, and multi-organ failure. (B) In elderly patients with numerous comorbidities that impair immune response, sepsis results in a blunted or absent hyper-inflammatory phase. Patients rapidly develop impaired immunity and an anti-inflammatory state, and may die from secondary infections. (C) Some patients with sepsis alternate between hyper- and hypo-inflammatory states, especially if they develop superimposing infections. They eventually become severely immunosuppressed and may die from secondary infections or organ failure.

In a post-mortem study, Torgersen *et al* determined that 80% of surgical patients admitted to an intensive care unit had unresolved septic foci; only 52 of 97 autopsy-confirmed pneumonias were appropriately diagnosed during their intensive-care admission. Additionally, peritonitis accounted for many unresolved septic foci. While such ongoing infections are not necessarily the main cause of death, the real cause of death and organ failure in most patients dying of sepsis is still unclear.

1.3 Immunology of Sepsis

1.3.1 Immunological Mechanisms in Sepsis

In sepsis, systemic exposure to pathogenic microbial lipids initiates a complex and dysregulated immune response [18, 52, 53]. Macrophages and antigen-presenting cells (APCs) recognize and phagocytose invading bacteria. These cells subsequently produce pro-inflammatory cytokines, including interleukin (IL)-1β, tumour necrosis factor (TNF), and IL-6, as well as chemokines such as IL-8 [18]. Following the recruitment of neutrophils and lymphocytes, and the resulting surge of more pro-inflammatory cytokines such as interferon (IFN)-γ [54-57], some patients develop an overwhelming hyper-inflammatory response with systemic physiological effects [18]. The intensity of this initial hyper-inflammatory phase is determined by factors such as pathogen virulence, bacterial load, host genetic factors, age, and patient comorbidities. In 30% of cases, mortality occurs within the first 72 hours because of the cytokine-storm-mediated inflammatory response that leads to septic shock and multiple organ failure.

Opposing anti-inflammatory processes are also concomitantly initiated to mitigate the pro-inflammatory state. Studies of circulating cytokines in 464 patients with community-

acquired infections demonstrated that, in addition to pro-inflammatory cytokines, concentrations of the potent anti-inflammatory cytokine interleukin 10 (IL-10) were significantly increased [58]: additionally, a high ratio of IL-10 to TNF-α correlated with mortality in these patients. Other studies have also documented a global cytokine depression in sepsis, with reduced production of pro- and anti-inflammatory cytokines [59-61]. When whole blood from patients with and without sepsis was stimulated with endotoxin, the production of TNF-α, IL-1β, and IL-6 from septic patients was less than 10‑20% of that found in non-septic patients [59]. Lipopolysaccharide-stimulated monocytes from septic patients had profoundly decreased production of TNF-α, IL-1β, and IL-6, compared to controls [60]. Similarly, when Sinistro *et al* stimulated blood monocytes from septic and non-septic patients, fewer than 5% of monocytes from the septic group produced cytokines, compared with roughly 15-20% of monocytes from non-septic patients [62]. In a study by Weighardt *et al*, postoperative sepsis was associated with defects in production of both proinflammatory and anti-inflammatory cytokines when monocytes were stimulated by lipopolysaccharide [63]. These results indicate that patients with sepsis either rapidly produce both pro- and anti-inflammatory cytokines, or produce a predominance of anti-inflammatory cytokines, or produce reduced levels of cytokines overall.

1.3.2 Immunosuppression in Sepsis

Immunosuppression also occurs in individual organs during sepsis. In a study by Boomer *et al* [64], lipopolysaccharide-stimulated splenocytes from patients with sepsis had reduced production of both proinflammatory and anti-inflammatory cytokines, less than 10% of that in patients without sepsis. Both spleens and lungs showed upregulated expression of selected inhibitory receptors including programmed cell death 1 (PD-1), expansion of suppressor cells (T regulatory cells and myeloid derived suppressor cells), and concomitant downregulation of

activation pathways [64]. These results confirm that sepsis decreases the response of cells of the innate and adaptive immune systems, and that multiple mechanisms of immunosuppression occur in different organs.

Critically-ill patients who have normal immunity before admission may become profoundly immune-compromised during protracted sepsis as a result of immunosuppression: weakly-virulent or opportunistic pathogens, such as *Stenotrophomonas*, *Acinetobacter*, *Enterococcus*, *Pseudomonas*, and *Candida*, especially affect septic patients with severely depressed host immunity [65, 66]. Additionally, the reactivation of cytomegalovirus (CMV) and herpes simplex virus (HSV) occurred in approximately 33% and 21%, respectively, of critically-ill patients with sepsis who were immune-competent prior to their infection [67, 68]. Meakins *et al* [69] noted that patients with sepsis and trauma had loss of delayed type hypersensitivity response to common recall antigens such as measles and mumps, a finding that correlated with mortality.

1.3.3 Apoptosis in Sepsis

Apoptosis of the innate and adaptive immune systems plays a critical role in the anti-inflammatory and immunosuppressive host response during sepsis. Hotchkiss *et al* observed a striking apoptosis-induced loss of cells of the innate and adaptive immune systems in the spleen, including $CD4^+$ and $CD8^+$ T cells, B cells, and dendritic cells [70, 71]. During a life-threatening infection when clonal expansion of lymphocytes should be occurring, the loss of immune cells is particularly striking, and occurs in all ages, including pediatric and neonatal patients with sepsis [72, 73]. In addition to the widespread apoptosis that occurs in the spleen during sepsis [70, 74-76], Le Tulzo *et al* observed a marked increase in apoptosis among circulating lymphocytes obtained from patients in septic shock compared to critically ill non-septic patients [77]. This

phenomenon is believed to lead to a profound and persistent lymphopenia that is associated with poor outcome.

Caspases are the key enzymes involved in apoptosis, and also play critical regulatory roles in the inflammatory response [78, 79]. They may be divided into two functionally distinct subfamilies: those involved in apoptosis (caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9 and caspase-10) and those related to cytokine processing and regulation of inflammation (caspase-1, caspase-4, caspase-5 and caspase-12) [80, 81]. The pro-inflammatory caspases, such as caspase-1 and caspase-5, are activated after assembly of an intracellular structure, designated the inflammasome, and mediate the cleavage and activation of several pro-inflammatory cytokines, including IL-1β and IL-18 [80].

Apoptosis may be induced through two different pathways: a death receptor-initiated caspase-8-mediated pathway and a mitochondrion-initiated caspase-9-mediated pathway [82, 83]. Either caspase-8 or caspase-9 can activate caspase-3, which is a crucial apoptotic protease in the apoptotic cell-death mechanism. Caspase-8 can be activated by several ligands of the different death receptors, including TNF-α, a key cytokine that increases in patients with sepsis, and CD95L (also known as FasL). The mitochondrial pathway can be activated by a diverse range of stimuli, including reactive oxygen species, radiation and chemotherapeutic agents [84].

Apoptosis contributes to immunosuppression during sepsis through the deletion of critical effector cells including T and B cells, and the induction of anergy (the inability of a lymphocyte to mount a complete response against a specific antigen) and T helper 2 (Th2)-cell responses in surviving immune cells. The apoptosis of T and B cells significantly impairs the adaptive immune response, and, by disabling the cross-talk between the adaptive and innate immune

systems, also impairs the latter [17, 64, 74]. The apoptosis-induced reduction in the number of dendritic cells (DCs), the most potent APCs, further compromises the innate and adaptive immune responses [85]. Apoptosis also induces anergy and T helper 2 (Th2)-cell responses in surviving immune cells [86, 87]. Furthermore, the uptake of apoptotic cells by macrophages and DCs stimulates the release of anti-inflammatory cytokines, including IL-10 and transforming growth factor-β (TGF-β), and suppresses the release of pro-inflammatory cytokines [87]. This potential link between the release of IL-10 by apoptotic cells and immune suppression in sepsis is underscored by studies showing that the circulating concentration of IL-10 is predictive of a fatal outcome in patients with sepsis [58, 88]. In addition, uptake of apoptotic cells by macrophages and DCs does not induce the expression of co-stimulatory molecules: therefore, T cells that come into contact with APCs that have ingested apoptotic cells might either become anergic or undergo apoptosis themselves [87].

1.3.4 Immunotherapy of Sepsis

Given the increasing knowledge about the mechanisms and effectors in sepsis, more than thirty clinical trials of immunotherapeutic agents were initiated. The results were disheartening: none showed any benefit and even worse, some drugs demonstrated reduced survival rates [89, 90] and were prematurely terminated. The paucity of knowledge about the molecular pathophysiology of sepsis, the inability of animal models to correctly mimic the pathophysiological processes leading to sepsis in humans, and the inability to account for the influence of risk factors such as age, nutrition, gender, and various other co-morbidities in patients [90] were identified as the key reasons for the failure of these trials.

Table 4: Immunotherapeutic agents that have failed in human sepsis trials.

Immunotherapeutic Target	Drug	Company	Result	Ref.
Treatment with anti-endotoxins				
Anti-endotoxin antibodies	Nebacumab	Withdrawn	Increased mortality	[91]
LPS analogs	Eritoran	Eisai	No effect on mortality	[92]
Treatment with antagonists to specific mediators				
TNF				
Anti-TNF antibodies	Afelimomab	Abbott	Marginal reduction in mortality	[93]
TNF receptors	Lenercept	Genentech	No effect on mortality	[94]
IL-1 or IL-1RA	Anakinra	Amgen	No effect in mortality	[95]
Coagulants				
Antithrombin	Antithrombin III	Grifols	No effect on mortality	[96]
Activated protein C	Drotrecogin alpha, rAPC	Eli Lilly	Increased mortality	[97]
Tissue factor pathway inhibitor	Tifacogin	Novartis	No effect on mortality	[98]
PAF				
PAF antagonists	Lexipafant	British Biotechnology Ltd.	No effect on mortality	[99]
PAF-acetylhydrolase	rPAF-AH		No effect on mortality	[100]
PLA2: PLA2 inhibitor	Varespladib	Anthera Pharmaceuticals	No survival benefit	[101]
Immunostimulation therapy				
Immunoglobulins	IVIG	N/A	No effect on mortality	[102]
Granulocyte colony-stimulating factor, IFN-γ Immunonutrition	Molgramostim	Zenotech	Reduced ventilator-dependent days and ICU stay	[103]
Nonspecific interventions				
Corticosteroid therapy	Steroids	N/A (Generic)	No effect on mortality	[104]
High-output hemofiltration	-	N/A	No evidence for use	[105]

Since most patients rapidly progress to an immunosuppressive state, the focus of immunotherapeutic approaches has shifted to the development of methods to augment host immunity. Granulocyte macrophage colony stimulating factor (GM-CSF), a cytokine that activates and induces production of neutrophils and monocytes or macrophages, has shown potential as a treatment agent in sepsis [106, 107]. Patients who were in the immunosuppressive phase of sepsis (as determined by persistent decreases in monocyte HLA-DR expression, a common abnormality in sepsis), were treated with GM-CSF and had restoration of HLA-DR expression, fewer ventilator-dependent days, and shorter hospital and intensive care unit days [106]. In a paediatric sepsis study, Hall *et al* [107] treated immunosuppressed patients with GM-CSF, which reduced the incidence of new nosocomial infections.

Another immunotherapeutic agent is interleukin 7 (IL-7), a cytokine that has been termed the "maestro of the immune system" because of its diverse effects on immunity [108-112]. IL-7 induces proliferation of naive and memory T cells, thereby supporting the replenishment of lymphocytes, which are relentlessly depleted during sepsis [64, 70, 71, 113]. In clinical trials at the National Cancer Institute (NCI), it caused a doubling of circulating $CD4^+$ and $CD8^+$ T cells, and an increase in size of spleen and peripheral lymph nodes by roughly 50% [110]. Similarly, IL-7 significantly increased the levels of circulating $CD4^+$ and $CD8^+$ T cells in HIV-infected patients with persistently low $CD4^+$ T cells despite effective viral suppression [114]. IL-7 therefore reverses profound lymphopenia, a major pathological abnormality in sepsis. IL-7 also has other additional actions that are highly beneficial in sepsis [109, 111, 112]: it increases the ability of T cells to become activated, potentially restoring functional capacity of hyporesponsive or exhausted T cells which typify sepsis [115, 116]; increases expression of cell-adhesion

molecules, which enhance trafficking of T cells to sites of infection [115] and increases T-cell receptor diversity, leading to more potent immunity against pathogens [111, 114].

IL-7 has shown efficacy both clinically and in animal models of infection. A case report of a patient with idiopathic low CD4 T cells with progressive multifocal leukoencephalopathy (PML) showed that IL-7 caused rapid increases in lymphocytes, decreased circulating JC virus, and led to disease resolution [117]. In mice that were chronically infected with lymphocytic choriomeningitis, IL-7 treatment enhanced T-cell recruitment to the infected site and increased T-cell numbers, thereby improving viral clearance [111]. Unsinger *et al* showed that IL-7 restored the delayed type hypersensitivity response, decreased sepsis-induced lymphocyte apoptosis, reversed sepsis-induced depression of IFN-γ (a cytokine that is essential for macrophage activation), and improved survival in murine polymicrobial sepsis [115]. IL-7 was also found to be beneficial in a fungal sepsis model that reproduces the delayed secondary infections typical of patients in intensive care units [118]. IL-7 was able to reverse sepsis-induced T-cell alterations in septic shock patients [116]. *Ex-vivo* treatment of patients' cells with IL-7 corrected multiple sepsis-induced defects including $CD4^+$ and $CD8^+$ T cell proliferation, IFN-γ production, STAT5 phosphorylation, and Bcl-2 induction to that of healthy controls. This functional restoration indicates that the IL-7 pathway remains fully operative during sepsis [116].

IL-7 is in clinical trials in patients with cancer, HIV-1, and PML. It has been well tolerated in more than 200 patients and, unlike IL-2, a closely-related cytokine, it rarely induces fever, capillary leak syndrome, or other clinical abnormalities associated with excessive pro-inflammatory cytokines [110, 119]. Because of its diverse beneficial effects on immunity and excellent safety record, investigators at the National Cancer Institute have consistently ranked IL-7 as one of the top potential immunotherapeutic molecules [120]. Because of its many

beneficial effects on immunity, reported efficacy in bacterial, fungal, and animal sepsis models, and clinical track record, IL-7 is believed to have enormous promise in the treatment of sepsis.

Another exciting immunomodulatory therapy that holds much potential in sepsis involves blockade of negative costimulatory molecules present on T cells. The negative costimulatory molecule PD-1 is inducibly expressed on $CD4^+$ and $CD8^+$ T cells [121, 122]. Signalling through PD-1 inhibits the ability of T cells to proliferate, produce cytokines, or perform cytotoxic functions. Persistent antigenic exposure as occurs in chronic viral infections such as HIV-1 and viral hepatitis leads to excessive PD-1 expression and exhausted T cells [123, 124]. Antibody blockade of PD-1 or its ligand (PD-L1) can reverse T-cell dysfunction and induce pathogen clearance [124]. Similarly, three independent groups showed that blockade of the PD-1 pathway improves survival in clinically relevant animal models of bacterial and fungal sepsis [125, 126]. PD-1 over-expression on circulating T cells from patients with sepsis correlated with decreased T-cell proliferative capacity, increased secondary nosocomial infections, and mortality [127]. Thus, expression of PD-1 or PD-L1 on circulating immune cells could function as a valuable biomarker for the selection of candidates for blockade therapy. Importantly, post-mortem study of patients with sepsis showed that PD-L1 was highly expressed on tissue parenchymal cells, including endothelial cells, thereby providing opportunity for pathway activation [128].

Another immunostimulatory cytokine receiving renewed interest as a potential therapeutic agent in sepsis is IFN-γ, a potent monocyte, macrophage, and NK cell activator, which produced encouraging results in a small trial of patients with sepsis. Docke *et al* [129] treated patients with sepsis whose monocytes had reduced HLA-DR expression and

produced decreased amounts of TNF-α after lipopolysaccharide stimulation. IFN-γ treatment reversed the sepsis-induced monocyte dysfunction and resulted in eight of nine patients successfully resolving the septic insult. Nalos *et al* reported on use of IFN-γ in a patient with persistent staphylococcal sepsis [130]. IFN-γ therapy resulted in increased monocyte expression of HLA-DR, increased numbers of IL-17-producing $CD4^+$ T cells, and clinical resolution of the sepsis. IFN-γ is approved for treatment of fungal sepsis in patients with chronic granulomatous disease. In a randomized controlled trial, Jarvis *et al* [131] treated HIV patients who had cryptococcal meningitis with IFN-γ: patients treated with IFN-γ had more rapid clearing of cerebrospinal fluid than control patients.

Other molecules in early stages of testing have also shown efficacy in clinically relevant animal models of sepsis. IL-15 is a pluripotent cytokine closely related to interleukin 7 [132] that also acts on $CD4^+$ and $CD8^+$ T cells to induce proliferation and prevent apoptosis. A potential advantage of IL-15 compared with IL-7 is its potent immunostimulatory and proliferative effects on natural killer (NK) cells and dendritic cells. These cells have important roles in fighting infection and are also severely depleted in sepsis. Inoue *et al* [132] reported that IL-15 blocked sepsis-induced apoptosis of $CD8^+$ T cells, NK cells, and dendritic cells, and improved survival in sepsis due to cecal ligation and puncture and in primary pseudomonas pneumonia. The B and T lymphocyte attenuator (BTLA) is an immunoregulatory receptor expressed by various innate and adaptive immune cells. Activation of BTLA induces a potent immunosuppressive effect on T cells and other immune cells. Adler *et al* [133] reported that BTLA null mice showed reduced parasitaemia and faster clearing of malaria in a murine model of infection. Results in the cecal ligation and puncture model of murine sepsis show similar protective effects: BTLA-null mice have increased survival and reduced organ injury compared with wild-type mice [133].

Lastly, anti-apoptotic therapies have also shown promise in early pre-clinical studies of sepsis [113, 134, 135]. Mice that overexpress B-cell lymphoma 2 (BCL-2; a protein known to protect against apoptosis mediated through the mitochondrial pathway) in T or B cells are almost completely protected from sepsis-induced lymphocyte apoptosis [113]. In rat cardiomyocytes exposed to endotoxin, Carlson *et al* observed that TNF-α-induced caspase activation that subsequently caused cardiac dysfunction [78]. Lancel *et al* also demonstrated that caspases also caused contractile dysfunction in cardiac myocytes exposed to endotoxin [79]; however, the broad-spectrum caspase inhibitor zVAD.fmk (N-benzlyoxycarbonyl-valylalanyl- aspartyl-fluoromethylketone) had a protective effect on endotoxin-exposed myocytes. zVAD.fmk also provided significant neuroprotection by reducing hippocampal neuronal cell death in pneumococcal meningitis [136], and improved survival in a mouse model of cecal ligation and puncture [134, 137]. Despite these favourable results, the use of caspase inhibitors for treating sepsis may not be feasible for multiple reasons. Firstly, it is necessary to have a persistent and nearly complete caspase inhibition to prevent cell death, because even a small amount of activated caspase-3 is sufficient to initiate genomic DNA breakdown and lead to apoptotic cell death [138]. Secondly, caspases have many functions in addition to their roles as cell-death proteases and regulators of inflammation, including being essential for lymphocyte activation, proliferation and protective immunity; blocking caspases therefore may have unintended negative consequences by blocking the patient's ability to mount an effective immune response. Despite the challenges of developing immune-based therapy for a disease as complex as sepsis, novel immune-adjuvants and immunomodulatory treatments offer hope in the battle against this disease.

1.4 Natural Killer T Cells and Sepsis

1.4.1 Characterization of Natural Killer T Cells

In the last decade, there has been increasing interest in natural killer T cells, a unique population of lymphocytes that plays a central role as effectors and regulators of the septic response by interacting with both the innate and adaptive immune systems [139].

NKT cells were originally defined in mice as a $CD4^{-}CD8^{-}$ population that co-expresses the T cell receptor (TCR) and NK1.1, a natural killer (NK) cell surface marker in certain mouse strains [140]. Subsequent studies, however, have shown that a subset of $NK1.1^{-}$ cells may also exhibit characteristics of NKT cells [141]. These cells can be broadly categorized into type I or invariant NKT (iNKT) cells and type II NKT cells [141]. Unlike conventional T cells which recognize peptide antigens presented in the context of the major Histocompatibility Complex (MHC) class I or II molecules, NKT cells recognize glycolipid antigens presented on the MHC class I-like molecule CD1d [141, 142]. CD1d is a member of a family of CD1 glycoprotein molecules expressed on various APCs associated with β2-microglobulin [142]. Type I and II NKT cells differ in the diversity of their TCRs and their known ligands. iNKT cells express semi-invariant α/β TCRs consisting of an invariant Vα14/Jα18 chain in mice (Vα24/Jα18 in humans; **Figure 2**) and a limited set of β chains [141]. Despite having well-characterized TCRs, the endogenous ligands of iNKT cells are ill defined. Alpha-galactosylceramide (α-GalCer), a synthetic glycosphingolipid (GSL) initially derived from a marine sponge, is the prototype agonist of iNKT cells and a powerful tool in studying the impact of NKT cell activation on microbial immunity [143]. In contrast to iNKT cells, type II NKT cells are nonresponsive to α-GalCer and possess a more diverse TCR repertoire [140, 141]. NKT cells promptly secrete large amounts of Th1 and Th2 cytokines including IFN-γ and IL-4, respectively, upon stimulation

[144, 145], leading to the activation of macrophages, B cells, NK cells, and dendritic cells as well as effector T cells (**Figure 3**). In addition to cytokine production, NKT cells also possess cytotoxic effector activity by way of lysis of target cells that is dependent on perforin and FasL [146, 147].

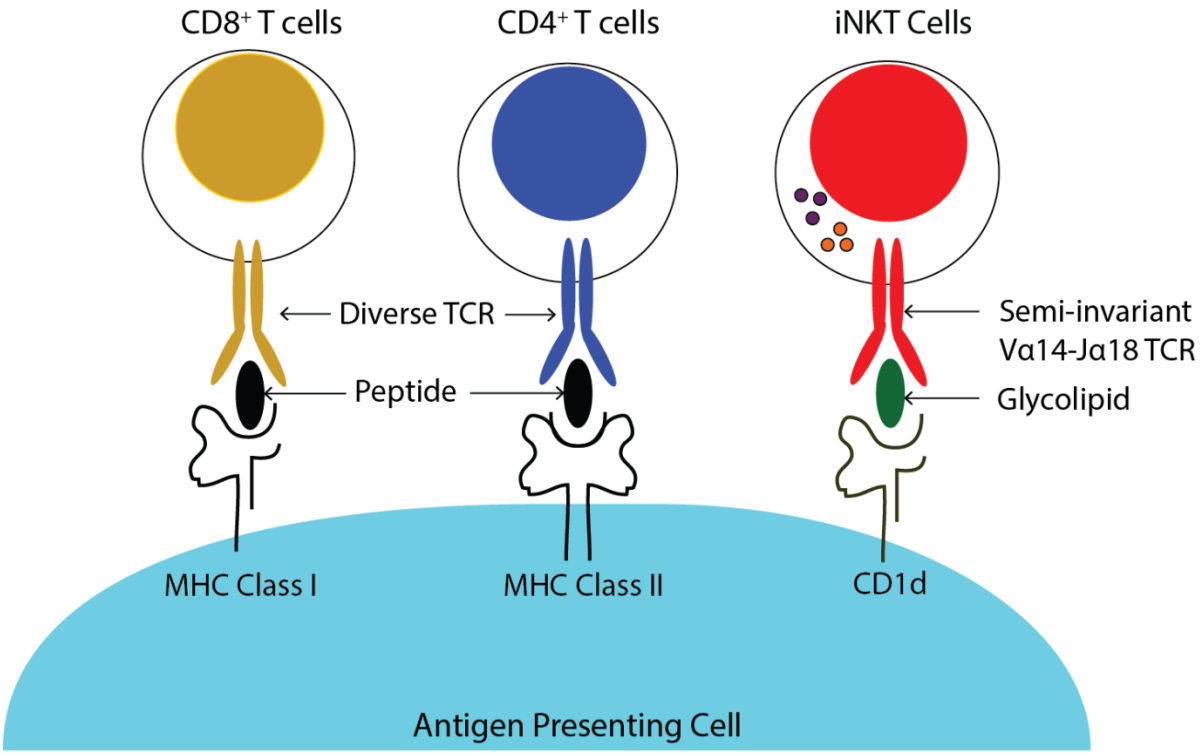

Figure 2: Antigens and Receptors of invariant Natural Killer T (iNKT) cells.

iNKT cells have a semi-invariant T cell receptor (TCR) with restricted antigen-binding ability, whereas conventional T cells possess diverse TCRs which can recognize a multitude of antigens. iNKT cells bind glycolipid antigens presented in the context of an MHC Class I-like molecule CD1d, in stark contrast to conventional $CD4^+$ and $CD8^+$ T cells which bind peptide antigens presented in MHC Class II and I molecules, respectively.

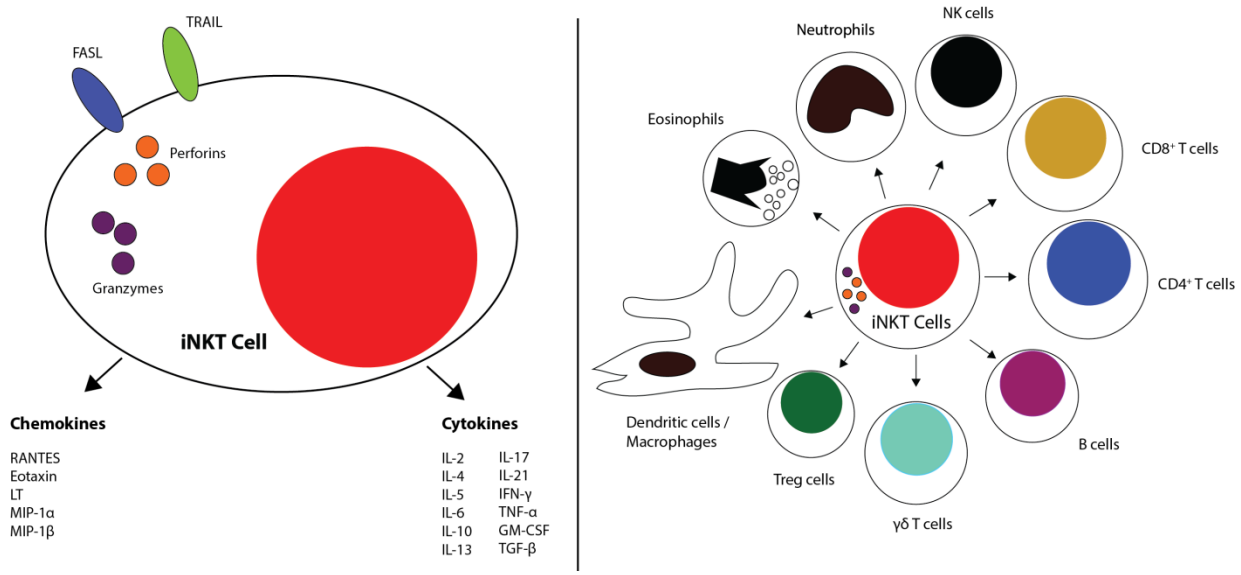

Figure 3: Factors released by invariant Natural Killer T (iNKT) cells.

iNKT cells rapidly release many cytokines and chemokines upon activation, which in turn have regulatory effects on numerous effector cells of the innate and adaptive immune systems.

Functional differences have been reported for NKT cells that differentially express CD4 [148, 149], NK1.1 [150-152] and other surface molecules [153] and may also exist between NKT cells from different organs [154, 155]. There is no clear consensus on this issue, but it is important to recognize that the composition and origin of NKT cells may affect their overall response. For that reason, it is highly recommended that functional assays be assessed in context of the origin and phenotype of NKT cells at the beginning and end of experiments.

1.4.2 NKT Cells in Experimental Animal Models

Various experimental models [156-161] have established NKT cells as the principal initiators of an excessive pro-inflammatory response that promotes lethality in sepsis and endotoxic shock. In mice, two consecutive injections of LPS stimulate $NK1.1^+$ T cells [162] to release large amounts of IFN-γ [163], which results in a lethal endotoxemia. LPS also activates and increases the cytotoxicity of NKT cells in the liver through IL-12 produced by Kupffer cells [164]. Additionally, when mice were depleted of NKT cells and NK cells by anti-NK1.1 antibody, the mice released less IFN-γ, and had reduced mortality when injected with LPS [156]: a similar result was observed in mice deficient in Beta 2-microglobulin ($\beta 2m^{-/-}$) and lacking most of their $NK1.1^+$ αβ T cells [156]. iNKT cell-deficient ($J\alpha 18^{-/-}$) mice also had a significant survival advantage when injected with LPS, with concurrently lower serum levels of IFN-γ [160] and TNF-α than wild-type C57BL/6 mice [157]. While IFN-γ produced by iNKT cells facilitates pathogen clearance [165], mortality remains unaffected [160], suggesting that the complete activation of the pro-inflammatory cascade, which is important for the proper clearance of infection, may have deleterious consequences when overly activated. In $J\alpha 18^{-/-}$ mice injected with LPS, NK cell activation and production of IFN-γ were also significantly reduced,

suggesting a role for iNKT cells in amplifying the immune response by rapidly activating other immune cell types [160].

In the cecal ligation and puncture (CLP) sepsis model, pre-treatment with monoclonal antibody (mAb) blocking CD1d was shown to reduce CLP-induced mortality compared to IgG-treated controls and to suppress plasma and splenic levels of the Th2 cytokine IL-10 [166]. Although CD1d mAbs were thought to have enhanced Th1 responses [167, 168], these studies looked at conditions in which there is deficient protective immunity by iNKT cells and examined the ability of these antibodies to bypass iNKT activation and directly stimulate $CD1d^{+}$ APCs. Similarly, $J\alpha18^{-/-}$ mice were used to show that iNKT cell deficiency significantly decreased septic mortality and ameliorated the systemic pro-inflammatory response [159].

Despite contradictory findings on the relative contribution of iNKT cells to a Th1 or Th2 response, these results consistently implicate a detrimental effect of NKT cell activation in polymicrobial sepsis. These studies constitute growing evidence for the large contribution of NKT cells to the dysregulated and overwhelming pro-inflammatory response in polymicrobial sepsis and endotoxic shock. Although many strong correlations have been made between septic mortality and NKT cell activation and cytokine production, researchers are still far from delineating and demonstrating the exact mechanism by which NKT cells participate in the septic immune response, or how their activity is regulated in general.

1.4.3 Mechanisms of iNKT Cell Activation

The mechanism by which iNKT cells are activated by microbial infection remains unclear. A “direct” pathway, in which the TCR of iNKT cells recognizes the glycosphingolipid cell-wall components of microbial pathogens such as *Sphingomonas* bacteria, has been reported

[169]. This early activation of iNKT cells appears to be important for bacterial clearance, because CD1d$^{-/-}$ and Jα18$^{-/-}$ mice were impaired in their ability to clear *Sphingomonas* [170]. Other studies have proposed a combination of two signals to culminate in IFN-γ secretion by iNKT cells: a weak response that is initially generated from the recognition of CD1d-presented endogenous glycosphingolipid antigens, followed by a stronger activation of the IL-12 receptor on iNKT cells by APC-derived IL-12 [171, 172]. IL-12 alone cannot activate iNKT cells when dendritic cells are absent, providing further evidence that recognition of self-ligand is an essential part of this indirect pathway [171, 172]. In a third mechanism, iNKT cells may be activated by IL-12 and IL-18 derived from APCs. In IL-12$^{-/-}$ and IL-18$^{-/-}$ mice, production of IFN-γ by iNKT cells was impaired in response to LPS, although not completely abrogated [160]. The addition of recombinant IL-12 or IL-18 to iNKT cells was sufficient to induce a measurable amount of IFN-γ production [160]. The addition of anti-CD1d Ab to co-cultures of iNKT cells with DCs and LPS also did not affect IFN-γ production [160]. All these pathways may be active during polymicrobial sepsis, due to the systemic release of many microbial stimuli, and may form part of a positive feedback loop whereby IFN-γ produced by iNKT cells further activates APCs [160]. Nevertheless, these mechanisms of iNKT cell activation quickly amplify the innate immune response to infection and contribute to the rapid development of the hyperinflammatory response in sepsis.

A remarkable and unique feature of iNKT cells is their ability to swiftly secrete copious amounts of Th1- and Th2-type cytokines after stimulation without *de novo* cytokine mRNA synthesis [173, 174]. iNKT cells contain preformed mRNA for these cytokines, which may explain the rapidity with which they are secreted. Hence, they are believed to be responsible for the first wave of cytokine release early in the course of immune responses, potentially shaping

the course of subsequent adaptive responses. The paradoxical ability of iNKT cells to either promote or suppress immune responses presents a classical "double-edged sword" dilemma of immune regulation [175], which can be attributed at least in part to the ability these cells to produce enormous quantities of Th1 cytokines, Th2 cytokines, or both.

Although natural killer (NK) cells are also known to be potent producers of IFN-γ and play an important role in promulgating sepsis by migrating to the peritoneal cavity and upregulating the pro-inflammatory activities of myeloid cells [176], studies have shown that NKT cells rapidly activate NK cells to initiate the inflammatory process: administration of α-GalCer rapidly activated NK cells to produce IFN-γ and upregulated their expression of the early activation marker CD69 within hours of exposure [177]. This provides more evidence for the pivotal role of NKT cells as a bridge between innate and adaptive immunity.

1.4.4 iNKT Cells in Human Populations

In mice, up to 30% of liver lymphocytes are NKT cells, but levels elsewhere are usually 0.1–1.0% [178, 179]. In humans, however, NKT cell frequencies are typically lower [180], and can vary 100-fold between healthy individuals. In the thymus, iNKT cell frequencies are at least 100-fold lower in humans [179], whereas only 1% of hepatic lymphocytes are NKT cells [181]. While the tetramer binds NKT cells in mice and humans, human NKT cells can also be identified with anti-Vα24 (or 6B11, an antibody specific for the CDR3 region of the TCRα chain used by human NKT cells) and anti-Vβ11 [182, 183]: a reliable Vα14-specific antibody is not available for mice.

As blood is often the only source of human NKT cells, findings are often extrapolated to NKT cells in general. This is a risky assumption because the frequency and function of NKT

cells from different organs may be unrelated [155, 180, 184]. In non-obese diabetic (NOD) mice, for example, systemic NKT cell deficiencies are evident in all locations except blood [180]. It is unclear whether a similar phenomenon exists for humans, but it cannot be assumed that blood NKT cells are representative of NKT cells in other organs, even from the same donor. Surprisingly, however, iNKT cells comprise 15% of hematopoietic cells obtained from human omentum, the highest frequency of cells found to date within any organ in the human body: this finding suggests that the omentum may play a key role in mediating immune-based responses to intra-abdominal pathology [185].

1.4.5 iNKT Cells as Targets for Immunotherapy

Given the ability of NKT cells to bridge innate and adaptive immunity and their extensive immunoregulatory roles, manipulation of these cells provides a promising therapeutic strategy for sepsis and inflammation. α-Galactosylceramide (α-GalCer), the prototype iNKT cell glycolipid ligand [186] has been used both experimentally and in several clinical trials in patients with malignancies such as melanoma [187-189] and viral diseases [189, 190]. Depending on the length and time of *in vivo* exposure to α-GalCer, iNKT cells may be strongly polarized towards a Th1-like phenotype [158, 191] or a Th2-like phenotype [192, 193]. While the Th1 phenotype appears to be mediated by dendritic cell (DC) maturation and its presentation of α-GalCer [158, 191], the Th2 phenotype, induced by repeated injection of α-GalCer, may be due to iNKT cell anergy [194, 195]: iNKT cells may have a blunted response to DC stimulation with reduced production of IFN-γ. Concomitant administration of α-GalCer and LPS protected mice from sepsis [196, 197], and was associated with significantly lower serum levels of Th1 cytokines (including IFN-γ and TNF-α), and higher levels of Th2 cytokines such as IL-10 [196, 197].

Recent availability of several αGalCer analogs that exhibit distinct immunomodulatory properties now allows for more comprehensive examination of iNKT cell function and the consequences of their manipulation in transplant recipients (**Figure 4**). KRN7000, a synthetic αGalCer, which selectively stimulates iNKT cells [186] and leads to the production of both pro- (Th1) and anti-inflammatory (Th2) cytokines, has been used in several models of allotransplantation. KRN7000 administration after γ irradiation and allogeneic bone marrow transplantation was reported to reduce morbidity and mortality associated with murine graft-versus-host disease in two independent studies [198, 199]. In contrast, treatment of cardiac allograft recipients with KRN7000 failed to prevent rejection in a BALB/c-to-B6 model [200]. It is possible that cytokine environments in which alloreactive T cells are primed in these models may be different. Irradiation shifts the cytokine profile of iNKT cells towards a Th2-promoting phenotype, which may contribute greatly to the beneficial effects of αGalCer in graft-versus-host disease models [199, 201]. In comparison, Th1- and Th2-type cytokines concomitantly produced after KRN7000 administration, may cancel each other out leading to no beneficial net effects in the above cardiac allograft model or other similar conditions.

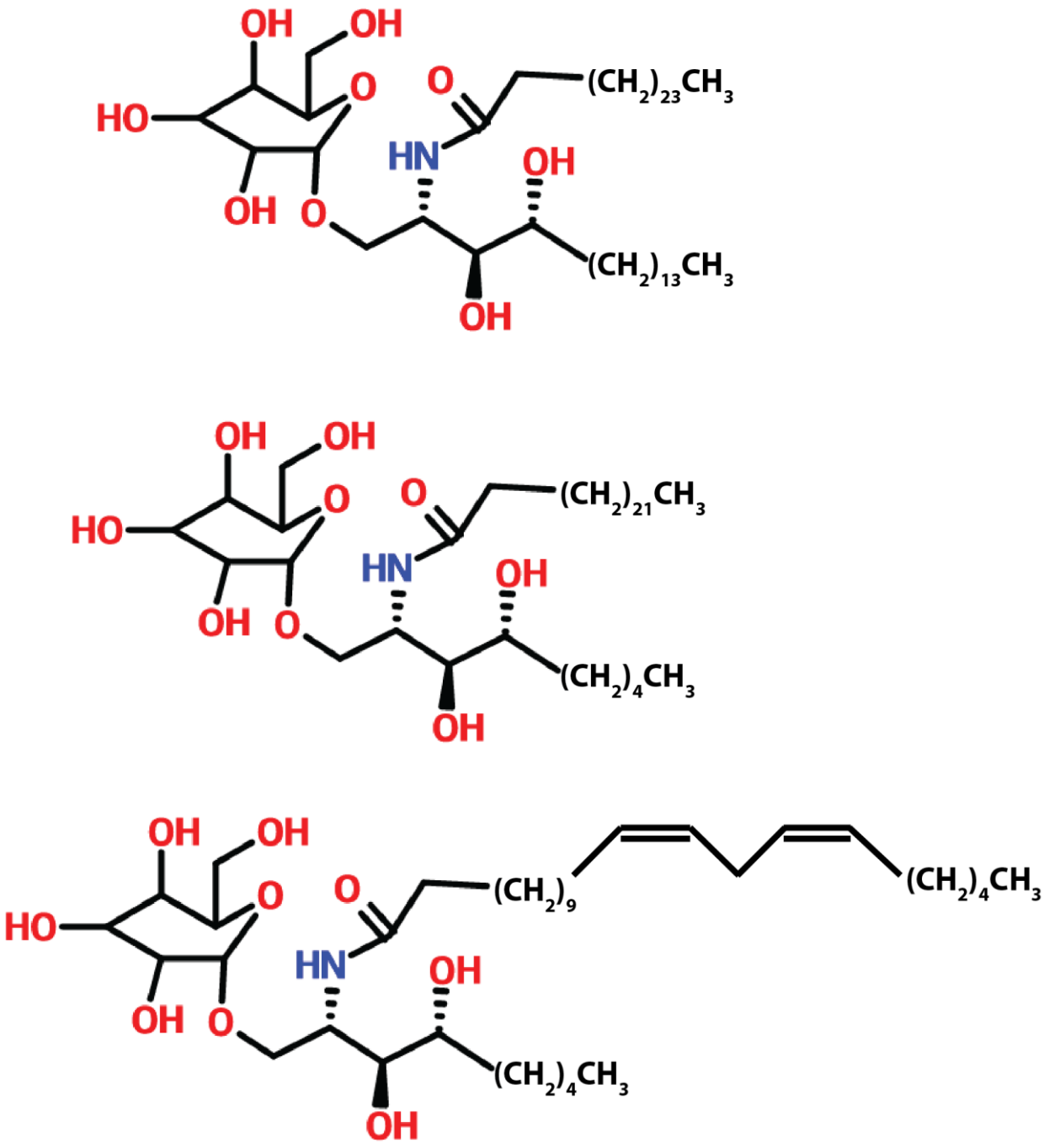

Figure 4: Select glycolipid agonists of iNKT cells.

From top to bottom, α-GalCer, OCH, and C20:2 are glycolipids which respectively skew the iNKT cell response towards a Th1, Th2, and Th2 phenotype.

OCH, a sphingosine-truncated analog of α-GalCer with known Th2-polarizing properties [202] has exerted promising effects in the treatment of several experimental autoimmune diseases [201, 203] where a destructive Th1-type response is suspected to play a role in the disease pathogenesis: by ameliorating autoreactive T cells [188], OCH mitigated disease severity in non-obese diabetic (NOD) mice [204] experimental autoimmune encephalomyelitis [202] and collagen-induced arthritis (CIA) [205, 206]. Walker *et al* demonstrated that treatment with OCH can not only prevent, but also cure disease symptoms in a humanized mouse model of citrullinated fibrinogen-induced RA [207]. OCH also delayed Th1-mediated cardiac allograft rejection in two mouse models [208].

Another promising Th2-biasing glycolipid is C20:2, a variant of α-GalCer consisting of a fatty acyl side chain truncated from C26 to C20 that has two sites of unsaturation at carbons 11 and 14. C20:2 induces Th2-biased cytokine production [209] *in vivo,* and has a similar binding affinity to CD1d as α-GalCer [210]. *In vivo,* C20:2 results in an overall reduced pro-inflammatory cytokine (IFN-γ) secretion, reduced iNKT cell expansion, and reduced activation of T, B and NK cells [209, 211]. Its effects, however, do not last beyond six hours [209]. When given in multiple doses, C20:2 significantly delayed and reduced the incidence of Type 1 Diabetes (T1D) in NOD mice [209, 211].

Tailoring the type of NKT cell stimulation to promote a pro- or anti-inflammatory response has exciting implications for the future potential of NKT cell-based therapies for various clinical conditions [212, 213]. Although more work is needed to determine what specific factors trigger NKT cells to assume a Th1 or Th2-like phenotype during sepsis, the work done in animal models so far provides insight on the contribution of NKT cells to inflammation and injury, and an important foundation upon which to build a more targeted immunotherapy.

1.5 The Greater Omentum

1.5.1 Anatomy and Embryology of the Greater Omentum

The greater omentum has been identified as a rich source of iNKT cells in humans [185], although little is known about its immune mechanisms. The greater omentum is a large fold of visceral peritoneum that hangs down from the stomach, and drapes over much of the small bowel, hanging as low as the pelvis [214]. Its descending and ascending portions fuse to form a four-layer vascular fatty apron, with a space contiguous with the lesser sac. Several prominent, hypertrophied folds within the greater omentum and adhesins between organs can be identified: the gastrocolic ligament between the stomach and the transverse colon; the gastrosplenic ligament that connects between the stomach and the spleen; and, occasionally, the splenorenal ligament which adheres the spleen to the left kidney. The right and left gastroepiploic vessels, which derive from the gastroduodenal and splenic arteries respectively, provide the blood supply to the greater omentum. The greater omentum develops from the dorsal mesentery that connects the stomach to the posterior abdominal wall. During gastric development, the stomach undergoes a 90° clockwise rotation along the axis of the embryo, so that structures posterior to the stomach are moved to the left, and anterior structures are moved to the right. As a result, the dorsal mesentery folds over on itself, forming a pouch with its blind end on the left side of the embryo. A second 90° clockwise rotation of the stomach along the frontal plane moves left-sided structures inferiorly, and right-sided structures superiorly. Consequently, the blind-ended sac (also called the lesser sac) formed by the dorsal mesentery is brought inferiorly, where it assumes its final position as the greater omentum. The greater omentum subsequently enlarges to drape over the majority of the small and large intestines.

In the mouse, the greater omentum consists of a band of intra-abdominal adipose tissue running from the distal spleen to the duodenal lobe of the pancreas [215, 216]. Murine and human greater omenta share many micro-anatomical features despite some differences in gross appearance [214]. In both, the omentum is a mobile structure that moves with gut peristalsis in a small volume of intra-peritoneal fluid. This movement is thought to be essential for the omentum to access injured or infected peritoneal surfaces where it adheres and facilitates repair processes: as the "abdominal policeman," the omentum restores order by surrounding the compromised site, sealing microperforations, localizing inflammation, limiting the spread of infection, and provoking revascularization and tissue regeneration [214, 215, 217]. Surgical transposition of omental pedicles or flaps to injured body sites has been used for over a century for this purpose [218, 219]. Because of the presence of peritoneal fluid, however, the omentum is also a common location for neoplastic intraperitoneal seeding and infectious processes.

1.5.2 Histopathology of the Greater Omentum

The omentum in both mice and humans is composed of two mesothelial sheets enclosing adipocytes embedded in loose connective tissue interspersed with islands of compact tissue, known as "milky spots" [214, 220]. These spots contain macrophages, B cells, T cells, mast cells and DCs [217], and are reactive structures that increase in size and number in response to peritoneal inflammatory stimuli. Among the intra-abdominal adipose tissues, the greater omentum is unique because it is thought to function in peritoneal surveillance and as an access route for blood leukocytes entering the resting [221, 222] and inflamed [220] peritoneal cavity. Milky spots develop a more organized structure where T and B cells segregate as in secondary lymphoid tissues [223, 224]. This structural organization originally led to the proposal that milky spots were themselves secondary lymphoid organs [224]. However, segregation of B and T cell

areas was not observed in the resting omentum [223, 225]. These findings have led to the view that the greater omentum is an inflammation-induced lymphoid structure that lacks the defining characteristics of secondary lymphoid tissues, such as resident professional APCs, permanence in basic structure, and segregation of B and T cell regions in the absence of antigenic stimulation or inflammation.

1.5.3 Immunology of the Greater Omentum

Although controversy still exists around its precise immunological definition, the omentum has been demonstrated to play an important role in both innate and adaptive immunity [221, 226]. For example, the fetal omentum is critical to the development of B cells, and may play an important role in the homeostasis of this cell population in adult mice and humans [227, 228]. In another study, the omentum was observed to contain large populations of stem cells known as myeloid-derived suppressor cells (MDSCs) when mice were subjected to intra-abdominal sepsis [229]: these cells secrete a variety of factors including those with immunosuppressive functions and provide a regenerative microenvironment for injured tissues to limit the area of damage and to mount a regenerative response [229]. Shah *et al* [229] suggest that the presence of functional mesenchymal stem cells (MSCs) is a part of the mechanism by which the omentum imposes tissue healing support on the damaged tissues. While expression of stem cell markers and angiogenic growth factors were previously identified as contributing factors to the regenerative properties of the omentum, Shah *et al* demonstrated that activated omentum has at least two functionally distinct groups of cells that can facilitate regeneration of damaged tissue: immunomodulatory $CD45^{+}Gr1^{+}$ MDSCs; and $CD45^{-}$ cells that have the ability to suppress Th17 cells. A striking feature of the omentum is that, unlike secondary lymphoid organs such as lymph nodes or spleen, it enlarges in response to foreign objects and acquires a

large number of immunomodulatory cells along with cells with stem cell function. This type of response has not been recognized for any other organ and is quite unique to the omentum.

1.6 Hypothesis

I hypothesize that expansion of the circulating iNKT cell population will occur in patients with sepsis and severe sepsis. I further hypothesize that the administration of Th2-polarizing glycolipid agonists of iNKT cells will significantly reduce sepsis severity by limiting the release and subsequent production of pro-inflammatory Th1 cytokines.

1.7 Specific Objectives

I developed several specific objectives in order to test my hypotheses:

1) Prospectively evaluate the frequency and proportion of circulating iNKT cells in patients admitted to the intensive care unit with sepsis, severe sepsis, or septic shock.
2) Develop and validate a mouse model of acute intra-abdominal sepsis that is characterized by a marked pro-inflammatory response and leads to early mortality.
3) Assess the effect of Th1- and Th2-polarizing iNKT cell agonists on sepsis severity in the animal model of sepsis.

Chapter 2: Materials and Methods

2.1 Ethics

All animal experimentation was carried out in strict accordance with the recommendations and guidelines established by the Canadian Council on Animal Care as well as institutional regulations. The protocols were approved by the Western University Animal Care and Veterinary Services (Approval number: 2008-034-01).

For the study involving human subjects, approval of the study protocol for both the scientific and ethical aspects was obtained from the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects (Approval number: REB103036).

2.2 Mice

Female C57BL/6J (B6) mice, 10–14 weeks of age, were purchased from Charles River Canada Inc. (St. Constant, Quebec, Canada). J$\alpha 18^{-/-}$ mice, which lack *i*NKT cells [230], were based on a B6 background and obtained from Dr. Luc Van Kaer (Vanderbilt University, Nashville, TN, USA). The development of the lymphoid organs in J$\alpha 18^{-/-}$ mice is macroscopically normal, and the numbers of total lymphocytes are not significantly different from J$\alpha 18^{+/+}$ mice with the exception of a complete loss of the Vα14 NKT subpopulation of NKT cells [230]. GFP-expressing transgenic mice are B6 mice with omnipresent enhanced GFP expression under the β-actin promoter, and were kindly provided by Dr. Stephen Kerfoot (Western University, London, Ontario) for a limited number of experiments.

Animals had an average weight of 22.5 g (range, 21-25 g) prior to the start of the experiments. Animal husbandry conditions included a room temperature of 23°C, humidity of 50%, and a 12-hour light-dark cycle (dark from 1900h to 0700h). Bedding in cages consisted of sawdust and wood shavings, while corn mash and water (in a stoppered-bottle with a nose-

activated nozzle) was available for mice to feed *ad libitum*. Cages also contained an igloo to allow nesting. Animals were housed with one to three cage mates, and were allowed to adapt to laboratory conditions for at least 3 days prior to experiments.

2.3 Mouse Intra-abdominal Sepsis Model

2.3.1 Preparation of Fecal Slurry

The solution used to cause IAS was made by the following procedure: fresh feces were collected from the lower cecum of euthanized donor mice, weighed, and mixed with a calculated volume of saline solution to give a fecal concentration of 90 mg/mL. To ensure reproducibility, the procedure was standardized by the use of fresh solution prepared from mice living in the same conditions as the experimental animals. The fecal solution (FS) was pressed through a 70-μm nylon mesh strainer (BD Biosciences, Franklin, NJ) to remove particulate matter.

2.3.2 Induction of Sepsis

For sepsis induction, each mouse was given an intraperitoneal (i.p.) injection of 0.5 mL of FS using a syringe and 27G needle (4 mg of FS per 1 g body weight). Sham mice were injected with sterile normal saline (NS). Pain (either from the injection or from FS) was assessed using facial expression as described by Langford *et al* [231], as well as body posture and vocalization. Analgesia was provided by a subcutaneous injection of buprenorphine (0.1mg/kg).

2.3.3 Monitoring of Mice

Monitoring of the health of the animals was conducted by two investigators every 2 hours after the induction of sepsis for 12 hours, and then every hour thereafter: one of the investigators was blinded to the treatment. Mice were evaluated while they were still in their cages (with the lids removed for better visualization).

2.3.4 Euthanasia of Mice

At the conclusion of the experiments, animals were sacrificed and post-mortem laparotomy was performed in order to collect tissues. Mice were anesthetized with 100 mg/kg ketamine (Bioniche Life Sciences, Belleville, ON) and 5 mg/kg xylazine (Bayer AG, Leverkusen, Germany), and euthanized by cardiac puncture using a 27G needle and 3-mL syringe.

2.4 Development of the Murine Sepsis Score

In this project, a scoring system (the murine sepsis score, MSS) was developed to assess and monitor disease severity, and serve as a humane surrogate to death as an endpoint. In a pilot study involving thirty mice that were given 90 mg/mL FS and observed over 24 hours, a veterinarian from the Animal Care and Veterinary Services (Ian Welch, ACVS, Western University), and two researchers from our group assessed the mice jointly using variables that have been described in the literature [231-233]. Certain variables such as temperature and weight loss did not change during the experimental timeline, while fewer than 5% of mice needed analgesia for pain immediately after the fecal slurry injection. Consequently, the final variables that were incorporated into the MSS (Table 5) included spontaneous activity, response to touch and auditory stimuli, posture, respiration rate and quality (laboured breathing or gasping), and appearance (i.e. degree of piloerection). Each of these variables are given a score between 0 and 4 (Table 5). Mice were euthanized if the MSS at any given time point was greater than 21, or if the points ascribed to respiratory rate or quality increased by more than 3.

Table 5: Murine Sepsis Score (MSS)

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 and moving
1- Mouse is moving but avoids standing upright
2- Mouse is slow moving
3- Mouse is not moving without provocation
4- Mouse does not move even when provoked

Response to stimulus

0- Mouse responds immediately to auditory stimulus or touch
1- Slow or no response to auditory stimulus, responsive to touch
2- No response to auditory stimulus, moves away in response to touch
3- No response to auditory stimulus, some movement in response to touch
4- No response to auditory stimulus, little or no movement in response to touch

Activity

0- Mouse is any of: eating, drinking, climbing, running, fighting
1- Mouse is moving around bottom of cage
2- Mouse is stationary with occasional investigative movements
3- Mouse is stationary
4- Mouse experiencing tremors, particularly in the hind legs

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 mily

Respiration rate

0- Normal, rapid mouse respiration
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, 0.5s between breaths)
4- Extremely reduced respiration (> 1s between breaths)

Respiration quality

0- Normal
1- Brief periods of laboured breathing
2- Laboured, no gasping
3- Laboured with intermittent gasps
4- Gasping

2.5 Glycolipids

Lyophilized OCH was generously supplied by the National Institutes of Health (NIH) Tetramer Core Facility (Atlanta, GA, USA). Each vial containing 0.2 mg of OCH was solubilized in 1 mL of sterile distilled water and stored as aliquots at 4°C until use. KRN7000 was synthesized, solubilized at 1 mg/ml in dimethylsulfoxide (DMSO) and stored as aliquots at −20°C until use [234]; the control vehicle was 2% DMSO in phosphate-buffered saline (PBS). C20:2 was synthesized as described previously [235] and dissolved in a vehicle solution containing PBS, 0.02% Tween 20, and 0.1% DMSO. The resulting stock solution was stored in aliquots at −20 °C. Aliquots were re-warmed and sonicated prior to use. For *in vivo* experiments, mice were injected i.p. with a single dose of glycolipid (4 μg/dose) [209]. In experiments where mice were induced with IAS, glycolipids were administered 15-20 minutes after the injection of fecal slurry to allow the animals to recover in between injections.

2.6 Antibodies

2.6.1 Mouse

Allophycocyanin (APC)-conjugated PBS-57-loaded and -unloaded CD1d tetramers for staining mouse iNKT cells were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA) [209]. Fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β (H57-597) and phycoerythrin (PE)-conjugated anti-CD69 (H1·2F3) monoclonal antibodies (mAbs) were purchased from eBiosciences (San Diego, CA, USA) or BD Biosciences (Mississauga, ON, Canada). PE-conjugated anti-B220/CD45R and anti-CD8 mAbs, as well as FITC-conjugated anti-CD3 mAbs were purchased from BD Biosciences (**Table 6**).

2.6.2 Human

APC-conjugated PBS-57-loaded and -unloaded CD1d tetramers for staining human iNKT cells were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA) while FITC-conjugated anti-CD3 (SK7), PE-conjugated anti-CD56 (B159), and peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD56 were obtained from BD Biosciences (**Table 6**).

Table 6: Antibodies used for flow cytometry in mouse and human studies.

Species	Target	Fluorochrome	Identifies	Source	Volume (µL)
Mouse	CD3	APC	T Cells	eBiosciences	1
	CD45R (B220)	APC	B Cells	eBiosciences	1
	TCRβ	FITC	T Cells	eBiosciences	1
	NK1.1	PE	NK and NKT Cells	eBiosciences	1
	CD1d tetramer-PBS-57 unloaded	APC	Control for iNKT Cells	NIH	1
	CD1d tetramer-PBS-57 loaded	APC	iNKT Cells	NIH	1
	F4/80	APC	Macrophages	eBiosciences	1
	Anti 7-AAD	-	Dead cells	Life Technologies	0.5
	Annexin V	FITC	Apoptotic Cells	eBioscience	2.5
Human	CD3	FITC	T Cells	eBioscience	1
	Vα24	PE	iNKT Cells	NIH	1
	CD1d tetramer-PBS-57 unloaded	APC	Control for iNKT Cells	NIH	1
	CD1d tetramer-PBS-57 loaded	APC	iNKT Cells	NIH	1
	CD56	PE	NK and NKT Cells	eBioscience	1

2.7 Bacterial CFU Counts

2.7.1 Tissue Homogenization for CFU

Whole hearts, lungs, and kidneys (left and right) were removed from euthanized mice, taking care to dissect away lymph nodes, and homogenized in 5 mL of PBS. Homogenates were serially diluted 1:10 in PBS and plated on bovine heart infusion (BHI) agar. Plates were grown aerobically at 37° overnight to determine tissue CFU.

2.7.2 Peripheral Blood CFU Determination

10 µL of intra-cardiac blood was collected in a syringe from the right ventricle during euthanasia and cardiac puncture, serially diluted 1:10 with PBS, and plated on BHI agar to determine blood CFU.

2.8 Preparation of mouse hepatic, splenic, and omental cell suspensions

To obtain hepatic lymphoid mononuclear cells, mice were euthanized, and livers were flushed with sterile PBS before they were harvested and pressed through a 40-µm nylon mesh. The resulting homogenate was washed in cold PBS, resuspended in a 33.75% Percoll PLUS solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and spun at 700 × g for 12 min at room temperature. The pelleted cells were then treated with ACK lysis buffer to remove erythrocytes and washed in cold PBS prior to staining.

To obtain omental lymphoid mononuclear cells, mice were euthanized, and the spleens, pancreas, and omenta were removed en-bloc and suspended in ice-cold PBS. The omenta floated above the spleen-pancreas complex and were removed and processed similar to the liver, as described above. Spleens were processed with a tissue homogenizer, and the resulting

homogenate was washed in cold PBS. The pelleted cells were treated with ACK lysis buffer for 4 minutes to remove erythrocytes, and washed in cold PBS prior to staining.

2.9 Adoptive Transfer of iNKT Cells into Jα18$^{-/-}$ Mice

Hepatic mononuclear cells and splenocytes were isolated, as previously described, from GFP-expressing transgenic mice. $CD4^+$ T cell populations were obtained using EasySep® Mouse $CD4^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) as per manufacturer's instructions. iNKT cells were further enriched by sorting with anti-TCRβ and CD1d tetramer on a FACSAriaIII flow cytometric cell sorter (London Regional Flow Cytometry Facility, London, Ontario). Cell populations were used only when purity was >95% as determined by flow cytometry. For the adoptive transfer experiments, 5×10^5 iNKT cells were transferred i.v. into Jα18$^{-/-}$ mice. Twelve hours after the transfer, mice were given IAS and monitored as already described.

2.10 Flow Cytometry

Mouse hepatic, splenic, and omental cells (1×10^6), and human peripheral blood mononuclear cells (PBMCs) and omental cells (1×10^6) were placed in fluorescence activated cell sorter (FACS) tubes (BD Biosciences, San Jose, CA, USA), and washed with cold FACS buffer [PBS + 2% fetal bovine serum (FBS) + 0.1% sodium azide]. Mouse cells were incubated with 5 μg/ml anti-mouse CD16/CD32 mAb (clone 2·4G2, Fc-block, eBiosciences) for 20 min on ice before staining with fluorescent mAbs or tetramer diluted in FACS buffer at 4°C for 30 min. Human cells were stained with fluorescent mAbs or tetramer diluted in FACS buffer at 4°C for 40 min.

Cells were then washed and flow cytometry was performed using FACSCanto II and FACSDiva software. Analyses were conducted using FlowJo software (Treestar, Ashland, OR, USA). The gating strategy used for analysis of apoptotic cells is shown in **Figure 5**.

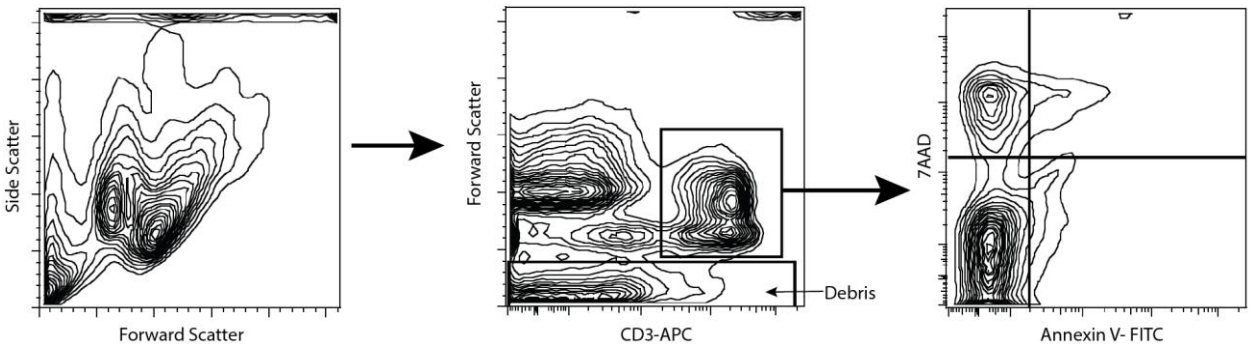

Figure 5: Gating strategy to identify the percentage of apoptotic and necrotic immune cell populations.

CD3-APC (for the detection of T cells) is shown as an example, but the same strategy was used for macrophages (F480-APC), B cells (B220-APC), and Natural Killer (NK) cells (NK1.1-PE).

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience, and were used to assess serum from experimental animals. Kits were run as specified by the manufacturer in Costar ELISA plates (Immunohistochemistry Technologies, Bloomington, MN). Plates were read at OD_{450} and at the reference OD_{570}.

2.12 Multiplex Cytokine Analysis

Serum was analyzed by bead-based multiplex assay for 32 different cytokines, chemokines, and growth factors (Eve Technologies, Calgary, Alberta, Canada) including granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, keratinocyte chemo-attractant (KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemotactic protein (MCP)-1, monocyte-colony stimulating factor (M-CSF), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF).

Multiplex data was visualized using a cytokine heat map that was generated using the web-based program Matrix2png [236]. The mean for each row of cytokine values was set at 0 with white representing values greater than 0, and brown lower than 0.

2.13 Serum Biochemistry

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lipase, expressed as U/L, as well as creatinine (expressed as mg/dL), glucose (mg/dL), and

albumin (expressed as mg/dL) were estimated using a commercially-available diagnostic kits (Catachem Inc., Oxford, CT) according to the manufacturer's instructions.

2.14 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from hepatic, splenic, and omental tissues using the TRIzol reagent (Invitrogen, Burlington, Ontario) and resuspended in nuclease-free water (Invitrogen). Quality control of samples was carried out using a Nanodrop ND-1000 spectrophotometer. cDNA was prepared using 1000ng of RNA by Superscript III RNase H^- Reverse Transcriptase with oligo dT priming (Invitrogen). Quantitative real-time PCR reactions were carried out in triplicate from every transcription reaction using the ABI Prism 7900HT apparatus (Perkin Elmer) with Taqman (Invitrogen) probes. The sequences of the primers and Taqman probes (Invitrogen) used in this study were as follows (GenBank Accession number S75451.1)[178]: Vα14: 5'−TGGGAGATACTCAGCAACTCTGG−3'; Jα18: 5'−CAGGTATGACAATCAGCTGAGTCC−3'; Vα14 Probe FAM: 5'−FAM-CACCCTGCTGGATGACACTGCCAC-TAMRA−3'. Quantitative analysis was performed by ΔΔCt method by using the Taqman GAPDH Gene Expression Assay (Invitrogen) as an internal control. The sequence that was examined for design of quantitative primers was 5'-GATGCTAAGCACAGCACGCTGCACATCACAGCCACCCTGCTGGATGACACTGCCAC CTACATCTGTGTGGTGGGGG//ATAGAGGTTCAGCCTTAGGGAGGCTGCATTTTGGAG CTGGGACTCAGCTGATTGTCATACCTGA-3' (// refers to the exon-splice site between Vα14 and Jα18 segments of the invariant TCR). Annealing temperatures for the primer-probe sets were at 60ºC for both the invariant TCR and GAPDH.

2.15 Histology

2.15.1 Organ Isolation

Mice were euthanized as described above. Organs were surgically removed without tool marks and were placed in 10-volumes of fresh 10% neutral buffered formalin (BDH, VWR, West Chester, PA, USA).

2.15.2 Histology Tissue Processing

Soft tissues (spleen, liver, intestine, peritoneum, and omentum) were further fixed in formalin for 48 hours at 4°C, changed daily. Organs were rinsed in 1× PBS before being re-suspended in 10-volumes of 1× PBS twice a day for three days, and washed in 10-volumes of 70% ethanol twice and stored in 70% ethanol until processed. Fixed tissues were placed in 4 mm Fisherbrand TRU-Flow tissue cassettes. Formalin-fixed cassetted tissues in 70% ethanol were sent to The Robarts Research Institute Molecular Pathology Core Facility for processing in preparation for embedding in wax. Cassettes were processed in Leica ASP300 fully enclosed paraffin wax tissue processor overnight and were transferred into a warm wax bath and embedded in paraffin wax. Embedded tissues were stored at room temperature until sectioning.

Tissues were sectioned on a microtome HM335E Microtome Leica in the Robarts Research Institute Molecular Pathology Core Facility using MB35 Premier Microtome blades (Thermo Scientific) into 5 micron sections. Serial sections were collected for head sections, and representative sections were cut for the spleen. Sections were mounted on Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Fair Lawn, NJ, USA) and were dried at 45°C for 48 hours prior to storage/staining.

2.15.3 Haematoxylin and Eosin Staining of Processed Tissue

Tissues were stained with haematoxylin and eosin in a Leica Autostainer XL. Slides were allowed to dry and Fisher Finest Premium Cover Glass (Fisher Scientific, Fair Lawn, NJ, USA) cover slips were affixed to the slides using Cytoseal 60 low viscosity mounting medium (Richard-Allen Scientific, Kalamazoo, MI, USA). Cover-slipped slides were dried for at least 24 hours horizontally before vertical storage.

2.15.4 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) Assay

This procedure was performed manually as described previously [237]. Briefly, 4-µm sections were deparaffinized in xylenes, rehydrated in graded alcohols, and rinsed in distilled water. Antigen unmasking was accomplished using freshly prepared Proteinase K solution (10 µg/mL) for 60 min at 37°C. After washing twice with distilled water, sections were incubated with TdT enzyme (75 U/mL) and digoxigenin-11-UTP (5 nmol) for 90min at 37°C. The slides were then washed in SSC buffer (150 mmol NaCL, 15 mmol sodium citrate, pH 7.0), followed by Tris-HCl buffer (10 mmol in 150 mmol NaCl, pH 8.2) for 1 min per wash. A blocking agent was used to prevent non-specific binding and sections were developed with a Fab fragment against digoxigenin linked to alkaline phosphatase and fast red chromogen. Sections were then washed and counter stained.

2.15.5 Histopathological Evaluation

Slides were evaluated in collaboration with two pathologists (Drs. Aaron Haig and Ian Welch, Department of Pathology, Western University, London, Ontario). After all slides were observed, evaluation criteria were determined for each tissue type. The relative number of lymphocytes present, the presence of cell necrosis and apoptosis, and other factors relating to

inflammatory change were assessed. The presence and severity of these findings were used to determine differences in histological pathology in the mice.

2.16 Patients

2.16.1 Inclusion Criteria

Patients aged 18 years and older with a diagnosis of severe sepsis or septic shock upon admission to the Medical-Surgical Intensive Care Unit (MS-ICU) at London Health Sciences Centre-University Hospital (LHSC-UH) and the Critical Care and Trauma Centre (CCTC) at London Health Sciences Centre-Victoria Hospital (LHSC-VH) were prospectively recruited from July 2012 to December 2012. The first day following ICU admission was considered day 1 in the analysis. Sepsis was defined as suspected infection in the presence of two or more systemic inflammatory response syndrome criteria [15]. Severe sepsis was defined as sepsis plus sepsis-induced organ dysfunction or tissue hypoperfusion [15]. Sepsis-induced hypotension was defined as systolic blood pressure (SBP) < 90 mmHg, mean arterial pressure < 70 mmHg or SBP decrease > 40 mmHg or < 2 SD below normal for age in the absence of other causes of hypotension. Septic shock was defined as hypotension (SBP < 90 mmHg) despite adequate fluid resuscitation (> 1,500 ml) or the use of vasoactive agents [15]. Severity of illness was assessed on the basis of two scores: the Acute Physiology and Chronic Health Evaluation II (APACHE II) score (**Table 7**) for the first 24 hours following diagnosis [238, 239]; and the Mannheim Peritonitis Score (**Table 8**) for patients with intra-abdominal sepsis [240].

To calculate the APACHE II score, twelve common physiological and laboratory values (temperature, mean arterial pressure, heart rate, respiratory rate, oxygenation (PaO2 or A-aDo2), arterial pH, serum sodium, serum potassium, serum creatinine, haematocrit, white blood cell

count and Glasgow Coma Score) are marked from 0 to 4, with 0 being the normal, and 4 being the most abnormal (**Table 7**). The sum of these values is added to a mark adjusting for patient age and a mark adjusting for chronic health problems (severe organ insufficiency or immunocompromised patients) to arrive at the APACHE II score.

Table 7: APACHE II Scoring System

Variable	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature (°C)	≥ 41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤ 29.9
Mean Arterial BP (mm Hg)	≥160	130-159	110-129		70-109		50-69		≤ 49
Heart Rate (min^{-1})	≥ 180	140-179	110-139		70-109		55-69	40-54	≤ 39
Respiratory Rate (min^{-1})	≥ 50	35-49		25-34	12-24	10-11	6-9		≤ 5
A-aPO_2 (if FiO_2>50%)	≥ 500	350-499	200-349		< 200				
PaO_2 (if FiO_2<50%)					> 70	61-70		55-60	< 55
Arterial pH	≥ 7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	< 7.15
Serum HCO_3^-	≥ 52	41-51.9		32-40.9	23-31.9		18-21.9	15-17.9	< 15
Serum Na^+ (mmol/L)	≥ 180	160-179	155-159	150-154	130-149		120-129	111-119	≤ 110
Serum K^+ (mmol/L)	≥ 7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		< 2.5
Serum Creatinine (g/dL)	≥ 3.5	2-3.4	1.5-1.9		0.6-1.4		< 0.6		
Hematocrit	≥ 60		50-59.9	46-49.9	30-45.9		20-29.9		< 20
WBC Count	≥ 40		20-39.9	15-19.9	3-14.9		1-2.9		< 1
Age (y)[1]					< 44		45-54	55-64	

[1] ADD: 5 points if age is 65-74 yrs; 6 points if age >75 yrs

Chronic Health Adjustment:

ADD: 2 points if patients have had elective surgery or for non-surgical patients. 5 points for emergency surgery

1. Biopsy-proven cirrhosis
2. New York Heart Association Class IV Congestive Heart Failure
3. Severe COPD (hypercapnic; requiring home O_2; pulmonary hypertension)
4. Chronic dialysis
5. Immune-compromised (HIV; immunosuppressive medications)

APACHE II score is calculated by adding all the points accumulated by a patient in the first 24 hours of his/her admission to the ICU.

Table 8: Mannheim Peritonitis Score (MPI)

Variable	Score
Age > 50 years	5
Female gender	5
Organ failure*	7
Malignancy	4
Pre-operative duration of peritonitis > 24 hours	4
Origin of sepsis not colonic	4
Diffuse generalized peritonitis	6
Exudate	6
Clear	0
Cloudy or purulent	6
Feculent	12

*Definitions of organ failure: Kidney: creatinine >177 µmol/L, urea > 167 µmol/L, oliguria < 20 mL/h; Lung: pO_2 < 50 mmHg; pCO_2 > 50 mmHg; Shock: hypodynamic or hyperdynamic; Intestinal obstruction (only if profound): Paralysis > 24h or complete mechanical ileus

2.16.2 Exclusion Criteria

Exclusion criteria were the presence of immunodeficiency or concomitant immunosuppressive therapy, pregnancy, Do Not Resuscitate (DNR) status and cardiac arrest. Informed consent was obtained directly from each patient or his or her legal representative before enrolment.

2.17 Microbiological Diagnostics

Standard cultures in biological samples guided by the presumptive source of the septic insult were performed to assess the presence of bacterial and fungal infection. Species identification and biotyping was conducted by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; MALDI Biotyper, Bruker Daltonics, Germany). Potentially contaminant microorganisms were not considered.

2.18 Isolation and Staining of Leukocytes from Human Peripheral Blood

Human blood was collected in heparinized vacuum tubes, diluted 1:1 with RPMI, layered over pre-warmed Ficoll and spun at 700 × *g* for one hour. PBMCs were removed by pipetting and washed in 40 ml of warm RPMI, pelleted for 5 minutes at 700 × *g*, and resuspended in cRPMI. Cells were assessed for viability by trypan blue. Cells were then washed and stained with anti-CD3ε-FITC (clone HIT3a, BD Pharmingen) and anti-CD69-PE (clone FN50, eBioscience) mAbs, along with human CD1d tetramer.

2.19 Statistical Analysis

All data were maintained in Microsoft Excel 2010 (Microsoft, Redmond, WA), and were analyzed using Graphpad Prism Version 5.01 (Graphpad, La Jolla, California). In all analyses, two-tailed *P* values less than 0.05 were considered statistically significant.

For murine experiments, statistical comparisons were performed using analysis of variance (ANOVA) or Mann-Whitney U test where appropriate. Survival curves were calculated by the Kaplan-Meier method.

Each of the seven variables measured as part of the MSS consisted of five possible scores (0 to 4). The internal consistency of the MSS and each of the variables was assessed by Cronbach's alpha. Inter-rater reliability of the MSS was also assessed by calculating the intraclass coefficient (ICC), comparing each assessor's independent scores for each mouse (sham and septic) at 2, 12, 14, 16, 18, 20, and 24 hours. Additionally, the ability of the MSS to discriminate between sham and septic mice was tested using the receiver operating characteristic (ROC) curve, and by quantifying the area under the curve (AUC) [241]. An AUC between 0.7 and 0.8 is classified as "acceptable," and an AUC between 0.8 and 0.9 is considered to have an "excellent" discrimination [241]. For the MSS, the score giving the best Youden index was determined to be the cutoff point [242]: the sensitivity, specificity, and positive and negative predictive values were calculated based on this score. To ensure that the MSS reflected the severity of the septic insult, correlations between the sepsis score and serum pro-inflammatory cytokine levels were performed by calculating the Pearson correlation coefficient (Pearson's r) with 95% confidence intervals.

Biochemistry and cytokine data obtained at all the time points for sham mice were pooled together and employed as a common control group, as Friedman's test indicated no differences over time. Data obtained from mice with IAS were considered independently. Differences between groups were analyzed by applying non-parametric ANOVA (Kruskal-Wallis) tests, followed by post-hoc pairwise multiple comparisons by Dunn's method. For cytokine analyses, data from multiple experiments were pooled and analyzed by one-way ANOVA with post-hoc

comparisons using Tukey's tests. Group sizes reported for data varied over time, reflecting the mortality rate in septic animals.

For human subjects, differences between groups were assessed using the Mann–Whitney U-test or Chi-square test for continuous and categorical variables, respectively. Survival curves were calculated by the Kaplan-Meier method.

Chapter 3: Results

3.1 Peripheral blood iNKT cells are elevated in patients with sepsis/severe sepsis

We first sought to determine if patients with sepsis had an altered frequency of iNKT cells in their peripheral blood compared to non-septic patients. We prospectively evaluated thirty patients who were admitted to the London Health Sciences Centre (LHSC) Critical Care and Trauma Centre (CCTC) for sepsis or non-sepsis-related critical illness; 23 patients were diagnosed with sepsis/severe sepsis, while 7 patients were non-septic trauma patients (**Table 9**). In the non-septic group, 3 patients (43%) had sustained traumatic head injuries and 4 patients (57%) had emergency surgery for trauma (2 liver resections; 1 abdominal aortic surgery; 1 spine stabilization operation). Groups were similar in age and severity of illness, as calculated by the APACHEII score [238]. However, the gender distribution was significantly different between the two groups, with a preponderance of males in the non-septic group ($p< 0.0001$). Most of the patients in the septic group had intra-abdominal sepsis (44%) or lower respiratory tract infections (39%) as confirmed by diagnostic tests. In 30% and 17% of septic patients respectively, a single Gram-positive or Gram-negative pathogen was identified, while multiple organisms were identified in 30% of the septic group. In 17% of septic patients, the microbial agent was not identified, while 1 patient (4%) had fungal candidemia (**Table 9**).

When lymphocyte subpopulations were assessed by flow cytometry and compared, the septic group had a higher median percentage of T cells among total lymphocytes (57.8% versus 36.7% in the non-septic group, $p= 0.039$) (**Table 10**; **Figure 6a** and **b**). Moreover, the iNKT:T cell ratio was significantly higher in the septic group (**Table 10**). Patients in the septic group stayed in hospital for a significantly longer time (25.2 days versus 12.8 days, $p= 0.045$ by Mann Whitney U test), although in-hospital mortality was similar between the two groups (**Table 10**).

Table 9: Demographics and clinical characteristics of study patients

Demographic and clinical characteristics	**Non-septic (n = 7)**	**Septic (n = 23)**	**P Value**
Median Age (years)	61	59	0.433
Gender			**< 0.0001**
Male	6	13	
Female	1	10	
Mean APACHEII Score	23	16	0.377
Comorbidities, n:			0.689
Cardiovascular Disease	4	4	
COPD	1	7	
Chronic Renal Failure or Dialysis	0	0	
Diabetes mellitus types 1 and 2	2	4	
Alcohol abuse	1	1	
Hypertension	3	7	
Neoplasia	1	4	
Obesity	2	1	
Diagnostic at ICU admission, n:			
Sepsis	-	14	-
Severe Sepsis	-	4	-
Septic Shock	-	0	-
Presumed Source of Infection, n:			
Lower respiratory tract/pneumonia	-	6	-
Urogenital	-	1	-
Intra-abdominal	-	9	-
Catheter- or device-Related	-	0	-
Skin (soft tissues)	-	2	-
Prosthesis	-	0	-
Central nervous system	-	0	-
Other/unknown	-	0	-
Documented microbial agent, n:			
Gram-positive	-	5	-
Gram-negative	-	4	-
Fungi	-	1	-
Polymicrobial	-	4	-
None/Unknown	-	4	-

Table 10: Outcomes of study patients.

Variable	Non-Septic (n = 7)	Septic (n = 23)	P Value
Median White Blood Cell Count	10.6	11.5	0.182
Lymphocytes, %[1]	16.2	17.6	0.252
Lymphocyte Subset Populations[2]			
T cells, %	36.7	57.8	**0.039**
NK cells, %	5.19	12.25	0.274
NKT cells, %	0.45	1.88	0.262
NKT:T cell ratio, %	0.011	0.029	0.274
iNKT cells, %	0.0041	0.00569	0.138
iNKT:T cell ratio, %	0.0090	0.020	**0.047**
Mean Hospital Stay (range), days	12.8 (0-38)	25.2 (4-55)	**0.045**
Mortality, n (%)	3 (43)	5 (28)	0.955
Cause of Mortality, n (%)			0.293
Multi-organ failure	1 (14)	4 (17)	
Cardiac arrest	1 (14)	0 (0)	
Withdrawal of care	1 (14)	1 (4.3)	

[1]Expressed as a percentage of the total sample analyzed on flow cytometry

[2]Expressed as a percentage of lymphocytes. Median populations are presented.

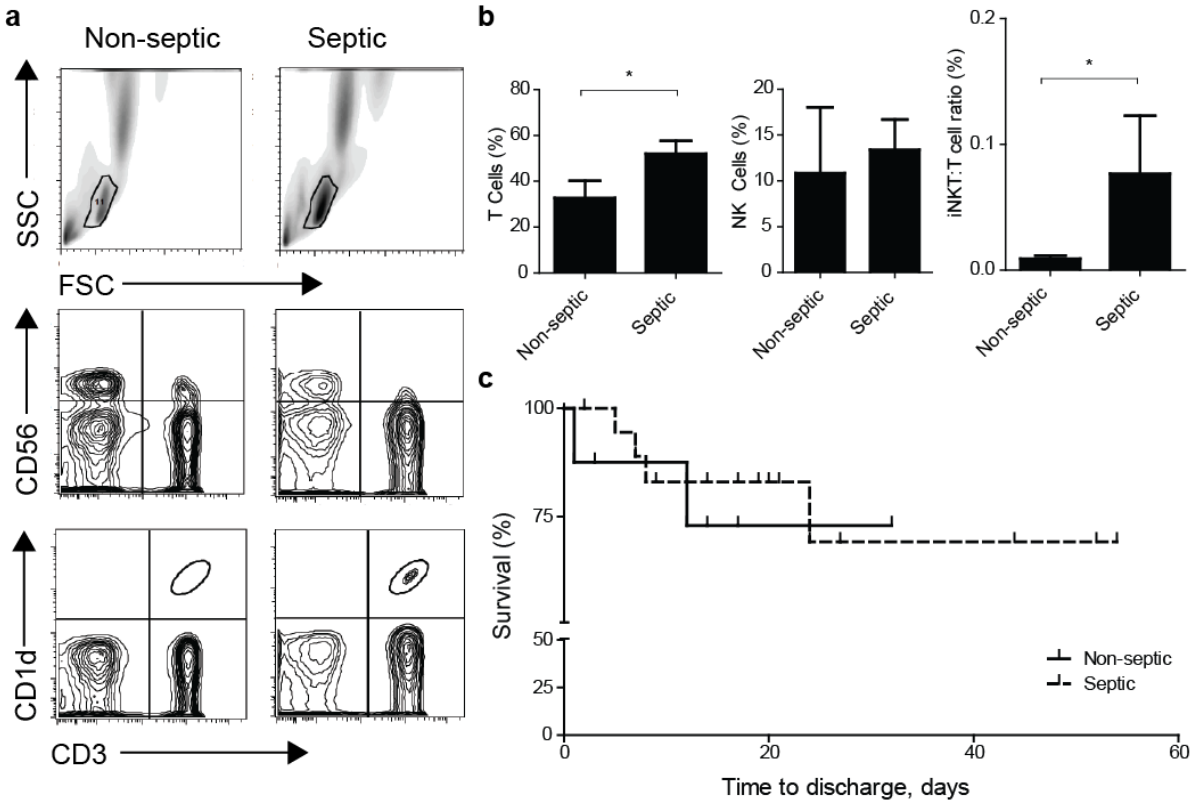

Figure 6: Characterization of iNKT cell populations in the sera of critically-ill patients.

(a) Representative flow cytometry plots of peripheral blood sampled from a septic and non-septic patient. (b) Histograms (median ± SEM) comparing frequency of T cells, NK cells, and iNKT:T cell ratios in septic and non-septic patients in the intensive care unit. *p< 0.05 by Mann Whitney U test (C) Kaplan-Meier survival curves from time of blood collection to time of discharge.

3.2 Validation of the MSS in a mouse model of IAS

We next sought to validate the MSS as a humane and effective surrogate to death as an endpoint in our mouse model of IAS, and correlate it with disease severity. A total of 300 mice were used in all the experiments that were conducted independently over a period of two years, to develop the mouse model of IAS and the MSS, and to validate the latter. Compared to sham-treated mice (n= 60), which had a survival of 100% throughout the experimental timeline, mice with IAS had a survival rate of 0% for 180 mg/mL FS (n= 20), 25% for 90 mg/mL FS (n= 200) and 40% for 45 mg/mL FS (n= 20) at 24 hours post-FIP induction (**Figure 7A**). For subsequent experiments, we used a FS concentration of 90 mg/mL to mimic the clinical mortality of 70-80% in severe untreated intra-abdominal sepsis [243, 244]: MSS for FIP mice at this concentration are shown in **Figure 7B**. Compared to sham-treated mice with a mean score of 1 after 24 hours, FIP-treated mice had significantly higher (p <0.0001) sepsis scores. Between 0 to 11 hours post-FIP induction, mouse scores remained relatively consistent as assessed by independent observers, with mild piloerection and decreased movement. After 12 hours, septic mice appeared to progressively manifest additional symptoms including decreased respiratory rate, increasingly laboured breathing and minimal response to auditory and tactile stimuli. Between 12 to 17 hours post-FIP induction, variability in sepsis severity scores was observed to be due to differences in respiratory rates and quality of breathing as well as response to tactile and auditory stimuli. The intra-class correlation coefficient for comparison between the blinded and non-blinded assessors of septic mice was 0.96 (95% CI: 0.92 - 0.98), indicating excellent inter-rater reliability. The Cronbach alpha coefficient was 0.92, indicating excellent internal consistency of the MSS.

For a concentration of 90 mg/mL FS, we calculated a mortality rate of 42% within 1 hour of attaining a score of 10, and a mortality of 75% within 2 hours of attaining a score of 10. Fifty-

seven percent of mice that reached a score of 15 died or had to be euthanized (as per ethics guidelines) within 1 hour, and 86% of mice that reached a score of 15 died within 2 hours. Based on the ROC curve generated for the MSS (**Figure 7D**), the AUC (95% confidence interval) was 0.825 (0.752 - 0.898) with a p value < 0.0001, suggesting that the scoring system has excellent discriminatory power. An MSS of 3 (Youden score of 0.61) was selected as the cut-off point for mice that progressed to severe sepsis post-FIP induction: the sensitivity (±95% C.I.) and specificity (±95% C.I.) of this score was 57% (47-67%) and 100% (82-100%), respectively.

When organs were homogenized and plated on agar, bacterial growth was observed in all tissues, including liver, spleen, heart, lung, and kidneys (**Figure 7C**). Consistent with the polymicrobial nature of the model, significant variations in colony size (ranging from 1-3 mm in diameter), colour (white, brown, and yellow), and CFU counts were observed. Bacterial counts were not observed in any organs recovered from sham mice (data not shown). We did not observe a correlation between sepsis score and CFU counts in FIP mice.

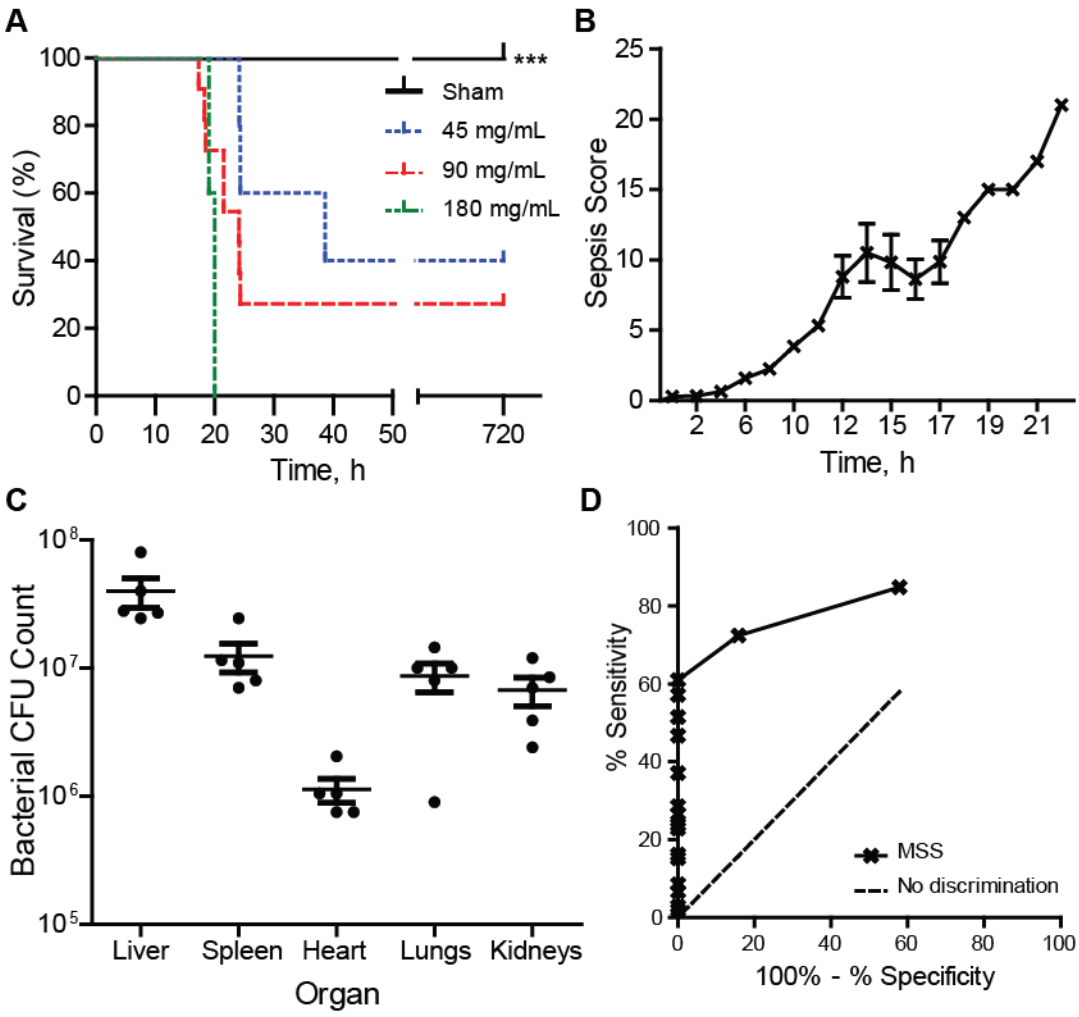

Figure 7: Characterization of a mouse model of acute intra-abdominal sepsis (IAS). (A) Mouse survival over time versus concentration of fecal solution (FS; *n* = 20 mice per concentration of FS). (B) Murine Sepsis Score (MSS) over time of mice that were administered 90 mg/mL FS (*n* = 20, 1 representative experiment). (C) Viable bacterial colony forming units (CFU) recovered from solid organs of mice treated with 90 mg/mL FS, at the time of euthanasia (*n* = 5). (D) Receiver-operator curve (ROC) evaluating the sensitivity and specificity of the MSS in differentiating healthy mice from those that progress to septic shock and death.

On necropsy, FIP mice were observed to have developed diffuse intestinal distension compared to uninfected control mice (**Figure 8**). In addition, FIP mice had peritoneal and mesenteric lymphadenopathy, and rarely developed abscesses. We also routinely observed the presence of a yellow fibrin film on the surfaces of the intra-abdominal organs, most notably overlying the liver and spleen. We did not identify any grossly visible areas of necrosis or ischemia within the organs. In mice that were euthanized due to severe respiratory distress, we observed minor pulmonary haemorrhage and the lungs appeared edematous.

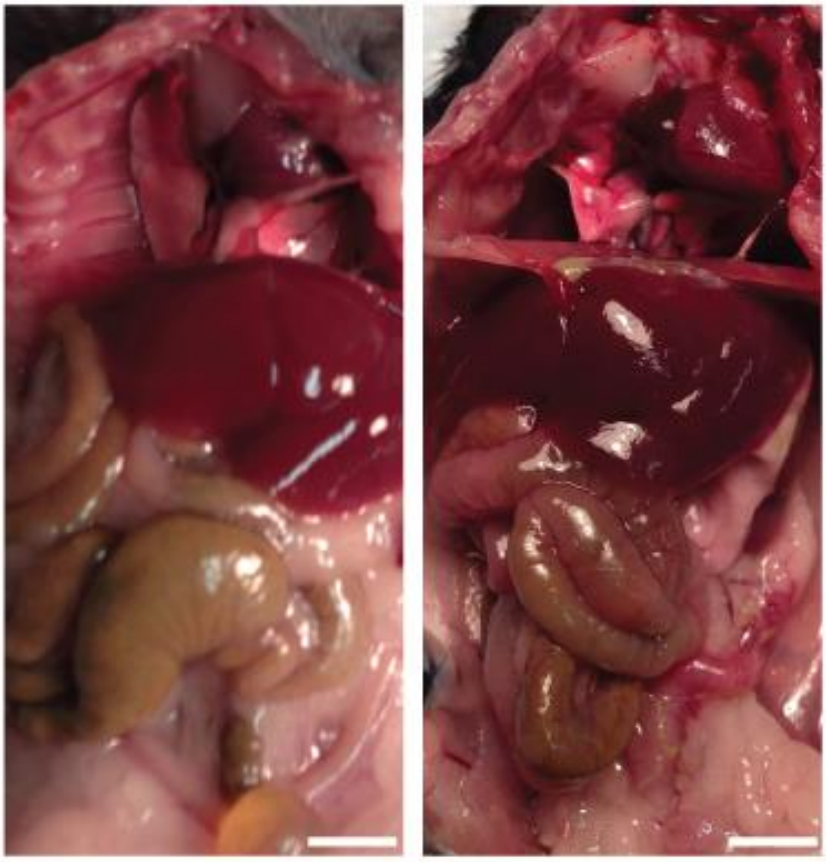

Figure 8: Necropsy of naïve and septic B6 mice.

Macroscopic intra-abdominal view of control (*left*) and 90mg/mL FIP mouse (*right*) at 24h reveals significant intestinal distension in the latter (size bar, 1 cm).

Serum biochemistry demonstrated significantly elevated AST and ALT levels in the FIP group at 6, 12, 18, and 24 hours post-sepsis induction ($p < 0.001$) compared to the sham group (**Figure 9A**). However, both AST and ALT levels peaked at 6 to 12 hours in septic mice: while the AST levels declined and rose again at 18 and 24 hours respectively, the ALT levels fell sharply at 18 and 24 hours. A linear correlation between liver transaminases and MSS was only significant for the first 12 hours of the experimental timeline, but was non-significant for the entire duration (24 hours) of the experiment. Serum glucose and creatinine did not demonstrate significant changes over time in the FIP group (**Figure 9B**, **C**). Serum albumin levels decreased significantly at 3 and 12 hours post-sepsis compared to the sham group ($p = 0.0057$ and $p = 0.018$, respectively), but there was no difference in albumin levels after 24 hours (**Figure 9D**). We observed a trend towards higher lipase levels at 24 hours post-sepsis but there was significant variability in lipase activity among individual mice in the FIP group (**Figure 9E**).

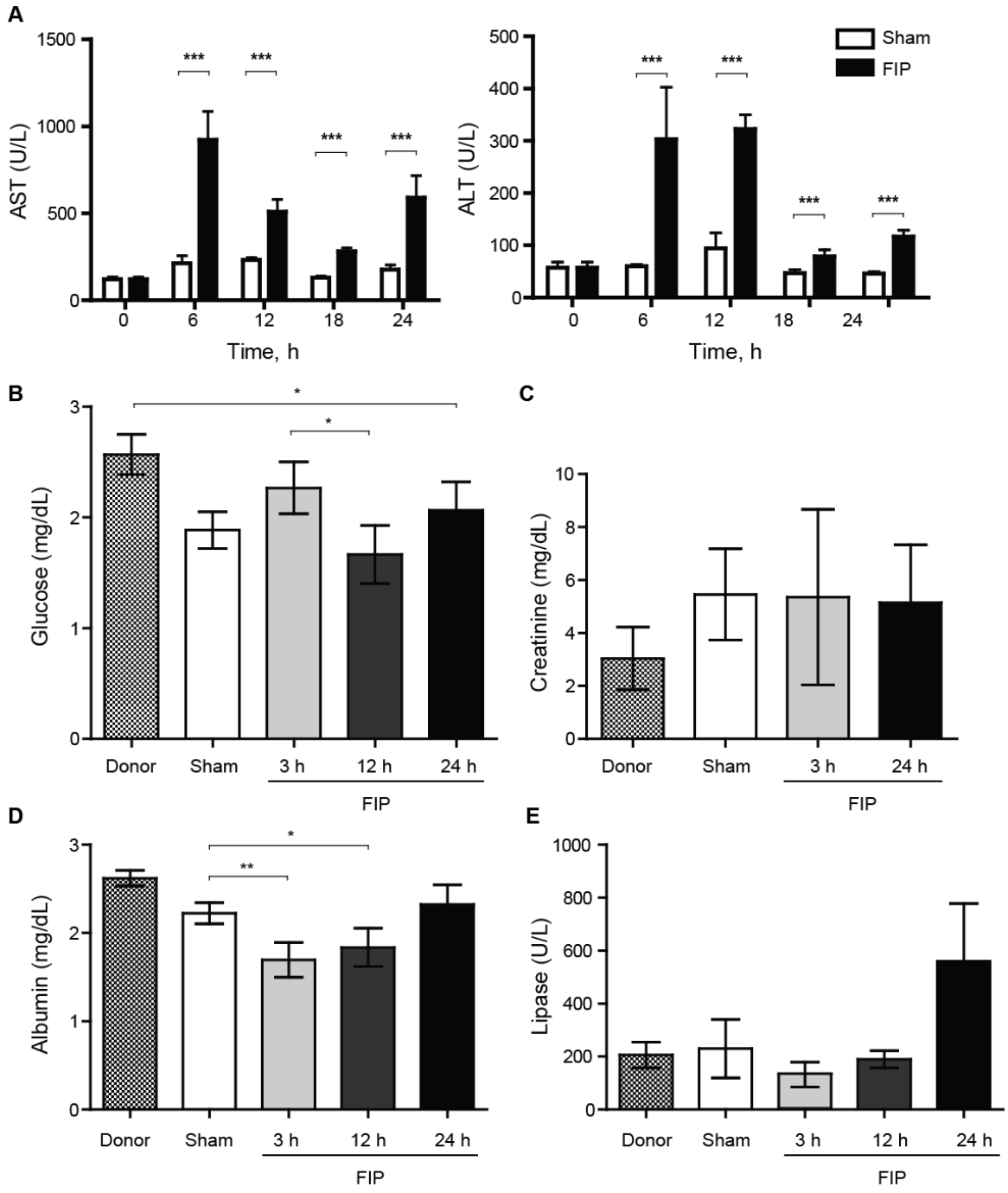

Figure 9: Serum biochemistry of B6 mice with IAS.

Blood serum biochemistry of (A) liver enzymes aspartate transaminase (AST) and alanine aminotransferase (ALT), (B) Glucose, (C) Creatinine, (D) Albumin, and (E) Lipase (n = 4 in donor group; n = 12 for sham group; $n \geq 4$ per group at 3 h, 12 h, 16 h, 18 h, and 24 h). Mean values shown with SEM error bars. * p <0.05, ** p <0.01, *** p <0.001.

Histological examination of the tissues demonstrated different degrees of pathology in various organs at 24 hours after FIP (**Figure 10**). In the lung, we observed mild edema in the alveolar spaces and leukocyte accumulation in the peripheries of pulmonary arterioles. In the liver of mice with FIP, parenchymal cells demonstrated vacuolization, limited necrosis, and loss of organization and structure. We also occasionally observed capsular edema and recruitment of inflammatory cells onto the liver surface. The spleen demonstrated significant changes post-sepsis, with expansion of the white pulp, and widespread cellular apoptosis, which was also confirmed by TUNEL staining (**Figure 10**). At higher concentrations of FS (180 mg/mL), pathological changes associated with damage and inflammation could be observed within 12 hours of insult (data not shown). We also observed pathological changes in the small intestine (**Figure 10**), characterized by the loss of goblet cells and loss of villi. We did not observe the accumulation of neutrophils or other leukocytes within the submucosa, but we occasionally observed necrosis and debris on the serosal surfaces of the gastrointestinal tract. We did not observe pathological changes in the hearts or brains of septic mice at 24 hours (data not shown).

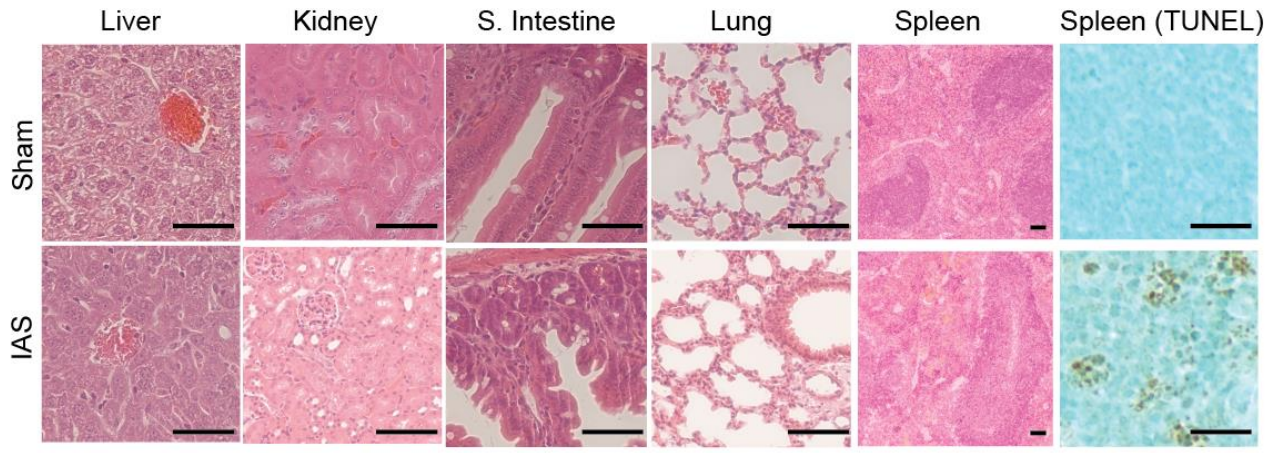

Figure 10: Histology of sham-treated and septic B6 mice.

Representative tissues from sham-treated and septic mice at 24h developed with TUNEL or haematoxylin and eosin (size bar, 50µm).

Analysis of cytokine levels by multiplex array showed a rapid, sustained, and significant increase of the putative markers of experimental sepsis, namely IL-1β, IL-6, IL-10, and TNF-α, in FIP mice over a 24-hour period ($p < 0.001$) versus the sham group (**Figure 11**). Additionally, we observed increased levels of eotaxin, M-CSF, MIG, MIP-1α, MIP-1β, MIP2, IL-5 and IL-15. IL-5 and IL-15 returned to baseline levels by 12 hours; however, IL-5 was detected at significantly increased levels at 24 hours, compared to the control group ($p < 0.001$). Results of additional analysed cytokines, which are well described in septic models, are shown in **Table 11**.

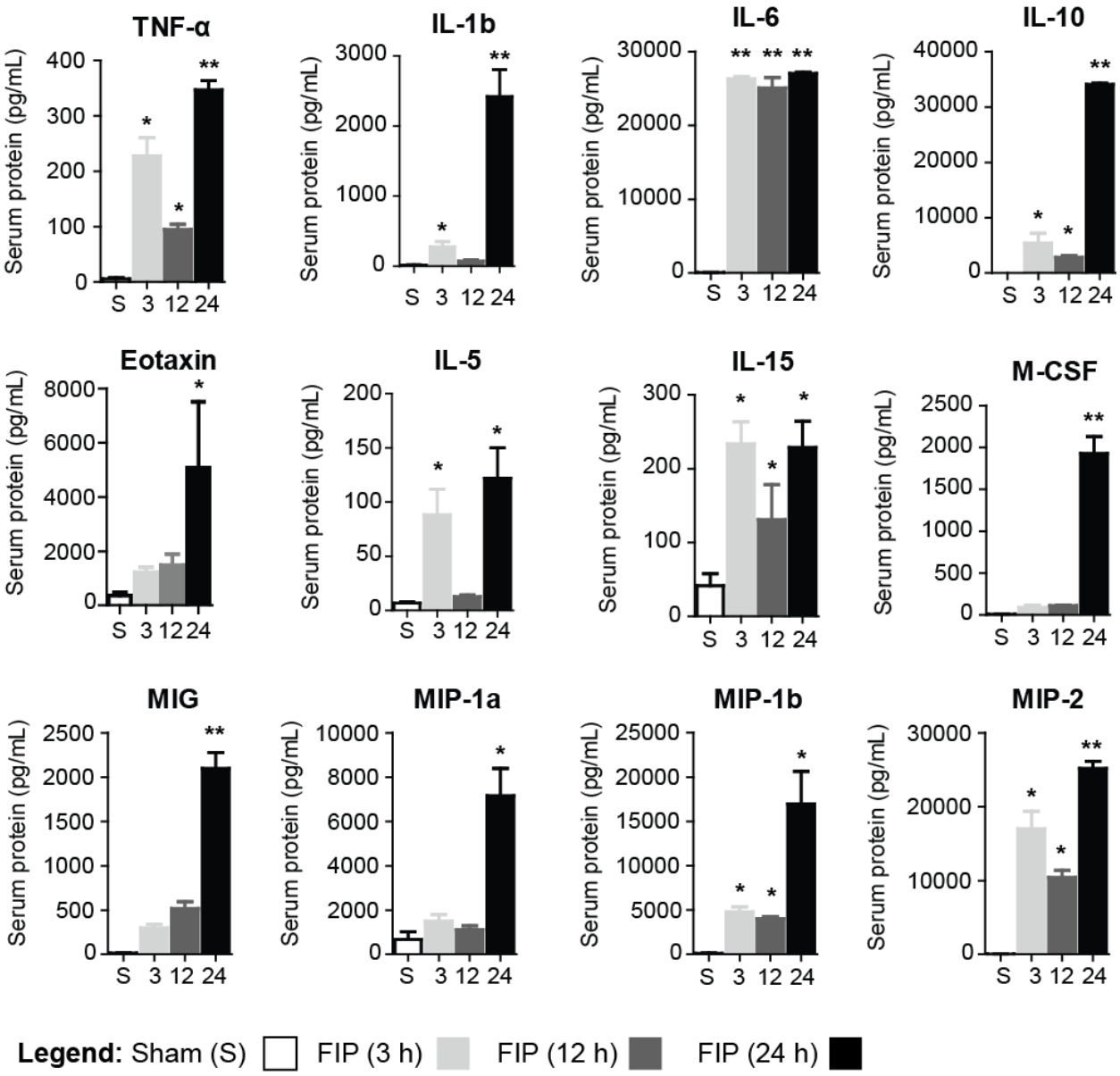

Figure 11: Serum cytokines and chemokines in septic B6 mice.

Sham and FIP (90 mg/mL) cytokine and chemokine levels (pg/mL) at 3, 12, and 24h post FIP induction. Mean serum protein concentrations ± SEM are shown (n = 12 for sham group; $n \geq 3$ for 3, 12, and 24h groups). *p <0.05, **p <0.01, ***p <0.001.

Table 11: Changes in concentrations of chemokines and cytokines in sham- and 90 mg/mL FS-treated mice with IAS.

Cytokine / Chemokine	Sham	IAS			P Value
		3h	12h	24h	
IL-1α	81.14 ± 22.79	313.91 ± 25.18	*ND*	1089.33 ± 141.89	<0.001
IL-12p40	10.14 ± 4.10	66.01 ± 13.74	0.91 ± 0.91	86.76 ± 8.97	<0.001
IL-12p70	39.81 ± 24.53	48.14 ± 17.62	28.78 ± 17.15	70.83 ± 36.10	*ns*
IL-13	218.93 ± 31.44	413.71 ± 17.57	315.23 ± 39.11	812.51 ± 171.84	<0.001
IL-17A	5.45 ± 1.13	403.32 ± 132.46	103.32 ± 33.20	1954.34 ± 538.63	<0.001
IP-10 (CXCL10)	59.42 ± 9.45	553.66 ± 223.84	155.26 ± 51.19	1059.87 ± 98.50	<0.001
KC (CXCL1)	556.53 ± 121.08	30212.64 ± 109.51	28136.57 ± 2005.17	30600.09 ± 84.61	<0.001
LIF	0.95 ± 0.46	60.67 ± 16.62	31.58 ± 6.64	377.38 ± 66.71	<0.001
G-CSF (CSF3)	1159.18 ± 289.34	39854.47 ± 224.65	39942.15 ± 207.13	39855.60 ± 335.01	<0.001
GM-CSF (CSF2)	46.33 ± 5.53	212.57 ± 19.99	92.79 ± 19.11	349.34 ± 29.02	<0.001
MCP-1	34.51 ± 6.32	3568.44 ± 1114.53	1182.31 ± 85.60	27602.38 ± 806.52	<0.001
RANTES (CCL5)	17.96 ± 4.15	106.74 ± 14.70	108.34 ± 19.58	1321.31 ± 147.91	<0.001
VEGF	2.67 ± 1.14	5.01 ± 0.81	13.75 ± 12.11	5.24 ± 2.19	*ns*

Mean concentrations ± SEM are given in pg/mL (n = 12 for sham mice; n = 3 in the 3h, 12h, and 24h FIP groups respectively; *ns*: not significant; *ND*: not determined).

3.3 iNKT cells are pathogenic in intra-abdominal sepsis

Given our finding of elevated iNKT cell proportions in human sepsis/severe sepsis, and the multiple studies that have demonstrated the pathogenicity of iNKT cells in animal models mimicking chronic polymicrobial sepsis [159], we studied iNKT cells in our mouse model of IAS. Since iNKT cells can rapidly produce pro- and/or anti-inflammatory cytokines in response to stimuli and shape the subsequent immune responses in various diseases [174, 235], we hypothesized that these cells would affect disease severity and survival in IAS. Compared to C57BL/6 (B6) mice, we observed a significant reduction in sepsis severity (**Figure 12a**) and mortality (**Figure 12b**) in J$\alpha 18^{-/-}$ mice, which selectively lack iNKT cells [230]. Whereas an intra-peritoneal injection of a fecal slurry solution (90 mg/mL) in B6 mice resulted in 100% mortality at 24 hours, the sham B6 and J$\alpha 18^{-/-}$ groups, which were injected with normal saline, as well as the septic J$\alpha 18^{-/-}$ group, remained alive. On necropsy, we observed discrete abscess collections overlying the intestines and liver in septic J$\alpha 18^{-/-}$ mice, whereas septic B6 mice developed intestinal distension and edema without abscess formation (**Figure 13**).

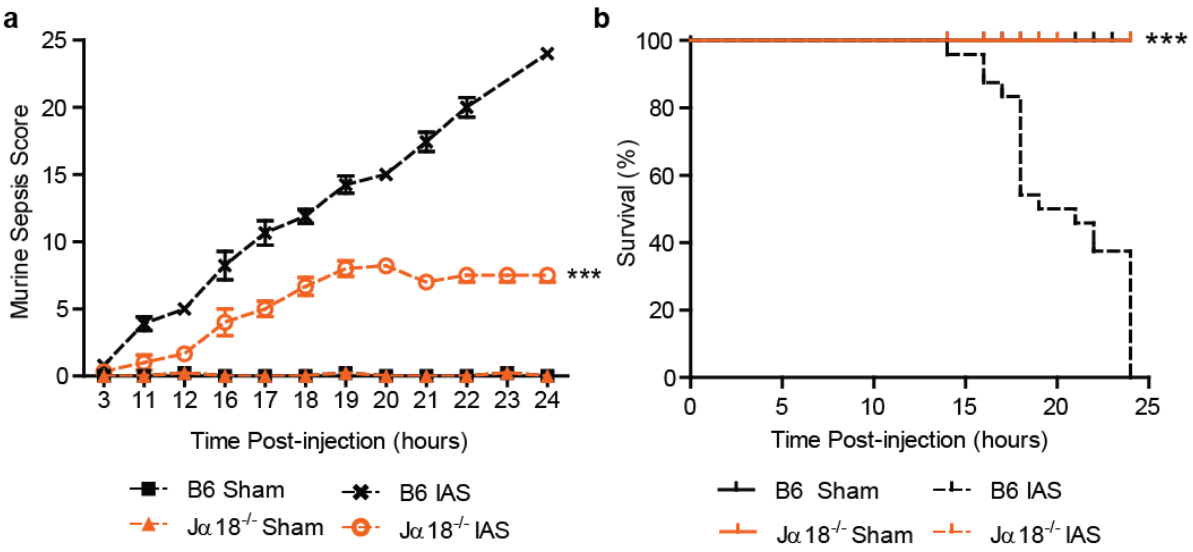

Figure 12: iNKT cells are pathogenic in intra-abdominal sepsis (IAS).

(a) B6 and iNKT-deficient Jα18$^{-/-}$ mice (*n*= 6) were injected with fecal slurry (90mg/mL) to induce IAS and monitored during the experimental timeline. Murine sepsis scores were significantly higher compared to sham-treated B6 and Jα18$^{-/-}$ mice (injected i.p. with normal saline [NS]) and Jα18$^{-/-}$ mice with IAS (*n*= 6 for sham B6 and Jα18$^{-/-}$ mice each, *n*= 10, *n*= 6 for septic B6 and Jα18$^{-/-}$ mice respectively). ***p< 0.001 by two-way ANOVA test. (b) Mortality for B6 mice with IAS were significantly higher than sham B6 and Jα18$^{-/-}$ mice, as well as septic Jα18$^{-/-}$ mice (*n*= 6 for sham B6 and Jα18$^{-/-}$ mice each, *n*= 10, *n*= 6 for septic B6 and Jα18$^{-/-}$ mice respectively). ***p< 0.001 by log-rank test

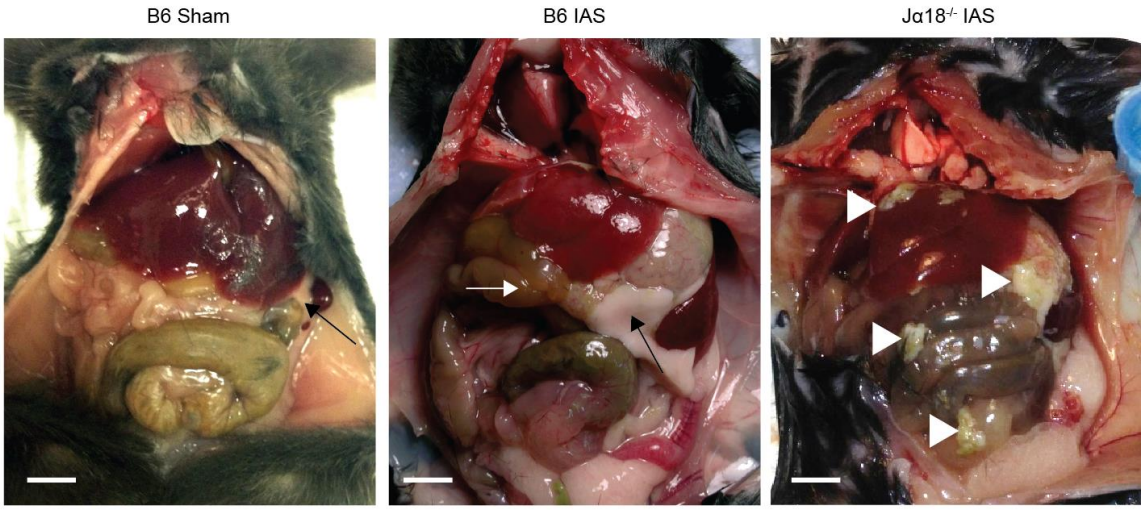

Figure 13: Necropsy of sham and septic B6 and Jα18$^{-/-}$ mice.

On necropsy, septic B6 mice had significant intestinal distension (white arrow) compared to sham-treated B6 mice. The omentum (black arrow) was also enlarged in septic B6 mice. Jα18$^{-/-}$ mice formed discrete abscesses (white arrowheads) overlying the liver and intestines, with no intestinal distension (size bar, 0.5 cm).

To assess whether iNKT cells were migrating to the omentum or proliferating within their native tissues, we adoptively transferred 5×10^5 GFP-expressing iNKT cells from transgenic mice into $J\alpha18^{-/-}$ mice through a tail-vein injection. After 18 hours, we induced sepsis by intraperitoneal administration of fecal slurry (90 mg/mL) and monitored mice for 24 hours. Mice that received iNKT cells fared worse than $J\alpha18^{-/-}$ that did not receive iNKT cells (**Figure 14**), with respect to disease severity. None of the mice in either group died at the end of the experimental timeline. Together, these results confirm the pathogenic nature of iNKT cells in IAS.

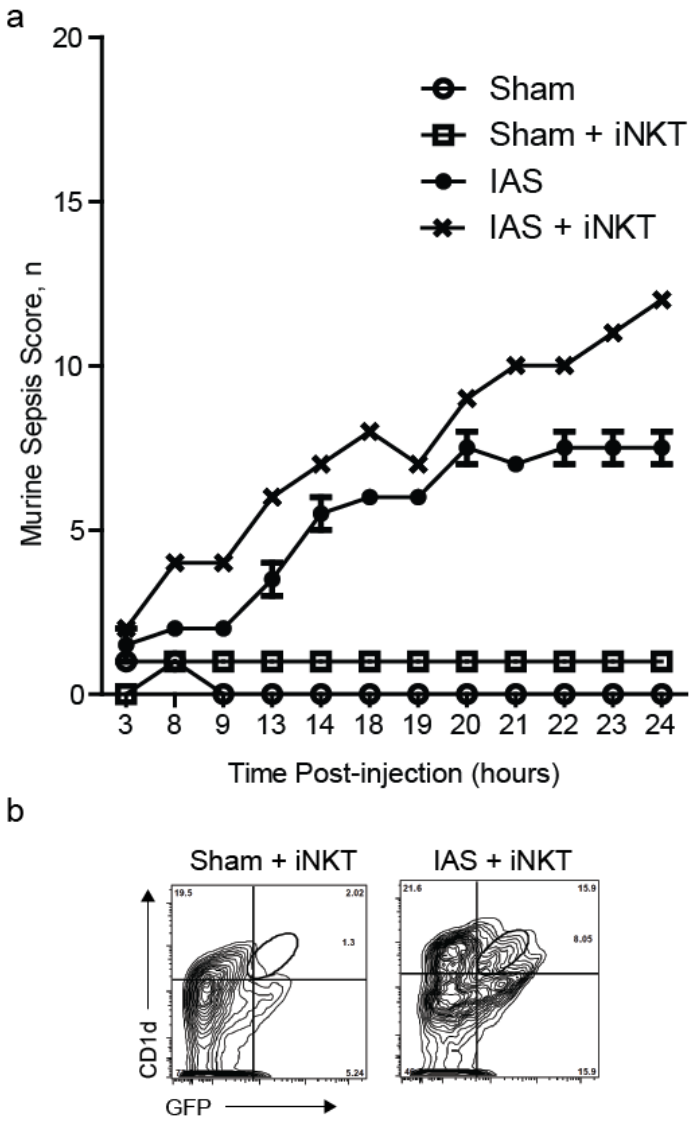

Figure 14: Adoptive transfer of iNKT cells into iNKT-deficient mice.

Splenic and hepatic iNKT cells were isolated and sorted from GFP-expressing transgenic mice, and injected i.v. into Jα18$^{-/-}$ mice. After 18 hours, mice were administered a fecal slurry (90 mg/mL) to induce IAS and monitored for 24 hours. Adoptive transfer of iNKT cells increased the severity of sepsis (a) compared to Jα18$^{-/-}$ mice that did not receive iNKT cells. (b) Adoptively-transferred iNKT cells moved into the omentum of Jα18$^{-/-}$ mice following IAS, as detected by flow cytometry, compared to adoptively-transferred iNKT cells in sham Jα18$^{-/-}$ mice.

3.4 Tissue-specific distribution of iNKT cells is altered in IAS

Previous animal studies using a model of chronic polymicrobial sepsis found that the frequency of hepatic iNKT cells declined significantly, whereas splenic iNKT cells remained unchanged [159]. We sought to determine whether a similar occurrence would be observed in acute IAS. Furthermore, we hypothesized that the omentum, which has been described as the "policeman of the abdomen" for its ability to migrate to and mitigate inflammatory reactions [229] may accommodate increased numbers of iNKT cells post-sepsis.

Using flow cytometry, we determined the frequencies of $TCR\beta^{+}CD1d$ tetramer^{-} conventional T cells and $TCR\beta^{+}CD1d$ tetramer^{+} iNKT cells in the spleen, liver and omentum. In the spleen (**Figure 15a**), the percentage of conventional T cells declined significantly post-sepsis from 44.5% to 31.2% (p= 0.0128). The percentage of splenic iNKT cells also reduced significantly post-sepsis from 1.18% to 0.33% (p= 0.0046). In the liver (**Figure 15a**), there was no difference in the tissue-specific distribution of iNKT or T cells. In the omentum (**Figure 15a**), the percentage of T cells increased significantly post-sepsis from 12.78% to 38% (p= 0.0095), and the percentage of iNKT cells were also significantly elevated post-sepsis from 0.58% to 5.5% (p= 0.040).

We also sought to quantify the transcriptional expression of the invariant TCR following IAS, because the surface receptors of iNKT cells (including the TCR and NK1.1) can be down-regulated upon activation [245, 246], and become undetectable by flow cytometry using standard reagents [245]. Using the Taqman assay with custom designed primers that overlap the invariant TCR Vα14-Jα18 splice site and amplify a portion of the TCR [173, 178], we observed significant increases in the transcriptional expression of the invariant TCR within the spleen, liver, and omentum post-sepsis (**Figure 15b**). Together, these results demonstrate that the tissue-specific

distribution of iNKT cells is altered significantly during IAS, and that the transcription of the invariant TCR is increased post-sepsis.

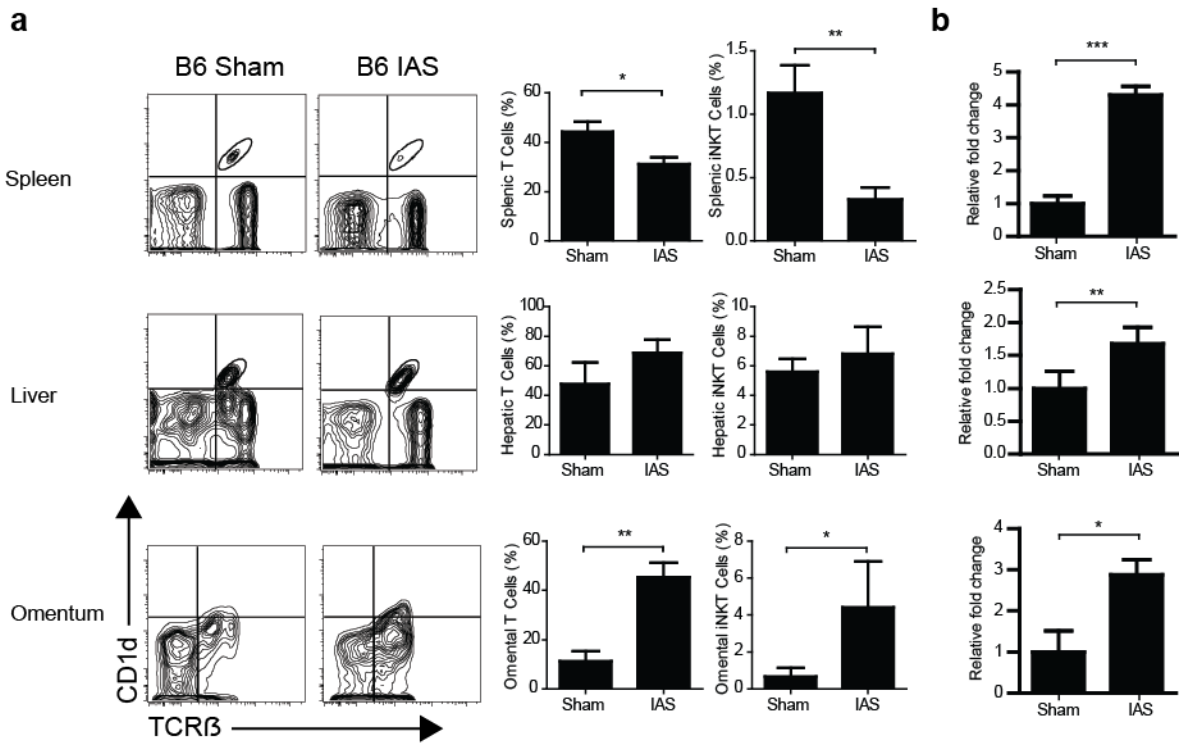

Figure 15: Tissue-specific distribution of iNKT cells is altered during IAS

(a) The distribution of T and iNKT cells in the spleen and omentum is altered significantly in IAS, but remains unchanged in the liver (*n*= 7, *n*= 10 in sham and IAS groups respectively). Percentages of cell populations are represented as means ± SEMs. *** $p<0.0001$, ** $p<0.001$, * $p<0.05$ by Mann Whitney U test (b) Quantitative RT-PCR detecting iNKT cells in the spleen, liver, and omentum.

3.5 Th2-polarized iNKT cells reduce disease severity in IAS

Multiple groups, including ours, have examined the use of glycolipids to modulate cytokine responses in iNKT cells, and ameliorate disease severity in mouse models of autoimmune diseases such as Type 1 Diabetes [204, 209] and rheumatoid arthritis [205-207]. Since the acute phase of intra-abdominal sepsis is primarily characterized by a marked pro-inflammatory or Th1-type response that contributes to mortality [14, 18, 56, 57, 70], we hypothesized that administration of a Th2-polarizing glycolipid would reduce disease severity in sepsis. OCH is an iNKT cell agonist which results in a Th2-biased cytokine profile when administered *in vivo* [202, 235]. Similar to previous studies by our group and others [209, 235, 247], we demonstrated that the i.p. injection of OCH into naïve B6 mice results in a rapid peak of serum IL-4 at 2 hours, and is then significantly reduced at 12 to 24 hours (**Figure 16**); in contrast, serum levels of the Th1 cytokine IFN-γ peaked at 12 hours, but was almost undetectable at 24 hours (**Figure 16**). The administration of the prototypical iNKT cell agonist KRN7000 [186] resulted in elevated serum levels of IFN-γ between 12 and 24 hours (**Figure 16**). The IL-4: IFN-γ ratio calculated based on the peak values of these cytokines was higher for OCH compared to KRN7000, confirming that OCH promotes a Th2-dominant cytokine response *in vivo.*

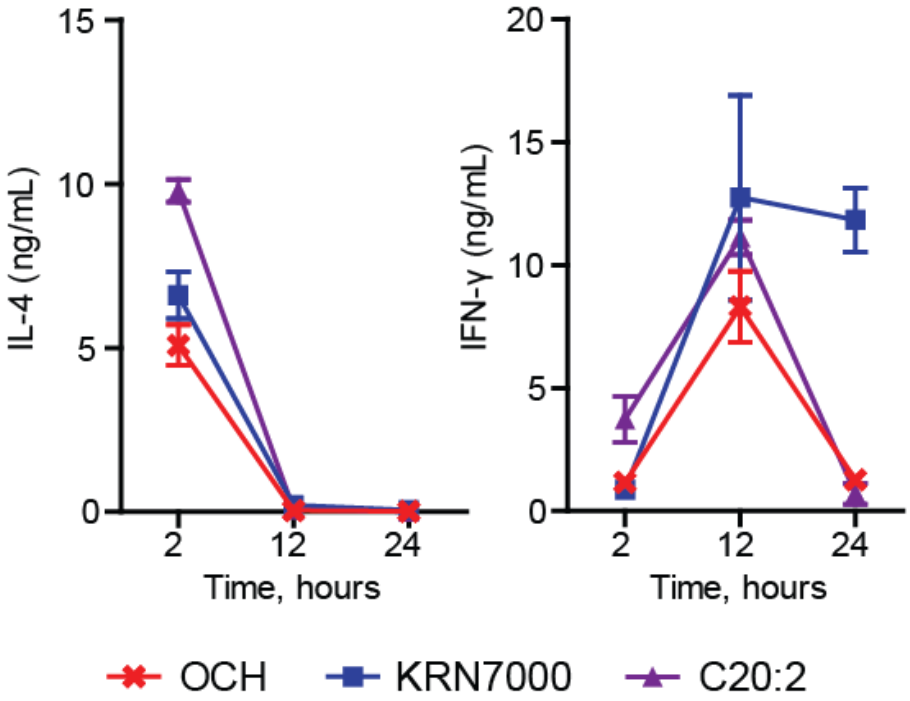

Figure 16: Effect of glycolipid agonists on cytokine expression in naïve B6 mice.

Naïve B6 mice were injected i.p. with 4 µg OCH, KRN7000 or C20:2, and bled at 2, 12, and 24 hours post-injection. Serum samples were assayed for IL-4 and IFN-γ by enzyme-linked immunosorbent assay (ELISA). Each data point shows mean (± SEM) of two or three mice from one representative experiment. Vehicle-treated mice had cytokine levels below limits of detection.

Treatment with OCH prolonged survival in septic mice compared to both vehicle and KRN7000 treatments (**Figure 17a**). Median survival for OCH-treated mice was 28 hours compared to 24 and 22 hours for vehicle- and KRN7000-treated mice, respectively ($p< 0.0001$ by log-rank test). Mice in the OCH group survived beyond 24 hours, whereas mortality for vehicle- and KRN7000-treated mice was 100% by 24 hours. OCH-treated mice (**Figure 17b**) also had a significantly lower MSS (± SEM) of 13 ± 0.53 after 24 h compared to vehicle- and KRN7000-treated mice with IAS (20 ± 0.33 and 18 ± 0.74 respectively, $p< 0.0001$ by two-way ANOVA with Bonferroni post-test). There were no statistical differences in MSS between the vehicle and KRN7000 treatments ($p= 0.8$ by two-way ANOVA with Bonferroni post-test).

The reduced MSS for OCH-treated mice derived from significant improvements in respiratory status, an important clinical predictor of mortality in sepsis [5, 16, 48, 248]. Most vehicle- and KRN7000-treated mice developed respiratory distress (laboured breathing and reduced respiratory rates) by 15 hours post-sepsis, unlike OCH-treated mice that continued to have relatively normal respiratory rates even at 24 hours. OCH-treated mice were also more responsive to auditory and touch stimuli whereas vehicle- and KRN7000- treated mice remained non-responsive and slow-moving or stationary. In addition, we did not observe any differences in disease severity between vehicle- and OCH-treated J$\alpha 18^{-/-}$ mice with IAS (**Figure 17c**), confirming that the beneficial effects of OCH on sepsis severity and mortality in B6 mice are linked to the specific modulation of iNKT cells. Vehicle-treated J$\alpha 18^{-/-}$ mice had a mean (± SEM) MSS of 8.7 (± 0.33) whereas OCH-treated J$\alpha 18^{-/-}$ mice had a mean (± SEM) MSS of 9.3 (± 0.33; $p= 0.10$ by two-way ANOVA with Bonferroni post-test).

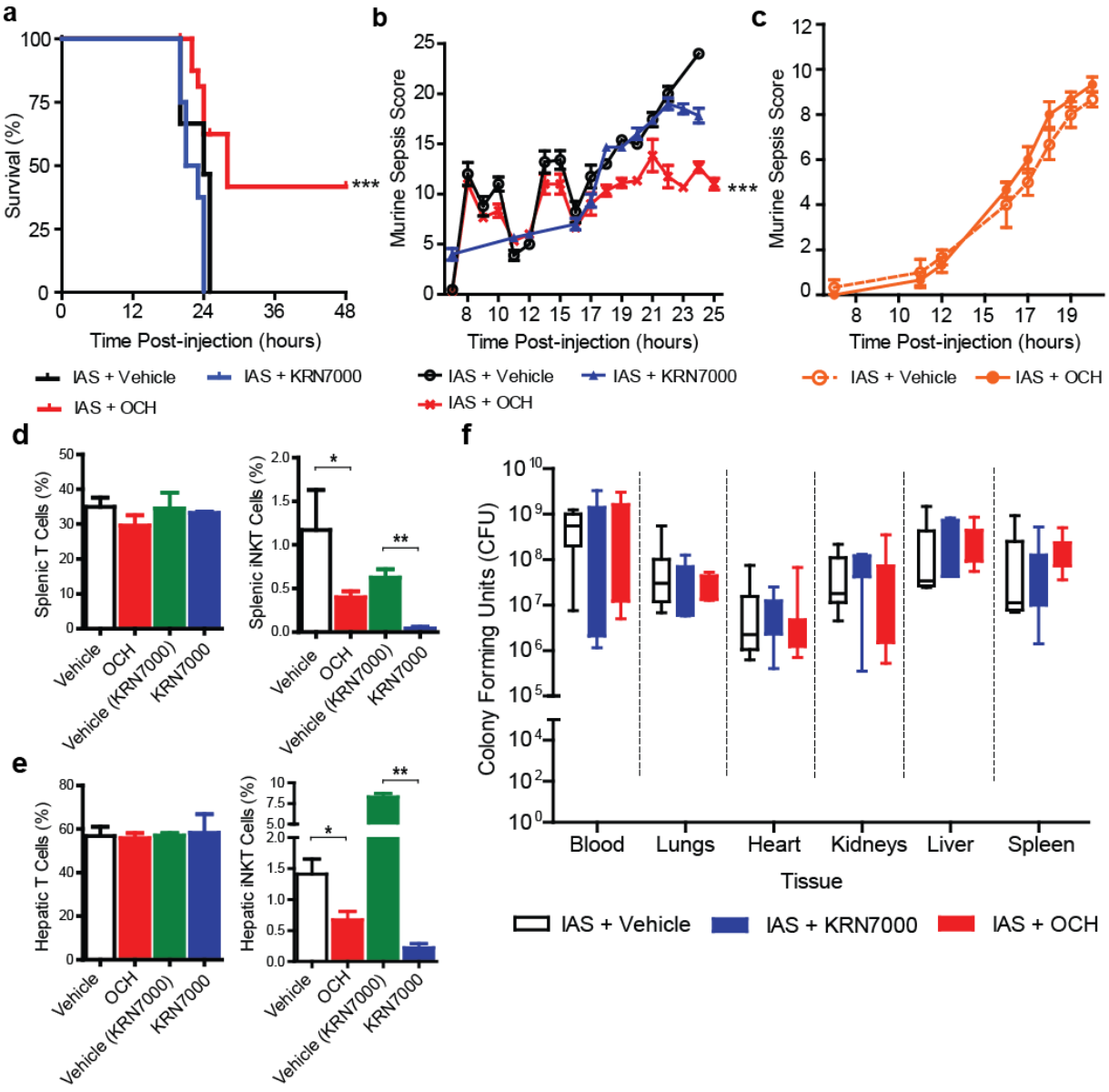

Figure 17: Th2-polarizing glycolipid OCH reduces disease severity in IAS.

(a) OCH-treated B6 mice had significantly prolonged survival compared to vehicle- and KRN7000-treated mice (*n*= 19, *n*= 15, *n*= 8 for OCH, vehicle, and KRN7000 groups respectively). ****p*< 0.001 by log-rank test (b) OCH-treated mice demonstrated significantly reduced disease severity compared to vehicle-treated and KRN7000-treated mice (*n*= 19, *n*= 15, and *n*= 8 mice respectively for OCH, KRN7000, and vehicle groups). ****p* <0.001 by two-way ANOVA with Bonferroni post-test. (c) iNKT-deficient Jα18$^{-/-}$ mice were given fecal slurry (FS;

90mg/mL) to induce intra-abdominal sepsis (IAS) and concomitantly treated with OCH or vehicle. Murine sepsis scores were similar between vehicle and OCH-treated mice (*n*= 3 per group). (d and e) Administration of OCH and KRN7000 resulted in significantly reduced detection of iNKT cells among septic B6 mice compared to vehicle treatments. The percentages of T cells remained unchanged with administration of iNKT-specific glycolipid agonists (*n*= 6, *n*= 4, *n*= 6, and *n*= 3 for vehicle, OCH, Vehicle (KRN7000) and KRN7000 groups respectively). **p* <0.05, ***p* <0.01 by Mann-Whitney U test. (f) Bacterial counts in blood and multiple organs were similar between vehicle-, OCH-, and KRN7000-treated mice with sepsis (*n*= 7-9 per group). Data are representative of at least three independent experiments.

Next, we analyzed the spleens and livers of septic mice treated with the glycolipid agonists but did not detect differences in splenic or hepatic T cell distributions (**Figure 17d** and **e** respectively). We did not determine any differences in splenic T cell distributions (**Figure 17d**) when septic mice were treated with OCH (29.7%), vehicle (35.0%), or KRN7000 (33.2%; p= 0.42). Hepatic T cell distribution was also unchanged between OCH (55.9%), vehicle (56.8%) and KRN7000 treatments (58.2%, p= 0.44) (**Figure 17e**). However, we had significantly reduced detection of iNKT cells in the spleen and liver following glycolipid treatment (**Figure 17d** and **e**). Splenic iNKT cells reduced from 1.2% in vehicle-treated mice to 0.30% in OCH-treated mice (p= 0.010) and 0.04% in KRN7000-treated mice (p= 0.0090). Hepatic iNKT cells reduced from 1.3% in vehicle-treated mice to 0.44% in OCH-treated mice (p= 0.0021) and 0.22% in KRN7000-treated mice (p= 0.0003). This likely reflects the down-regulation of the surface TCR that occurs with administration of glycolipid agonists, as shown previously by our group and others [[207, 235, 247, 249]; **Figure 17e**). In particular, we observed a significantly lower detection of iNKT cells following KRN7000 treatment compared to treatment with OCH. The differential degree to which the glycolipids down-regulate the surface TCR is a reflection of their differential binding kinetics to iNKT cells. While OCH and KRN7000 down-regulate the surface TCR within 4-12 hours post administration, KRN7000 is approximately 10-fold more potent at down-regulating the TCR after 24 hours [249], leading to the results we observed in **Figure 17**.

Anti-inflammatory processes are concomitantly initiated to mitigate pro-inflammatory states in sepsis, both systemically [58-61], and in individual organs [64]. These immunosuppressive mechanisms decrease the responsiveness of cells of the innate and adaptive immune systems, thereby increasing susceptibility to opportunistic and additional infections [65-68]. Importantly, we observed that the use of OCH, which significantly reduced the production

of the pro-inflammatory cytokine IFN-γ [207, 209, 235], did not worsen the microbial load of septic mice, compared to vehicle and KRN7000 treatments (**Figure 17f**). Therefore, administration of the Th2-polarizing glycolipid OCH did not result in overt susceptibility to microbial infection. Additionally, OCH-treated mice that survived to 48 hours demonstrated a significantly lower bacterial count in all tested organs, compared to OCH-treated mice that died at 24 hours (data not shown). Sham mice, as expected, did not demonstrate bacterial organ counts (data not shown).

Lastly, we tested the effect of a second Th2-polarizing glycolipid C20:2 on disease severity in IAS, to confirm whether the Th2-biased modulation of iNKT cells was responsible for ameliorating disease severity. C20:2 is a potent agonist with a capacity to bind and activate iNKT cells that is significantly stronger than OCH [209, 235]; administration of C20:2 in naïve B6 mice also results in a more pronounced Th2 response at 24 hours than OCH [209, 235] (**Figure 18**). When septic B6 mice were treated with C20:2, we observed a significant reduction in MSS between 20 and 24 hours compared to vehicle-treated mice (**Figure 18**), with improved respiratory status at observed time points. These results confirm the novelty of manipulating iNKT cells into a Th2-biased state for the mitigation of disease severity in IAS. However, the MSS continued to rise in C20:2-treated mice, in contrast to OCH, where the MSS reached a plateau (**Figure 17b**). Based on these results, we elected to focus on OCH and the means by which it improves mortality in IAS.

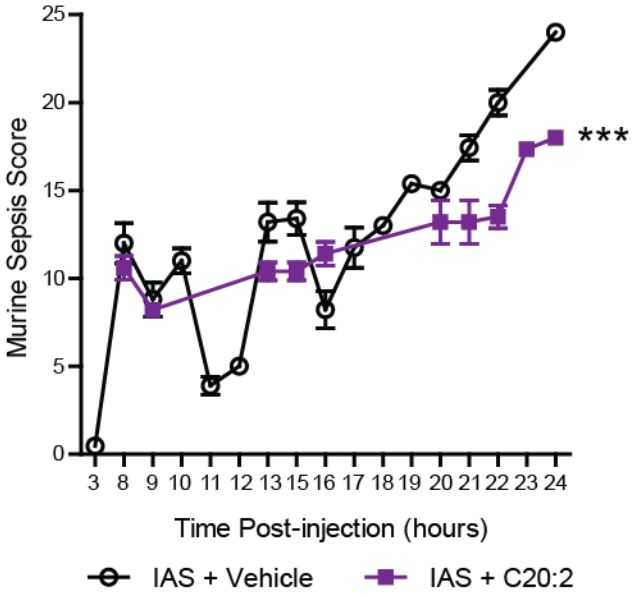

Figure 18: Murine Sepsis Scores for septic B6 mice treated with C20:2.

Mice were injected with fecal slurry and C20:2 or vehicle solution and monitored for 24 hours (*n*= 5, *n*= 15 mice for C20:2 and vehicle groups respectively). ***p<0.001 by two-way ANOVA test.

3.6 The pro-inflammatory cytokine profile in IAS is ameliorated by administration of OCH

In order to further understand the impact of the glycolipid agonists on the septic response, we assessed the concentrations of 32 cytokines and chemokines from the sera and spleens of vehicle-, OCH-, and KRN7000-treated septic mice, as well as sham treated mice (**Figure 19a-c**, **Table 12**, and **Table 13**). In the serum, mean concentrations of IL-17 was significantly lower in the OCH-treated mice compared to KRN7000-treated mice (p= 0.041 by one way ANOVA with post-hoc Tukey's multiple comparison test). The concentration of IL-13 was higher in the sera of OCH-treated mice compared to KRN7000-treated mice (p= 0.0403 by one way ANOVA with post-hoc Tukey's multiple comparison test). In the spleen, IFN-γ, IL-3, IL-4, IL-17, and TNF-α were significantly elevated in the KRN7000-treated group compared to the OCH-treated group. Therefore, the administration of OCH significantly reduces the levels of pro-inflammatory cytokines in IAS.

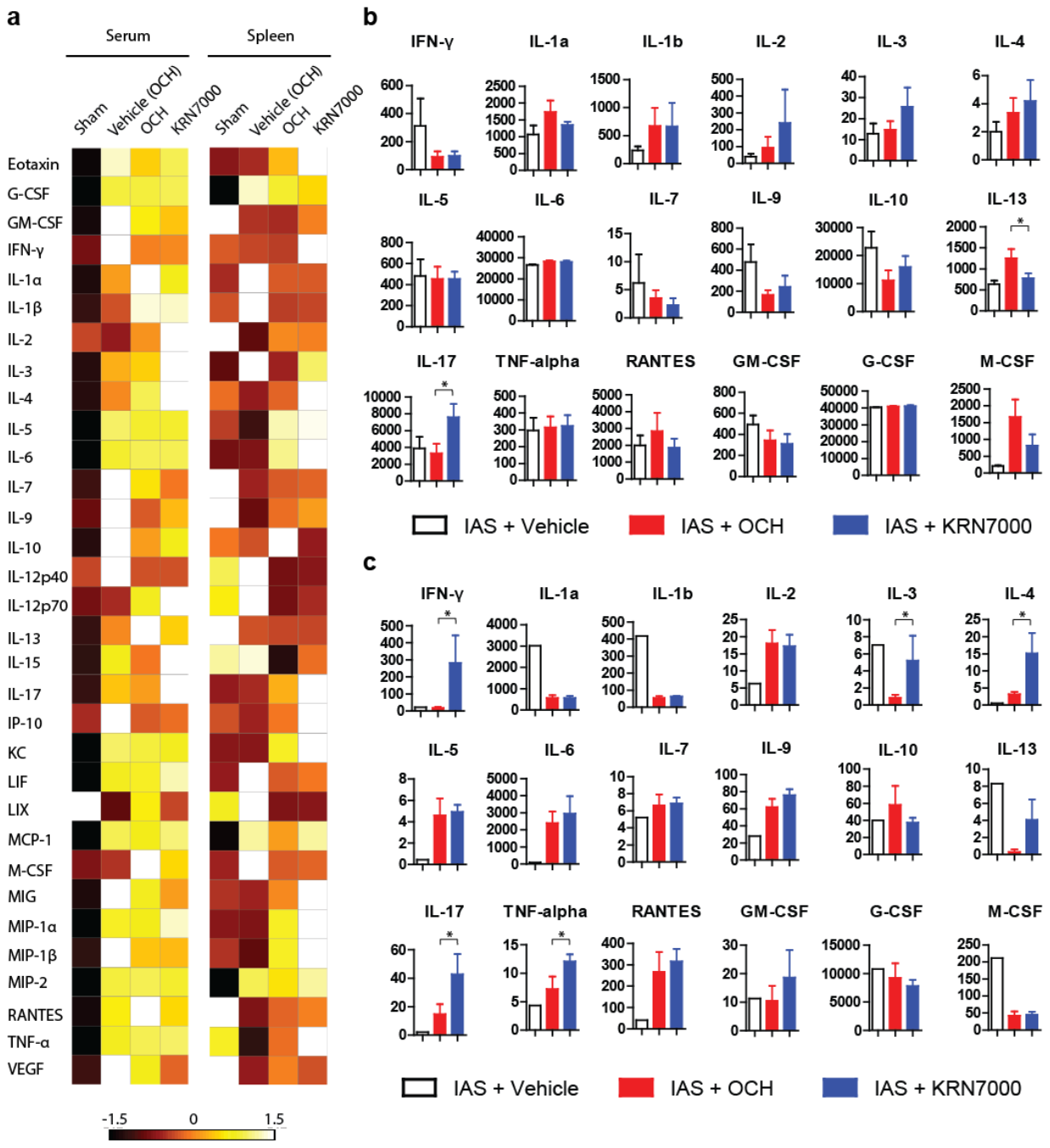

Figure 19: Cytokine levels in the sera and spleens of septic B6 mice.

(a) Sera and spleen homogenates from vehicle-, OCH-, and KRN7000-treated B6 mice with intra-abdominal sepsis (IAS) were analyzed at 24 hours for 32 inflammatory cytokines by multiplex array, and displayed as a heat map (*n*= 4 mice per group). Concentrations of iNKT cell-specific cytokines are shown from sera (b) and spleen homogenates (c) of septic mice treated

with vehicle, OCH, or KRN7000 (*n*= 4-8 per group). Concentrations of cytokines are shown in pg/mL. **p* <0.05, ***p* <0.01, ****p* <0.001 by one-way ANOVA with post-hoc Tukey's multiple comparison test. Data are representative of at least three independent experiments.

Table 12: Mean serum chemokines and cytokine concentrations (± SD) in B6 mice treated with vehicle, OCH, or KRN7000.

Cytokine / Chemokine	Sham (n = 4)	IAS			P Value
		Vehicle (n = 4)	OCH (n = 8)	KRN7000 (n = 6)	
Eotaxin	302.2 ± 223.2	7735 ± 6174	5411 ± 3145	6841 ± 4311	0.6538
G-CSF	283.4 ± 47.1	40342 ± 72	40868 ± 877.5	41081 ± 989.8	0.3992
GM-CSF	48.8 ± 15.7	495 ± 169	343.6 ± 268.1	311.8 ± 221.7	0.4723
IFN-γ	8.4 ± 14.5	311 ± 392	91.93 ± 110.5	98.85 ± 77.52	0.1852
IL-1a	179.1 ± 179.2	1070 ± 524	1737 ± 958.7	1345 ± 241.6	0.3030
IL-1b	12.3 ± 12.6	234 ± 141	671.6 ± 897.4	662.4 ± 1019	0.6749
IL-2	140.4 ± 172.8	40 ± 34	94.43 ± 178.2	239.7 ± 487.5	0.5581
IL-3	-	13 ± 9.5	14.70 ± 11.33	25.55 ± 22.56	0.3637
IL-4	0.16 ± 0.28	2 ± 1.4	3.363 ± 2.944	4.197 ± 3.656	0.5371
IL-5	2.57 ± 1.97	483.0 ± 320.8	455.5 ± 331.2	455.0 ± 167.2	0.9853
IL-6	21.4 ± 0.25	26529 ± 792.8	28164 ± 1356	27887 ± 1658	0.1765
IL-7	-	6.243 ± 10.07	3.533 ± 3.946	2.275 ± 2.917	0.5461
IL-9	178.3 ± 202.6	479.9 ± 334.5	165.3 ± 130.2	243.7 ± 259.9	0.1134
IL-10	-	22733 ± 11469	11187 ± 9980	15875 ± 9750	0.2135
IL-12 (p40)	-	3731 ± 7247	100.5 ± 44.28	107.3 ± 60.38	0.4602
IL-12 (p70)	16.0 ± 3.9	24.68 ± 10.31	104.2 ± 88.68	161.6 ± 218.7	0.3824
IL-13	270.6 ± 273.0	636.1 ± 165.2	1246 ± 626.3	775.1 ± 275.1	**0.0403**
IL-15	155.6 ± 174.8	321.1 ± 203.0	215.5 ± 139.1	447.1 ± 205.7	0.0775
IL-17	1.58 ± 2.42	3893 ± 2716	3301 ± 3184	7578 ± 3822	0.1858
IP-10	52.4 ± 41.3	28253 ± 43784	3332 ± 4097	4227 ± 2912	0.2907
KC	389.7 ± 369.3	29810 ± 1057	28626 ± 2871	27521 ± 3275	0.1489
LIF	-	289.0 ± 196.6	275.0 ± 137.2	343.4 ± 149.8	0.4501

LIX	2261 ± 3298	1848 ± 329.6	4842 ± 3144	2868 ± 518.2	0.1108
MCP-1	28.76 ± 32.32	23343 ± 14846	21132 ± 10003	24296 ± 8448	0.7748
M-CSF	3.93 ± 0.70	203.9 ± 48.19	1667 ± 1461	812.4 ± 828.1	0.1115
MIG	15.4 ± 10.1	8189 ± 3622	6101 ± 4333	4268 ± 4484	0.3813
MIP-1a	163.6 ± 108.3	5312 ± 3389	5456 ± 6949	6918 ± 6845	0.7355
MIP-1b	88.96 ± 120.2	18193 ± 12436	9737 ± 8622	9510 ± 8843	0.7290
MIP-2	14.8 ± 6.3	21982 ± 10040	22283 ± 5986	21423 ± 3911	0.9759
RANTES	16.1 ± 18.7	1993 ± 1201	2838 ± 3051	1870 ± 1304	0.7007
TNF-a	0 ± 0	296.2 ± 151.8	314.4 ± 182.1	323.3 ± 155.3	0.9688
VEGF	3.7 ± 3.7	37.10 ± 54.55	24.45 ± 46.15	13.70 ± 19.06	0.9220

All concentrations are in pg/mL. Comparison was made by one-way ANOVA with post-hoc Tukey's multiple comparison test. P values are shown for comparison tests between OCH- and KRN7000-treated mice.

Table 13: Mean concentrations of chemokines and cytokines in spleen homogenates (± SD) of septic mice treated with vehicle, OCH, or KRN7000.

Cytokine / Chemokine	Vehicle (n = 1)	OCH (n = 7)	KRN7000 (n = 6)	P Value (OCH v. KRN7000)
Eotaxin	167.17	654.3 ± 503.6	1560 ± 268.2	**0.0023**
G-CSF	10794.18	9244 ± 6763	7813 ± 2679	0.5338
GM-CSF	11.35	10.49 ± 13.94	18.68 ± 23.47	0.5867
Ifn-γ	22.49	17.91 ± 12.77	281.7 ± 394.8	**0.0221**
IL-1α	3007.60	561.5 ± 318.2	562.2 ± 234.7	0.9372
IL-1β	417.44	54.16 ± 25.40	63.01 ± 12.34	0.4452
IL-2	6.31	18.05 ± 10.46	17.27 ± 8.107	0.7308
IL-3	7.03	0.8543 ± 0.9072	5.222 ± 7.079	**0.0264**
IL-4	0.49	3.127 ± 1.943	15.12 ± 14.78	**0.0256**
IL-5	0.44	4.594 ± 4.254	4.938 ± 1.595	0.4452
IL-6	87.59	2396 ± 1794	2962 ± 2484	0.6282
IL-7	5.22	6.637 ± 3.395	6.862 ± 1.686	0.5338
IL-9	28.04	61.75 ± 26.23	76.06 ± 16.90	0.4452
IL-10	40.05	58.01 ± 59.15	37.45 ± 13.97	0.9452
IL-12 (p40)	268.12	12.29 ± 11.93	22.36 ± 17.07	0.7092
IL-12 (p70)	12.22	3.837 ± 3.359	4.762 ± 2.433	**0.0003**
IL-13	8.28	0.3000 ± 0.6708	4.053 ± 5.847	0.2448
IL-15	31.90	19.22 ± 19.61	24.94 ± 14.15	0.5477
IL-17	2.19	14.80 ± 18.33	42.80 ± 34.79	**0.0367**
IP-10	3.05	483.9 ± 439.2	2507 ± 756.3	**0.0088**
KC	79.33	2591 ± 2041	4814 ± 2651	**0.0035**
LIF	108.17	15.71 ± 13.44	22.30 ± 5.966	0.5477
LIX	533.31	107.9 ± 112.7	123.1 ± 166.4	0.2163
MCP-1	1251.65	781.4 ± 702.4	1234 ± 166.7	**0.0053**
M-CSF	210.76	42.50 ± 32.72	45.35 ± 20.29	0.9452

MIG	17.77	1870 ± 2010	7776 ± 7656	0.0596
MIP-1α	140.71	318.1 ± 216.2	462.9 ± 143.5	**0.0167**
MIP-1β	23.01	410.0 ± 282.5	698.6 ± 138.1	**0.0026**
MIP-2	5915.82	4937 ± 3253	6480 ± 3191	**0.0134**
RANTES	40.36	267.1 ± 225.6	316.2 ± 143.5	0.6991
TNF-α	4.37	7.239 ± 5.841	12.10 ± 3.006	0.1979
VEGF	3.16	7.460 ± 1.522	5.652 ± 1.646	**0.0049**

All concentrations are in pg/mL. Comparison was made between OCH and KRN7000-treated mice by unpaired two-tailed t test.

3.7 Treatment with OCH significantly reduces splenocyte apoptosis in IAS

We next sought to elucidate the reason for the improved survival among septic mice that were treated with OCH. When we performed histopathological analysis on the spleen, liver, and omentum of septic B6 mice treated with KRN7000 or OCH (**Figure 20**), we found a significant reduction of apoptotic cells within the spleens of OCH-treated mice compared to vehicle- and KRN7000-treated mice. The presence of karyorrhexic nuclei within clusters of cells with eosinophilic cytoplasm was observed in the white pulp of the spleen by hematoxylin and eosin staining, and subsequently confirmed as apoptotic cells by TUNEL staining, particularly in vehicle- and KRN7000-treated mice (**Figure 20**). Based on histopathological scoring by a pathologist blinded to the treatment, OCH-treated mice had mild apoptosis, whereas vehicle-treated and KRN7000-treated mice had moderate and severe apoptosis respectively (**Figure 21**).

In the omentum of vehicle- and KRN7000-treated mice, we noted a significant increase in lymphocytes whereas fewer lymphocytes were observed in the omentum of OCH-treated mice (**Figure 20**). We did not observe overt differences in liver histopathology among vehicle-, OCH-, and KRN-treated mice. When we examined the histology of C20:2-treated septic mice, we observed a decrease in apoptosis compared to KRN7000-treated mice **(Figure 22)**. However, the degree of apoptosis in C20:2-treated mice was higher than OCH-treated mice with IAS.

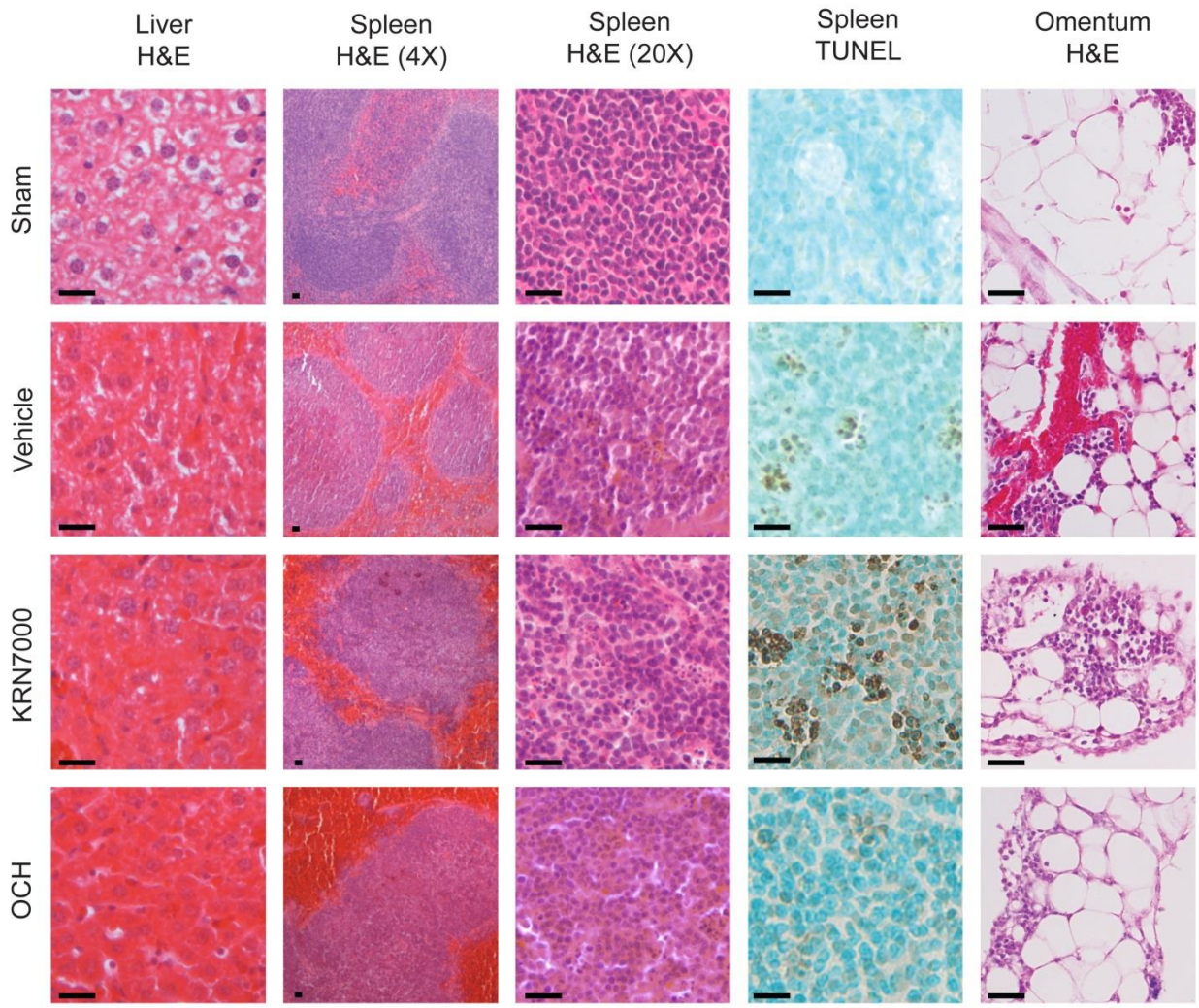

Figure 20: Histopathology of septic B6 mice treated with glycolipid agonists of iNKT cells. Treatment with OCH significantly reduced apoptosis within the spleen compared to vehicle- and KRN7000-treated mice with intra-abdominal sepsis (IAS), both by hematoxylin and eosin staining, as well as TUNEL staining. Lymphocyte migration to the omentum is also partially ameliorated in OCH-treated mice compared to vehicle- and KRN7000-treated mice. There were no histopathological differences in the liver. Images are representative of 4 animals per treatment group (*n*= 4 slides per animal; size bar, 50 μm).

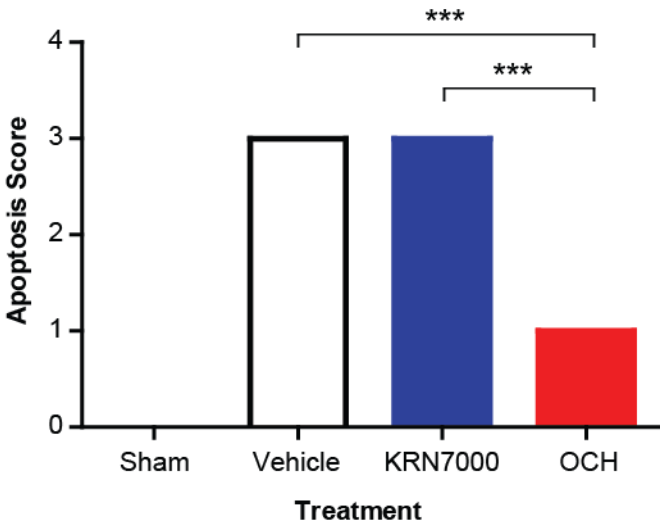

Figure 21: Histopathological scoring of splenic apoptosis in B6 mice with IAS. Histopathological scoring of the degree of apoptosis observed within the spleens of sham and septic B6 mice treated with vehicle, KRN7000, or OCH (*n*= 4 animals per treatment group; *n*= 4 slides per animal). Apoptosis was defined histologically by the presence of cell clusters with nuclear shrinkage (karyorrhexis), dark eosinophilic cytoplasm, intact plasma membrane, and relative paucity of surrounding inflammatory cells within the splenic follicles on H&E staining. Scores assigned to each animal by a blinded independent pathologist were as follows: 0 for complete absence of apoptosis; 1 for mild presence of apoptosis (0-15% per follicle); 2 for moderate apoptosis (16-30% per follicle); and 3 for severe apoptosis (31-45% per follicle). *** $p < 0.0001$ by two-tailed Mann Whitney U test.

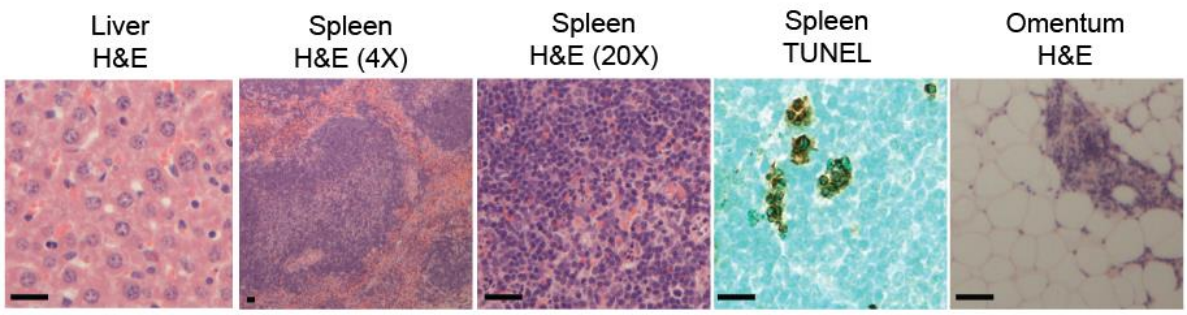

Figure 22: Histopathology of C20:2-treated B6 mice with IAS.

C57BL/6 (B6 mice) were injected intraperitoneally with 500 µL of FS (90 mg/mL) to induce IAS, and concomitantly injected with 4 µg of the glycolipid C20:2. Mice were sacrificed at 24 hours, and the liver, spleen, and omentum were removed and processed for histopathological analysis. These images are representative of 5 septic B6 mice that were treated with C20:2 (size bar, 25 µm).

We then performed flow cytometry on spleens harvested from vehicle-, OCH- and KRN7000-treated mice with IAS to determine the immune cell populations that had undergone apoptosis (**Figure 23**). Treatment with OCH significantly reduced the apoptosis of T and B cells compared to vehicle- and KRN7000-treated mice. However, there were no differences in the frequency of apoptotic macrophages between the KRN7000 and OCH groups, although both treatments reduced the frequency of apoptosis significantly compared to vehicle-treated mice. With respect to NK cell apoptosis, we observed a trend toward reduced apoptosis in KRN7000-treated mice. Together, these results demonstrate that different glycolipid agonists of iNKT cells differentially mitigate the apoptosis of splenic lymphocytes, but not NK cells and macrophages. Moreover, Th2-polarizing glycolipids significantly reduce lymphocyte apoptosis within the spleen, a critical predictor of mortality in severe sepsis and septic shock [71, 84, 134].

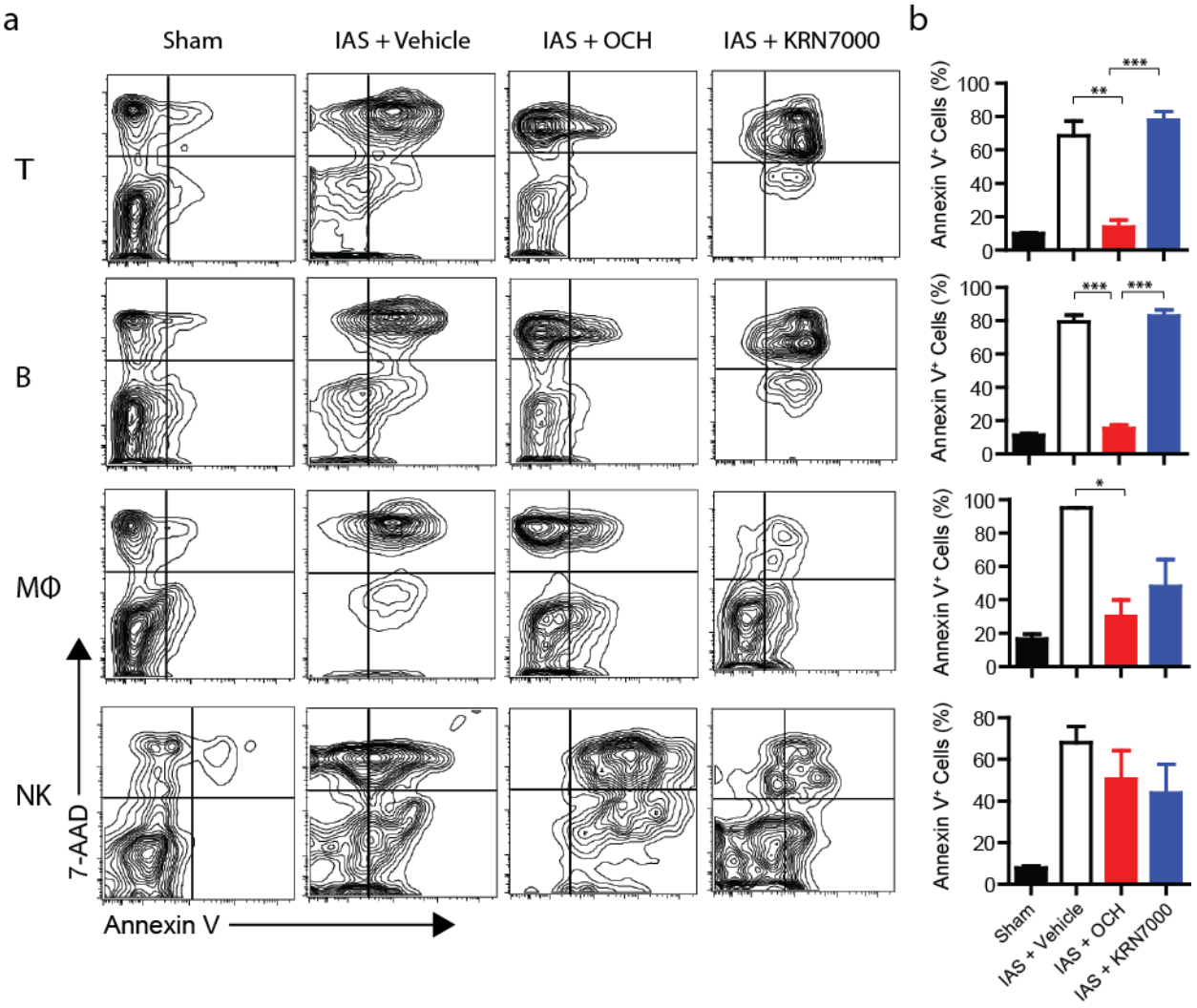

Figure 23: Analysis of apoptotic cell populations in the spleens of septic B6 mice.

(a) Splenocytes from sham and septic B6 mice treated with OCH, KRN7000, or vehicle were stained for T, B, and Natural Killer (NK) cells, and macrophages, and further stained for Annexin V (a marker for early apoptosis) and 7-AAD viability dye. (b) Early and late apoptotic cells (Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ cells, respectively) were quantified and compared between treatments. OCH treatment significantly reduced apoptosis among T and B cells, as well as macrophages, but not NK cells (n= 3-6 mice per group). *p <0.05, **p <0.01, ***p <0.001 by one-way ANOVA with post-hoc Tukey's multiple comparison test. Data are representative of 3 independent experiments.

Chapter 4: Discussion

4.1 Discussion

iNKT cells exert profound and diverse regulatory functions in health and disease, bridging the innate and adaptive defense mechanisms in a variety of immune responses [141, 155, 172]. Here, we demonstrate that patients with sepsis/severe sepsis have significantly elevated proportions of iNKT cells and that OCH, a Th2-polarizing glycolipid agonist of iNKT cells, profoundly reduces disease severity in IAS, with significantly reduced lymphocyte apoptosis within the spleen. These findings introduce iNKT cells as potential therapeutic targets for the treatment of sepsis.

In this thesis, we elected to utilize the fecal-induced peritonitis (FIP) model to simulate IAS in a reproducible and reliable fashion, and to mimic the clinical presentation and prognosis of acutely ill patients with IAS. Animal models that can reliably replicate the clinical characteristics of sepsis are essential for the study of this disease and for the development of novel diagnostic biomarkers [250], and new therapeutic strategies [3, 10, 18, 250-252]. The failure of therapies that showed promise during the preclinical stages, but yielded little benefit in human trials [251, 253], have highlighted the shortcomings of conventional animal models of sepsis [251, 252, 254]. The cecal ligation and puncture (CLP) model, often considered the "gold standard" among polymicrobial sepsis models [161, 232, 251, 253, 255], establishes a mixed bacterial infection with an inflammatory source of necrotic intestinal tissue [255-257]. It involves the performance of a laparotomy, ligation of the cecum in a non-obstructing manner, and puncture of the ligated portion to allow fecal content to leak into the normally sterile peritoneal cavity [161]. Despite its widespread use, however, the CLP model and its modifications, such as cecal ligation and incision (CLI) and colon ascendens stent peritonitis (CASP), is acutely dependent on operator technique [232, 251, 258, 259]: the percentage of

cecum that is ligated (and thus the amount of necrosis that is induced), the amount of the microbial dose that enters the peritoneum (which depends on the number and size of punctures and the pressure exerted on the cecum), the size of the incisions made in the skin and abdominal muscle, and even the use of different anaesthetic agents can considerably alter the outcome after CLP, precluding comparison between studies [161, 251, 255, 256]. Moreover, the CLP model more closely resembles intra-abdominal abscess formation [255, 257], which represents a different host response [255, 257] compared to our model.

The FIP model is also advantageous to use because the protocol can be easily modified to alter disease severity and outcome, by simply varying the concentration of feces in solution. A further striking advantage is that the preparation of the fecal solution and the injection can be highly standardized; therefore, our protocol provides a controlled setting that minimizes the number of variables influencing outcome, and resolves the inter-operator inconsistency associated with the CLP, CLI, and CASP models. In addition, an identical microbial load and composition is given to each mouse, avoiding the potential inconsistency of each individual mouse's microbiota leaking from the ligated cecum. The need for anesthesia is also obviated with the FIP model, removing another confounding factor that has been shown to alter innate immunity in animal models such as CLP [260, 261].

We consistently demonstrated bacterial growth in every major organ in septic mice, with similar tissue CFUs in independently performed experiments. While this study was limited by the inability to culture and identify strictly anaerobic microorganisms, we observed colonies that varied in size, shape, and color, confirming the polymicrobial nature of the infection.

In this study, we also developed a robust and comprehensive scoring system with high specificity and sensitivity in predicting severe sepsis and mortality during the experimental timeline. Our scoring system reliably predicts 1-hour and 2-hour mortality when clinical scores are greater than 10, with excellent discriminatory capacity. To our knowledge, the development of a sepsis score that can reliably predict acute mortality is novel among studies using animal models of acute sepsis and septic shock. Mice that attained a clinical score of 3 had 100% specificity for dying from sepsis during the experimental timeline. Although the murine sepsis score was developed in conjunction with our model, it may also potentially be used for other models of sepsis, including CLP.

Histologically, we observed changes at the organ level that were inconsistently reported in the CLP model, likely because of surgical variability in the latter technique. In the lung, we did not observe extravasation of red cells and accumulation of inflammatory cells into the air spaces as described by Zingarelli *et al* [262]. Doi *et al* did not observe any histological changes in other organs with CLP [263], although they noted that CLP caused renal tubular damage mainly consisting of tubular vacuolization [264, 265]. Surprisingly, while we observed gross intestinal distension in FIP treated mice where the intestinal tract manifested subtle histopathological changes that have been described in other sepsis models, including the loss of glandular structure and intestinal epithelial villi, edema of the lamina propria, capillary hemorrhage, ulceration and apoptosis [266, 267]. Apoptosis was most evident in the spleen, as confirmed by TUNEL staining. As we demonstrated in later experiments during this thesis, T and B lymphocytes, NK cells, and macrophages all underwent apoptosis, corroborating similar results shown in the CLP model by Hotchkiss *et al* [70, 71]. These results corroborate human studies that indicate that apoptotic factors modulate lymphocyte and monocyte activity [71, 268],

with significant implications for immunosuppression and mortality in sepsis. The biochemistry data consistently suggests hepatic and possibly pancreatic dysfunction may be contributing to mortality, while the overwhelming cytokine dysfunction may also play a significant role, as suggested by other studies [268].

The putative cytokine markers of sepsis, including IL-1β, IL-6, IL-10 and TNF-α [18, 269], increased significantly during the experimental timeline. For what we believe is the first time, however, we also demonstrate that levels of eotaxin, IL-5, IL-15, M-CSF, MIG, MIP-1α, MIP-1β, and MIP-2 rise significantly during sepsis. Eotaxin and IL-5 are associated with eosinophil recruitment and function [270], although eotaxin inhibits neutrophil recruitment during the acute inflammatory phase of sepsis [271]. IL-15, M-CSF, MIG, MIP-1α, MIP-1β and MIP-2 promote cellular differentiation, activity, survival, recruitment and chemotaxis [272-274], confirming the complexity of the dysregulated immune response during sepsis.

Corroborating other studies that demonstrate pathogenicity of iNKT cells in sepsis [156, 157, 159], iNKT-deficient Jα18$^{-/-}$ mice were resistant to IAS in this study. We also show that the tissue-specific distribution of iNKT cells is altered during IAS, with significant reductions in the spleen, and a concomitant rise in the omentum. The human omentum has been described as the "policeman of the abdomen" for its ability to adhere to sites of intra-abdominal pathology and prevent widespread pathogen contamination [219, 225]. Similarly, the murine omentum has been shown to facilitate the regeneration of damaged tissues [229]. These results, as well as the findings of Lynch *et al*, who demonstrated that the human omentum contained a rich reservoir of iNKT cells [185], prompted us to examine the murine omentum, wherein we observed a significant increase in iNKT cells post-sepsis. Our observation that the omentum became enlarged during IAS correlates with findings by Shah *et al* [229], and represents a unique feature

of this organ that has not been noted in other secondary lymphoid structures such as lymph nodes or spleens. T cells were also noted to be significantly increased in the omentum during IAS, corroborating observations made by Carlow *et al* [215] in a cecal ligation and puncture (CLP) model of polymicrobial sepsis. This was also confirmed by adoptive transfer experiments: iNKT cells that were transferred intravenously into iNKT-deficient mice moved into the omentum during IAS. While Barral and associates demonstrated that iNKT cells exit the spleen and enter the bloodstream in response to infection [275], the omentum may serve as a potential conduit for iNKT cells to help facilitate intra-abdominal immune response during IAS. Although it was beyond the scope of this study, we are currently investigating the frequency, function, and phenotype of omental iNKT cells in human sepsis to determine their role in regulating disease severity, and evaluate their prognostic significance.

Our results with respect to the tissue-specific distribution of iNKT cells post-sepsis contrast the findings of Hu *et al* [159], who demonstrated a significant reduction in hepatic iNKT cells but no changes in the frequency of splenic iNKT cells, in the CLP model. We propose that splenic iNKT cells mobilize more readily during acute sepsis compared to hepatic iNKT cells, since a recent study by Barral *et al* [275] showed that splenic iNKT cells patrol the red pulp and marginal zones of the spleen, rapidly sample blood-borne antigens, and display migratory capabilities. This may explain our observed changes in splenic iNKT cell frequency post-sepsis, and additionally suggests that the iNKT cells we detected in the omentum post-sepsis may have originated from the spleen, given that the two organs are physically attached to each other [229].

Glycolipid ligands of iNKT cells have been used successfully in experimental models of autoimmune diseases [203-205, 207, 212] and solid-organ transplantation [208], as well as in clinical trials of viral infections and various types of cancer [189, 190]. KRN7000 [186] reduces

morbidity and mortality associated with murine graft-versus-host disease [198, 199], while OCH mitigated disease severity in non-obese diabetic mice [204], experimental autoimmune encephalomyelitis [202], and collagen-induced arthritis [205, 206]. OCH also prevented disease symptoms in a humanized mouse model of citrullinated fibrinogen-induced inflammatory arthritis [207], and delayed Th1-mediated cardiac allograft rejection in mice [208].

In our study, we show that the administration of OCH ameliorated the severe pro-inflammatory Th1-type response associated with IAS and reduced mortality. Although pro-inflammatory cytokines such as IFN-γ and TNF-α contribute to immune responses against bacterial infections [276], elevated levels of these cytokines are also associated with poor outcomes and decreased survival in sepsis [[277, 278];(Shrum *et al*, submitted)]. As confirmed in this study, the treatment of septic mice with KRN7000 resulted in a Th1-type response at 24 hours [141, 235, 279] and did not affect disease severity. In addition, elevated levels of the Th2 cytokine, IL-13, may be contributing to the significant improvements in respiratory status and disease severity that we observed in OCH-treated mice. A potent anti-inflammatory cytokine [280, 281], IL-13 is produced in large quantities by alveolar macrophages in the lung during polymicrobial sepsis [280], and has been shown to protect mice from endotoxic shock when administered *in vivo* [282]. Since a compromised respiratory status significantly increases morbidity and mortality in sepsis [16, 48, 248], the selective Th2-biased modulation of iNKT cells may provide a novel strategy to prevent this complication in the first place.

Using serum ELISA from healthy B6 mice that were administered OCH at various time points, we demonstrated that the ratio of IL-4 to IFN-γ was significantly elevated compared to naïve B6 mice (Figure 14) with OCH administration. We also showed that in both naïve (Figure 14) and septic B6 mice (Figure 14) that received OCH or KRN7000, we could not detect iNKT

cells in the liver or spleen by flow cytometry. These results also confirm the activity of KRN7000 and OCH as previously described [283]: the TCR of iNKT cells is downregulated when activated by KRN7000, thereby reducing the detection of iNKT cells by surface antigen staining [235, 283]. The reduced detection of hepatic and splenic iNKT cells in OCH-treated mice also confirms its pharmacokinetic action, down-regulating surface TCR within 4 to 12 hours of glycolipid administration as previously reported [249]. Although treatment with KRN7000 resulted in potent down-regulation of iNKT-cell TCR, as observed in other studies [198, 235, 283], there was no effect on sepsis severity in our study. A synthetic analogue of α-GalCer that has been used in most experimental studies [283], KRN7000 is a high-affinity ligand that induces the release of Th1- and Th2-type cytokines simultaneously [140, 141], although a single injection of KRN7000 leads to a Th1-type response after 24 hours [140, 141].

Following the initial hyperinflammatory response of sepsis is a prolonged immunosuppressive phase that may lead to secondary infections [65-68]. Although pro-inflammatory cytokines such as IFN-γ and TNF-α are associated with increased morbidity and mortality in sepsis [277, 284-286], they also contribute to immune responses against bacterial infections [276, 287]; IFN-γ, in particular, has been shown to reverse the altered immune status of monocytes in human sepsis [129]. Thus, one concern with Th2-polarizing agonists of iNKT cells is that this may further dysregulate protective immunity, leading to the potential uninhibited growth of bacterial pathogens. However, we did not observe an increase in microbial load within the blood and organs of OCH-treated mice compared to vehicle-treated mice with IAS. Since OCH is a less potent agonist than KRN7000, with lower binding affinity for the invariant TCR compared to the latter [235, 249], the administration of a single dose of OCH may have affected only a portion of iNKT cells, thereby abrogating rather than eliminating the pro-inflammatory

response. In addition, other immune cells which are not directly affected by glycolipid administration may continue to participate in bacterial clearance, including NK cells, which also produce significant amounts of IFN-γ [276]. Any differences in microbial counts between KRN7000- and vehicle-treated mice may have been masked by the excessive pro-inflammatory response that is inherent in our sepsis model (Shrum *et al*, submitted). Lastly, our study also confirms that the manipulation of iNKT cells alone can dramatically alter outcomes in sepsis, given that iNKT-deficient mice are resistant to mortality from sepsis, and disease severity was unaffected by glycolipid treatment in these animals.

Interestingly, the use of C20:2, another Th2-polarizing glycolipid that is significantly more potent at inducing a Th2 bias compared to OCH [165, 209, 288] and supresses downstream NK cell function [235], also mitigated sepsis severity significantly and reduced splenocyte apoptosis. Unlike OCH, however, mice treated with C20:2 continued to worsen, although their MSS remained lower than vehicle-treated mice at most observed time-points, suggesting that while the Th2-biased manipulation of iNKT cells may be a viable therapeutic strategy in sepsis, the use of a drug that is too potent may have unintended immunosuppressive consequences [84]: balancing the pro-inflammatory response with OCH may therefore be better at improving survival in IAS, rather than suppressing it with C20:2.

For what we believe is the first time, we demonstrate that Th2-polarized iNKT cells significantly reduce apoptosis within the spleen, particularly among T and B lymphocytes, and macrophages. Apoptosis contributes to immunosuppression during sepsis through the deletion of critical effector cells including T and B cells, and the induction of anergy in surviving immune cells. The loss of T and B cells significantly impairs the adaptive immune response, and, by disabling the cross-talk between the adaptive and innate immune systems, also impairs the latter

[17, 64, 74]. Hotchkiss *et al* observed a striking apoptosis-induced loss of cells of the innate and adaptive immune systems in the spleen during sepsis, including $CD4^+$ and $CD8^+$ T cells, B cells, and dendritic cells [70, 71]. The marked increase in apoptosis among circulating lymphocytes [77] is also believed to contribute to the profound and persistent lymphopenia that is strongly associated with mortality during sepsis.

The uptake of apoptotic cells also stimulates immune tolerance by inducing the release of anti-inflammatory cytokines, including IL-10 and transforming growth factor-β (TGF-β), and suppressing the release of pro-inflammatory cytokines [87]. This potential link between the release of IL-10 by apoptotic cells and immune suppression in sepsis is underscored by studies showing that the circulating concentration of IL-10 is predictive of a fatal outcome in patients with sepsis [58, 88]. In the sera of OCH-treated mice, the levels of IL-10 appear to be lower than vehicle- and KRN7000-treated mice, although splenic levels of IL-10 are higher in OCH-treated mice compared to KRN7000-treated mice. Additionally, the uptake of apoptotic cells by macrophages and DCs does not induce the expression of co-stimulatory molecules: therefore, T cells that come into contact with APCs that have ingested apoptotic cells might either become anergic or undergo apoptosis themselves [87]. Therefore, the significant reduction in splenic apoptosis may prevent T cell anergy in OCH-treated mice. Interestingly, apoptosis of NK cells appeared to be reduced by treatment with OCH and KRN7000, although the trend is more pronounced for the latter. Since NK cells also produce significant amounts of IFN-γ [276], their apoptosis in the spleens of vehicle- and OCH-treated mice may explain the reduced levels of splenic IFN-γ in these two groups.

Interleukin-17 is significantly lower in the sera and spleen of OCH-treated mice compared to KRN7000- and vehicle-treated mice. This cytokine has been strongly implicated in

promoting mortality during sepsis, both in animal and human studies, by causing the Th17 response. Moreover, it has been shown that blockade of apoptosis impairs the release of IL-17 and the subsequent Th17 response in a *Citrobacter rodentium* infectious colitis model [289]. Indeed, blockade of IL-17 has been shown to improve mortality in several animal models of sepsis [290, 291]. IL-17 is also involved in the apoptosis of PMNs [292] and also mediates cardiomyocyte apoptosis [293]. Therefore, reduction of IL-17A levels may have significant implications in improving cardiovascular output during septic shock, although it was beyond the scope of our current study to assess this. Nevertheless, the evidence from our study therefore suggests an alternative method to reduce mortality by manipulation of regulatory T cells without using end-target effector drugs.

We have also demonstrated that the proportion of circulating iNKT cells is elevated early in the septic process for critically-ill patients, corroborating a recently published study by Heffernan *et al* [294]. Given their propensity to rapidly produce significant quantities of pro- and/or anti-inflammatory cytokines, the increased proportion of iNKT cells suggests that they may be playing a prominent role in promulgating the immune response in septic patients. Furthermore, we observed that the proportion of iNKT cells is not increased in patients who have sustained significant inflammatory responses due to trauma, suggesting that these cells may be specifically responding to microbial pathogens in humans. Consequently, the detection of increased numbers of iNKT cells may also serve as an important biomarker to differentiate septic from non-septic patients early in the disease process, thereby facilitating rapid and targeted interventions for the disease.

There are several limitations to this study. We did not assess the impact of iNKT cell modulation on the function or activity of other immune cells within the spleen or liver. Since

iNKT cells play an important regulatory role in the context of immune responses and may be a potential target for therapy in sepsis, an evaluation of their downstream effects on immune cells will be necessary. One particular aspect of immune cell function in the context of Th2 modulation is that of anergy. A significant proportion of septic patients die from additional nosocomial infections post-admission, and immunosuppression secondary to T cell anergy has been proposed as a primary reason for this occurrence. The risk of dying after a septic episode rises significantly within the first year, and the risk of dying after surviving an episode of severe sepsis is significantly elevated for the next five years. A study by Perl *et al* [6] found that only 40% of severe sepsis patients were alive after 4 years, while only 20% of severely septic patients were alive within 8 years after leaving the hospital. Additionally, a study by Benjamim and colleagues showed that mice treated successfully following CLP died from reinfection when they were exposed to pulmonary *Aspergillosis* several weeks later [295]. Therefore, sepsis is not only an acute disease with acute clinical consequences, but also a syndrome that can cause chronic medical problems. Consequently, proliferation and activation assays of OCH- and KRN7000-treated mice will be necessary to determine whether the lymphocytes can still mount an effective immune response.

4.2 Conclusion

Given the failure of many immunotherapeutic drugs in the treatment of sepsis [89, 90], alternative agents have been sought to combat this disease with some success [106-112]. Our results indicate that Th2-polarized iNKT cells reduce disease severity in IAS by mitigating lymphocyte and macrophage apoptosis within the spleen, likely through reduction of IL-17. Moreover, circulating iNKT cells are increased in critically-ill patients with sepsis compared to

non-septic patients, and therefore they may be a potent therapeutic target in the treatment of sepsis.

References

1. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ: **Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992**. *Chest* 2009, **136**(5 Suppl):e28.
2. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: **Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care**. *Critical care medicine* 2001, **29**(7):1303-1310.
3. Parrillo JE, Parker MM, Natanson C, Suffredini AF, Danner RL, Cunnion RE, Ognibene FP: **Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy**. *Annals of internal medicine* 1990, **113**(3):227-242.
4. Hoyert DL, Arias E, Smith BL, Murphy SL, Kochanek KD: **Deaths: final data for 1999**. *National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System* 2001, **49**(8):1-113.
5. Wheeler AP, Bernard GR: **Treating patients with severe sepsis**. *The New England journal of medicine* 1999, **340**(3):207-214.
6. Perl TM, Dvorak L, Hwang T, Wenzel RP: **Long-term survival and function after suspected gram-negative sepsis**. *JAMA : the journal of the American Medical Association* 1995, **274**(4):338-345.
7. Heyland DK, Hopman W, Coo H, Tranmer J, McColl MA: **Long-term health-related quality of life in survivors of sepsis. Short Form 36: a valid and reliable measure of health-related quality of life**. *Critical care medicine* 2000, **28**(11):3599-3605.
8. Chalfin DB, Holbein ME, Fein AM, Carlon GC: **Cost-effectiveness of monoclonal antibodies to gram-negative endotoxin in the treatment of gram-negative sepsis in ICU patients**. *JAMA : the journal of the American Medical Association* 1993, **269**(2):249-254.
9. Brun-Buisson C, Meshaka P, Pinton P, Vallet B: **EPISEPSIS: a reappraisal of the epidemiology and outcome of severe sepsis in French intensive care units**. *Intensive care medicine* 2004, **30**(4):580-588.
10. Bone RC: **Sepsis, the sepsis syndrome, multi-organ failure: a plea for comparable definitions**. *Annals of internal medicine* 1991, **114**(4):332-333.
11. Avolio M, Diamante P, Modolo ML, De Rosa R, Stano P, Camporese A: **Direct Molecular Detection of Pathogens in Blood as Specific Rule-In Diagnostic Biomarker in Patients With Presumed Sepsis - Our Experience on a Heterogeneous Cohort of Patients With Signs of Infective SIRS**. *Shock (Augusta, Ga)* 2014.
12. Dremsizov T, Clermont G, Kellum JA, Kalassian KG, Fine MJ, Angus DC: **Severe sepsis in community-acquired pneumonia: when does it happen, and do systemic inflammatory response syndrome criteria help predict course?** *Chest* 2006, **129**(4):968-978.
13. Rangel-Frausto MS, Pittet D, Costigan M, Hwang T, Davis CS, Wenzel RP: **The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study**. *JAMA : the journal of the American Medical Association* 1995, **273**(2):117-123.

14. Alberti C, Brun-Buisson C, Chevret S, Antonelli M, Goodman SV, Martin C, Moreno R, Ochagavia AR, Palazzo M, Werdan K *et al*: **Systemic inflammatory response and progression to severe sepsis in critically ill infected patients**. *American journal of respiratory and critical care medicine* 2005, **171**(5):461-468.
15. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G: **2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference**. *Critical care medicine* 2003, **31**(4):1250-1256.
16. Dellinger RP, Carlet JM, Masur H, Gerlach H, Calandra T, Cohen J, Gea-Banacloche J, Keh D, Marshall JC, Parker MM *et al*: **Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock**. *Critical care medicine* 2004, **32**(3):858-873.
17. Hotchkiss RS, Monneret G, Payen D: **Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach**. *The Lancet Infectious Diseases* 2013, **13**(3):260-268.
18. Hotchkiss RS, Karl IE: **The pathophysiology and treatment of sepsis**. *The New England journal of medicine* 2003, **348**(2):138-150.
19. De Miguel-Yanes JM, Andueza-Lillo JA, Gonzalez-Ramallo VJ, Pastor L, Munoz J: **Failure to implement evidence-based clinical guidelines for sepsis at the ED**. *The American journal of emergency medicine* 2006, **24**(5):553-559.
20. Gao F, Melody T, Daniels DF, Giles S, Fox S: **The impact of compliance with 6-hour and 24-hour sepsis bundles on hospital mortality in patients with severe sepsis: a prospective observational study**. *Critical care (London, England)* 2005, **9**(6):R764-770.
21. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M: **Early goal-directed therapy in the treatment of severe sepsis and septic shock**. *The New England journal of medicine* 2001, **345**(19):1368-1377.
22. **Practice parameters for hemodynamic support of sepsis in adult patients in sepsis. Task Force of the American College of Critical Care Medicine, Society of Critical Care Medicine**. *Critical care medicine* 1999, **27**(3):639-660.
23. Sebat F, Johnson D, Musthafa AA, Watnik M, Moore S, Henry K, Saari M: **A multidisciplinary community hospital program for early and rapid resuscitation of shock in nontrauma patients**. *Chest* 2005, **127**(5):1729-1743.
24. Jones AE, Troyer JL, Kline JA: **Cost-effectiveness of an emergency department-based early sepsis resuscitation protocol**. *Critical care medicine* 2011, **39**(6):1306-1312.
25. Micek ST, Roubinian N, Heuring T, Bode M, Williams J, Harrison C, Murphy T, Prentice D, Ruoff BE, Kollef MH: **Before-after study of a standardized hospital order set for the management of septic shock**. *Critical care medicine* 2006, **34**(11):2707-2713.
26. Kortgen A, Niederprum P, Bauer M: **Implementation of an evidence-based "standard operating procedure" and outcome in septic shock**. *Critical care medicine* 2006, **34**(4):943-949.
27. Otero RM, Nguyen HB, Huang DT, Gaieski DF, Goyal M, Gunnerson KJ, Trzeciak S, Sherwin R, Holthaus CV, Osborn T *et al*: **Early goal-directed therapy in severe sepsis and septic shock revisited: concepts, controversies, and contemporary findings**. *Chest* 2006, **130**(5):1579-1595.

28. Houck PM, Bratzler DW, Nsa W, Ma A, Bartlett JG: **Timing of antibiotic administration and outcomes for Medicare patients hospitalized with community-acquired pneumonia**. *Archives of internal medicine* 2004, **164**(6):637-644.
29. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, Suppes R, Feinstein D, Zanotti S, Taiberg L *et al*: **Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock**. *Critical care medicine* 2006, **34**(6):1589-1596.
30. Groeneveld ABJ, Thijs, L.G: **Diagnosis: from clinical signs to haemodynamic evaluation**. London, England: WB Saunders; 2000.
31. Weinstein MP, Towns ML, Quartey SM, Mirrett S, Reimer LG, Parmigiani G, Reller LB: **The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1997, **24**(4):584-602.
32. Vincent JL, Gerlach H: **Fluid resuscitation in severe sepsis and septic shock: an evidence-based review**. *Critical care medicine* 2004, **32**(11 Suppl):S451-454.
33. Roberts I, Alderson P, Bunn F, Chinnock P, Ker K, Schierhout G: **Colloids versus crystalloids for fluid resuscitation in critically ill patients**. *Cochrane database of systematic reviews (Online)* 2004(4):CD000567.
34. Schortgen F, Lacherade JC, Bruneel F, Cattaneo I, Hemery F, Lemaire F, Brochard L: **Effects of hydroxyethylstarch and gelatin on renal function in severe sepsis: a multicentre randomised study**. *Lancet* 2001, **357**(9260):911-916.
35. **Human albumin administration in critically ill patients: systematic review of randomised controlled trials. Cochrane Injuries Group Albumin Reviewers**. *BMJ (Clinical research ed)* 1998, **317**(7153):235-240.
36. Finfer S, Bellomo R, Boyce N, French J, Myburgh J, Norton R: **A comparison of albumin and saline for fluid resuscitation in the intensive care unit**. *The New England journal of medicine* 2004, **350**(22):2247-2256.
37. Guidet B, Mosqueda GJ, Priol G, Aegerter P: **The COASST study: cost-effectiveness of albumin in severe sepsis and septic shock**. *Journal of critical care* 2007, **22**(3):197-203.
38. Hebert PC, Wells G, Blajchman MA, Marshall J, Martin C, Pagliarello G, Tweeddale M, Schweitzer I, Yetisir E: **A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group**. *The New England journal of medicine* 1999, **340**(6):409-417.
39. Silliman CC, Ambruso DR, Boshkov LK: **Transfusion-related acute lung injury**. *Blood* 2005, **105**(6):2266-2273.
40. Rivers EP, Kruse JA, Jacobsen G, Shah K, Loomba M, Otero R, Childs EW: **The influence of early hemodynamic optimization on biomarker patterns of severe sepsis and septic shock**. *Critical care medicine* 2007, **35**(9):2016-2024.
41. Beale RJ, Hollenberg SM, Vincent JL, Parrillo JE: **Vasopressor and inotropic support in septic shock: an evidence-based review**. *Critical care medicine* 2004, **32**(11 Suppl):S455-465.

42. Levy MM, Macias WL, Vincent JL, Russell JA, Silva E, Trzaskoma B, Williams MD: **Early changes in organ function predict eventual survival in severe sepsis**. *Critical care medicine* 2005, **33**(10):2194-2201.
43. Hannemann L, Reinhart K, Grenzer O, Meier-Hellmann A, Bredle DL: **Comparison of dopamine to dobutamine and norepinephrine for oxygen delivery and uptake in septic shock**. *Critical care medicine* 1995, **23**(12):1962-1970.
44. Jakob SM, Ruokonen E, Takala J: **Effects of dopamine on systemic and regional blood flow and metabolism in septic and cardiac surgery patients**. *Shock (Augusta, Ga)* 2002, **18**(1):8-13.
45. Barrett LK, Singer M, Clapp LH: **Vasopressin: mechanisms of action on the vasculature in health and in septic shock**. *Critical care medicine* 2007, **35**(1):33-40.
46. Russell JA, Walley KR, Singer J, Gordon AC, Hebert PC, Cooper DJ, Holmes CL, Mehta S, Granton JT, Storms MM *et al*: **Vasopressin versus norepinephrine infusion in patients with septic shock**. *The New England journal of medicine* 2008, **358**(9):877-887.
47. **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network**. *The New England journal of medicine* 2000, **342**(18):1301-1308.
48. Amato MB, Barbas CS, Medeiros DM, Magaldi RB, Schettino GP, Lorenzi-Filho G, Kairalla RA, Deheinzelin D, Munoz C, Oliveira R *et al*: **Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome**. *The New England journal of medicine* 1998, **338**(6):347-354.
49. Van den Berghe G, Wilmer A, Hermans G, Meersseman W, Wouters PJ, Milants I, Van Wijngaerden E, Bobbaers H, Bouillon R: **Intensive insulin therapy in the medical ICU**. *The New England journal of medicine* 2006, **354**(5):449-461.
50. Barochia AV, Cui X, Vitberg D, Suffredini AF, O'Grady NP, Banks SM, Minneci P, Kern SJ, Danner RL, Natanson C *et al*: **Bundled care for septic shock: An analysis of clinical trials**. *Critical care medicine* 2010, **38**(2):668-678.
51. Monneret G, Venet F, Pachot A, Lepape A: **Monitoring immune dysfunctions in the septic patient: A new skin for the old ceremony**. *Molecular Medicine* 2008, **14**(1-2):64-78.
52. Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG: **The pathogenesis of sepsis**. *Annual review of pathology* 2011, **6**:19-48.
53. Munford RS: **Severe sepsis and septic shock: the role of gram-negative bacteremia**. *Annual review of pathology* 2006, **1**:467-496.
54. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A: **Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia**. *Nature* 1987, **330**(6149):662-664.
55. Dinarello CA: **Therapeutic strategies to reduce IL-1 activity in treating local and systemic inflammation**. *Current opinion in pharmacology* 2004, **4**(4):378-385.
56. Riedemann NC, Guo RF, Ward PA: **Novel strategies for the treatment of sepsis**. *Nature medicine* 2003, **9**(5):517-524.
57. Ulloa L, Tracey KJ: **The "cytokine profile": a code for sepsis**. *Trends in molecular medicine* 2005, **11**(2):56-63.

58. Van Dissel JT, Van Langevelde P, Westendorp RGJ, Kwappenberg K, Frölich M: **Anti-inflammatory cytokine profile and mortality in febrile patients**. *Lancet* 1998, **351**(9107):950-953.
59. Ertel W, Kremer JP, Kenney J, Steckholzer U, Jarrar D, Trentz O, Schildberg FW: **Downregulation of proinflammatory cytokine release in whole blood from septic patients**. *Blood* 1995, **85**(5):1341-1347.
60. Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM: **Dysregulation of in vitro cytokine production by monocytes during sepsis**. *Journal of Clinical Investigation* 1991, **88**(5):1747-1754.
61. Rigato O, Salomao R: **Impaired production of interferon-gamma and tumor necrosis factor-alpha but not of interleukin 10 in whole blood of patients with sepsis**. *Shock (Augusta, Ga)* 2003, **19**(2):113-116.
62. Sinistro A, Almerighi C, Ciaprini C, Natoli S, Sussarello E, Di Fino S, Calò-Carducci F, Rocchi G, Bergamini A: **Downregulation of CD40 ligand response in monocytes from sepsis patients**. *Clinical and Vaccine Immunology* 2008, **15**(12):1851-1858.
63. Weighardt H, Heidecke CD, Emmanuilidis K, Maier S, Bartels H, Siewert JR, Holzmann B: **Sepsis after major visceral surgery is associated with sustained and interferon-γ-resistant defects of monocyte cytokine production**. *Surgery* 2000, **127**(3):309-315.
64. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman Ii SD, Kreisel D, Krupnick AS *et al*: **Immunosuppression in patients who die of sepsis and multiple organ failure**. *JAMA - Journal of the American Medical Association* 2011, **306**(23):2594-2605.
65. Otto G, Sossdorf M, Claus R, Rodel J, Menge K, Reinhart K, Bauer M, Riedemann N: **The late phase of sepsis is characterized by an increased microbiological burden and death rate**. *Critical Care* 2011, **15**(4):R183.
66. Kollef KE, Schramm GE, Wills AR, Reichley RM, Micek ST, Kollef MH: **Predictors of 30-day mortality and hospital costs in patients with ventilator-associated pneumonia attributed to potentially antibiotic-resistant gram-negative bacteria**. *Chest* 2008, **134**(2):281-287.
67. Limaye AP, Kirby KA, Rubenfeld GD, Leisenring WM, Bulger EM, Neff MJ, Gibran NS, Huang ML, Santo Hayes TK, Corey L *et al*: **Cytomegalovirus reactivation in critically ill immunocompetent patients**. *JAMA : the journal of the American Medical Association* 2008, **300**(4):413-422.
68. Luyt CE, Combes A, Deback C, Aubriot-Lorton MH, Nieszkowska A, Trouillet JL, Capron F, Agut H, Gibert C, Chastre J: **Herpes simplex virus lung infection in patients undergoing prolonged mechanical ventilation**. *American journal of respiratory and critical care medicine* 2007, **175**(9):935-942.
69. Meakins JL, Pietsch JB, Bubenick O, Kelly R, Rode H, Gordon J, MacLean LD: **Delayed hypersensitivity: indicator of acquired failure of host defenses in sepsis and trauma**. *Annals of surgery* 1977, **186**(3):241-250.
70. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, Buchman TG, Karl IE: **Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction**. *Critical care medicine* 1999, **27**(7):1230-1251.
71. Hotchkiss RS, Tinsley KW, Swanson PE, Schmieg RE, Jr., Hui JJ, Chang KC, Osborne DF, Freeman BD, Cobb JP, Buchman TG *et al*: **Sepsis-induced apoptosis causes**

progressive profound depletion of B and CD4+ T lymphocytes in humans. *Journal of immunology (Baltimore, Md : 1950)* 2001, **166**(11):6952-6963.
72. Felmet KA, Hall MW, Clark RSB, Jaffe R, Carcillo JA: **Prolonged lymphopenia, lymphoid depletion, and hypoprolactinemia in children with nosocomial sepsis and multiple organ failure**. *Journal of Immunology* 2005, **174**(6):3765-3772.
73. Toti P, De Felice C, Occhini R, Schuerfeld K, Stumpo M, Epistolato MC, Vatti R, Buonocore G: **Spleen depletion in noenatal sepsis and chorioamnionitis**. *American Journal of Clinical Pathology* 2004, **122**(5):765-771.
74. Hotchkiss RS, Monneret G, Payen D: **Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy**. *Nature reviews Immunology* 2013, **13**(12):862-874.
75. Zhang L, Cardinal JS, Pan P, Rosborough BR, Chang Y, Yan W, Huang H, Billiar TR, Rosengart MR, Tsung A: **Splenocyte apoptosis and autophagy is mediated by interferon regulatory factor 1 during murine endotoxemia**. *Shock (Augusta, Ga)* 2012, **37**(5):511-517.
76. Zhu J, Wang J, Sheng Y, Zou Y, Bo L, Wang F, Lou J, Fan X, Bao R, Wu Y *et al*: **Baicalin improves survival in a murine model of polymicrobial sepsis via suppressing inflammatory response and lymphocyte apoptosis**. *PloS one* 2012, **7**(5):e35523.
77. Le Tulzo Y, Pangault C, Gacouin A, Guilloux V, Tribut O, Amiot L, Tattevin P, Thomas R, Fauchet R, Drenou B: **Early circulating lymphocyte apoptosis in human septic shock is associated with poor outcome**. *Shock (Augusta, Ga)* 2002, **18**(6):487-494.
78. Carlson DL, Willis MS, White DJ, Horton JW, Giroir BP: **Tumor necrosis factor-alpha-induced caspase activation mediates endotoxin-related cardiac dysfunction**. *Critical care medicine* 2005, **33**(5):1021-1028.
79. Lancel S, Joulin O, Favory R, Goossens JF, Kluza J, Chopin C, Formstecher P, Marchetti P, Neviere R: **Ventricular myocyte caspases are directly responsible for endotoxin-induced cardiac dysfunction**. *Circulation* 2005, **111**(20):2596-2604.
80. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA *et al*: **Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis**. *Nature* 1995, **376**(6535):37-43.
81. Martinon F, Tschopp J: **Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases**. *Cell* 2004, **117**(5):561-574.
82. Roy S, Nicholson DW: **Cross-talk in cell death signaling**. *The Journal of experimental medicine* 2000, **192**(8):F21-25.
83. Marsden VS, Strasser A: **Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more**. *Annual review of immunology* 2003, **21**:71-105.
84. Hotchkiss RS, Nicholson DW: **Apoptosis and caspases regulate death and inflammation in sepsis**. *Nature reviews Immunology* 2006, **6**(11):813-822.
85. Lederer JA, Rodrick ML, Mannick JA: **The effects of injury on the adaptive immune response**. *Shock (Augusta, Ga)* 1999, **11**(3):153-159.
86. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I: **Immunosuppressive effects of apoptotic cells**. *Nature* 1997, **390**(6658):350-351.
87. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM: **Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine**

production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *The Journal of clinical investigation* 1998, **101**(4):890-898.
88. Gogos CA, Drosou E, Bassaris HP, Skoutelis A: **Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options**. *The Journal of infectious diseases* 2000, **181**(1):176-180.
89. Cohen J, Opal S, Calandra T: **Sepsis studies need new direction**. *The Lancet Infectious Diseases* 2012, **12**(7):503-505.
90. Vincent JL, Sun Q, Dubois MJ: **Clinical trials of immunomodulatory therapies in severe sepsis and septic shock**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2002, **34**(8):1084-1093.
91. Sprung CL, Eidelman LA, Pizov R, Fisher CJ, Jr., Ziegler EJ, Sadoff JC, Straube RC, McCloskey RV: **Influence of alterations in foregoing life-sustaining treatment practices on a clinical sepsis trial. The HA-1A Sepsis Study Group**. *Critical care medicine* 1997, **25**(3):383-387.
92. Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, Wittebole X, Dugernier T, Perrotin D, Tidswell M *et al*: **Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial**. *JAMA : the journal of the American Medical Association* 2013, **309**(11):1154-1162.
93. Rondon E, Venkataraman R: **Afelimomab led to a modest mortality benefit in patients with severe sepsis and elevated interleukin-6 levels**. *Critical care (London, England)* 2005, **9**(5):E20.
94. Abraham E, Laterre PF, Garbino J, Pingleton S, Butler T, Dugernier T, Margolis B, Kudsk K, Zimmerli W, Anderson P *et al*: **Lenercept (p55 tumor necrosis factor receptor fusion protein) in severe sepsis and early septic shock: a randomized, double-blind, placebo-controlled, multicenter phase III trial with 1,342 patients**. *Critical care medicine* 2001, **29**(3):503-510.
95. Opal SM, Fisher CJ, Jr., Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA *et al*: **Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group**. *Critical care medicine* 1997, **25**(7):1115-1124.
96. Warren BL, Eid A, Singer P, Pillay SS, Carl P, Novak I, Chalupa P, Atherstone A, Penzes I, Kubler A *et al*: **Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial**. *JAMA : the journal of the American Medical Association* 2001, **286**(15):1869-1878.
97. Marti-Carvajal AJ, Sola I, Lathyris D, Cardona AF: **Human recombinant activated protein C for severe sepsis**. *Cochrane database of systematic reviews (Online)* 2012, **3**:CD004388.
98. Abraham E, Reinhart K, Opal S, Demeyer I, Doig C, Rodriguez AL, Beale R, Svoboda P, Laterre PF, Simon S *et al*: **Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial**. *JAMA : the journal of the American Medical Association* 2003, **290**(2):238-247.
99. Suputtamongkol Y, Intaranongpai S, Smith MD, Angus B, Chaowagul W, Permpikul C, Simpson JA, Leelarasamee A, Curtis L, White NJ: **A double-blind placebo-controlled study of an infusion of lexipafant (Platelet-activating factor receptor antagonist) in**

patients with severe sepsis. *Antimicrobial agents and chemotherapy* 2000, **44**(3):693-696.
100. Opal S, Laterre PF, Abraham E, Francois B, Wittebole X, Lowry S, Dhainaut JF, Warren B, Dugernier T, Lopez A *et al*: **Recombinant human platelet-activating factor acetylhydrolase for treatment of severe sepsis: results of a phase III, multicenter, randomized, double-blind, placebo-controlled, clinical trial**. *Critical care medicine* 2004, **32**(2):332-341.
101. Zeiher BG, Steingrub J, Laterre PF, Dmitrienko A, Fukiishi Y, Abraham E: **LY315920NA/S-5920, a selective inhibitor of group IIA secretory phospholipase A2, fails to improve clinical outcome for patients with severe sepsis**. *Critical care medicine* 2005, **33**(8):1741-1748.
102. K BL: **Polyclonal intravenous immunoglobulin for the prophylaxis and treatment of infection in critically ill adults**. *The Canadian journal of infectious diseases = Journal canadien des maladies infectieuses* 2002, **13**(2):100-106.
103. Orozco H, Arch J, Medina-Franco H, Pantoja JP, Gonzalez QH, Vilatoba M, Hinojosa C, Vargas-Vorackova F, Sifuentes-Osornio J: **Molgramostim (GM-CSF) associated with antibiotic treatment in nontraumatic abdominal sepsis: a randomized, double-blind, placebo-controlled clinical trial**. *Archives of surgery (Chicago, Ill : 1960)* 2006, **141**(2):150-153; discussion 154.
104. Bruno JJ, Dee BM, Anderegg BA, Hernandez M, Pravinkumar SE: **US practitioner opinions and prescribing practices regarding corticosteroid therapy for severe sepsis and septic shock**. *Journal of critical care* 2012, **27**(4):351-361.
105. Borthwick EM, Hill CJ, Rabindranath KS, Maxwell AP, McAuley DF, Blackwood B: **High-volume haemofiltration for sepsis**. *Cochrane database of systematic reviews (Online)* 2013, **1**:CD008075.
106. Meisel C, Schefold JC, Pschowski R, Baumann T, Hetzger K, Gregor J, Weber-Carstens S, Hasper D, Keh D, Zuckermann H *et al*: **Granulocyte-macrophage colony-stimulating factor to reverse sepsis-associated immunosuppression: a double-blind, randomized, placebo-controlled multicenter trial**. *American journal of respiratory and critical care medicine* 2009, **180**(7):640-648.
107. Hall M, Knatz N, Vetterly C, Tomarello S, Wewers M, Volk H, Carcillo J: **Immunoparalysis and nosocomial infection in children with multiple organ dysfunction syndrome**. *Intensive care medicine* 2011, **37**(3):525-532.
108. Morre M, Beq S: **Interleukin-7 and immune reconstitution in cancer patients: a new paradigm for dramatically increasing overall survival**. *Targ Oncol* 2012, **7**(1):55-68.
109. Mackall CL, Fry TJ, Gress RE: **Harnessing the biology of IL-7 for therapeutic application**. *Nature reviews Immunology* 2011, **11**(5):330-342.
110. Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, Stetler-Stevenson M, Morton KE, Mavroukakis SA, Morre M *et al*: **IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells**. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2006, **29**(3):313-319.
111. Pellegrini M, Calzascia T, Toe JG, Preston SP, Lin AE, Elford AR, Shahinian A, Lang PA, Lang KS, Morre M *et al*: **IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology**. *Cell* 2011, **144**(4):601-613.

112. Kasten KR, Prakash PS, Unsinger J, Goetzman HS, England LG, Cave CM, Seitz AP, Mazuski CN, Zhou TT, Morre M *et al*: **Interleukin-7 (IL-7) treatment accelerates neutrophil recruitment through gamma delta T-cell IL-17 production in a murine model of sepsis**. *Infection and immunity* 2010, **78**(11):4714-4722.
113. Hotchkiss RS, Swanson PE, Knudson CM, Chang KC, Cobb JP, Osborne DF, Zollner KM, Buchman TG, Korsmeyer SJ, Karl IE: **Overexpression of Bcl-2 in transgenic mice decreases apoptosis and improves survival in sepsis**. *Journal of Immunology* 1999, **162**(7):4148-4156.
114. Levy Y, Sereti I, Tambussi G, Routy JP, Lelievre JD, Delfraissy JF, Molina JM, Fischl M, Goujard C, Rodriguez B *et al*: **Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2012, **55**(2):291-300.
115. Unsinger J, McGlynn M, Kasten KR, Hoekzema AS, Watanabe E, Muenzer JT, McDonough JS, Tschoep J, Ferguson TA, McDunn JE *et al*: **IL-7 promotes T cell viability, trafficking, and functionality and improves survival in sepsis**. *Journal of immunology (Baltimore, Md : 1950)* 2010, **184**(7):3768-3779.
116. Venet F, Foray AP, Villars-Mechin A, Malcus C, Poitevin-Later F, Lepape A, Monneret G: **IL-7 restores lymphocyte functions in septic patients**. *Journal of immunology (Baltimore, Md : 1950)* 2012, **189**(10):5073-5081.
117. Patel A, Patel J, Ikwuagwu J: **Treatment of progressive multifocal leukoencephalopathy and idiopathic CD4+ lymphocytopenia**. *The Journal of antimicrobial chemotherapy* 2010, **65**(12):2489-2492.
118. Unsinger J, Burnham CA, McDonough J, Morre M, Prakash PS, Caldwell CC, Dunne WM, Jr., Hotchkiss RS: **Interleukin-7 ameliorates immune dysfunction and improves survival in a 2-hit model of fungal sepsis**. *The Journal of infectious diseases* 2012, **206**(4):606-616.
119. Morre M, Beq S: **Interleukin-7 and immune reconstitution in cancer patients: a new paradigm for dramatically increasing overall survival**. *Target Oncol* 2012, **7**(1):55-68.
120. Cheever MA: **Twelve immunotherapy drugs that could cure cancers**. *Immunological reviews* 2008, **222**:357-368.
121. Keir ME, Butte MJ, Freeman GJ, Sharpe AH: **PD-1 and its ligands in tolerance and immunity**. *Annual review of immunology* 2008, **26**:677-704.
122. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N *et al*: **Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice**. *Science (New York, NY)* 2001, **291**(5502):319-322.
123. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C *et al*: **PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression**. *Nature* 2006, **443**(7109):350-354.
124. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ: **The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection**. *Nature immunology* 2007, **8**(3):239-245.

125. Huang X, Venet F, Wang YL, Lepape A, Yuan Z, Chen Y, Swan R, Kherouf H, Monneret G, Chung CS *et al*: **PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(15):6303-6308.
126. Zhang Y, Zhou Y, Lou J, Li J, Bo L, Zhu K, Wan X, Deng X, Cai Z: **PD-L1 blockade improves survival in experimental sepsis by inhibiting lymphocyte apoptosis and reversing monocyte dysfunction**. *Critical care (London, England)* 2010, **14**(6):R220.
127. Guignant C, Lepape A, Huang X, Kherouf H, Denis L, Poitevin F, Malcus C, Cheron A, Allaouchiche B, Gueyffier F *et al*: **Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock patients**. *Critical care (London, England)* 2011, **15**(2):R99.
128. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD, 2nd, Kreisel D, Krupnick AS *et al*: **Immunosuppression in patients who die of sepsis and multiple organ failure**. *JAMA : the journal of the American Medical Association* 2011, **306**(23):2594-2605.
129. Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W: **Monocyte deactivation in septic patients: restoration by IFN-gamma treatment**. *Nature medicine* 1997, **3**(6):678-681.
130. Nalos M, Santner-Nanan B, Parnell G, Tang B, McLean AS, Nanan R: **Immune effects of interferon gamma in persistent staphylococcal sepsis**. *American journal of respiratory and critical care medicine* 2012, **185**(1):110-112.
131. Jarvis JN, Meintjes G, Rebe K, Williams GN, Bicanic T, Williams A, Schutz C, Bekker LG, Wood R, Harrison TS: **Adjunctive interferon-gamma immunotherapy for the treatment of HIV-associated cryptococcal meningitis: a randomized controlled trial**. *AIDS (London, England)* 2012, **26**(9):1105-1113.
132. Inoue S, Unsinger J, Davis CG, Muenzer JT, Ferguson TA, Chang K, Osborne DF, Clark AT, Coopersmith CM, McDunn JE *et al*: **IL-15 prevents apoptosis, reverses innate and adaptive immune dysfunction, and improves survival in sepsis**. *Journal of immunology (Baltimore, Md : 1950)* 2010, **184**(3):1401-1409.
133. Adler G, Steeg C, Pfeffer K, Murphy TL, Murphy KM, Langhorne J, Jacobs T: **B and T lymphocyte attenuator restricts the protective immune response against experimental malaria**. *Journal of immunology (Baltimore, Md : 1950)* 2011, **187**(10):5310-5319.
134. Hotchkiss RS, Tinsley KW, Swanson PE, Chang KC, Cobb JP, Buchman TG, Korsmeyer SJ, Karl IE: **Prevention of lymphocyte cell death in sepsis improves survival in mice**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(25):14541-14546.
135. Wesche-Soldato DE, Swan RZ, Chung CS, Ayala A: **The apoptotic pathway as a therapeutic target in sepsis**. *Current Drug Targets* 2007, **8**(4):493-500.
136. Braun JS, Novak R, Herzog KH, Bodner SM, Cleveland JL, Tuomanen EI: **Neuroprotection by a caspase inhibitor in acute bacterial meningitis**. *Nature medicine* 1999, **5**(3):298-302.
137. Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender P, Xanthoudakis S, Roy S, Black C, Grimm E *et al*: **Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte**. *Nature immunology* 2000, **1**(6):496-501.

138. Methot N, Huang J, Coulombe N, Vaillancourt JP, Rasper D, Tam J, Han Y, Colucci J, Zamboni R, Xanthoudakis S *et al*: **Differential efficacy of caspase inhibitors on apoptosis markers during sepsis in rats and implication for fractional inhibition requirements for therapeutics**. *The Journal of experimental medicine* 2004, **199**(2):199-207.
139. Balato A, Unutmaz D, Gaspari AA: **Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions**. *The Journal of investigative dermatology* 2009, **129**(7):1628-1642.
140. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG: **NKT cells: facts, functions and fallacies**. *Immunology today* 2000, **21**(11):573-583.
141. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L: **NKT cells: what's in a name?** *Nature reviews Immunology* 2004, **4**(3):231-237.
142. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB: **Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells**. *Nature* 1994, **372**(6507):691-694.
143. Natori T, Morita M, Akimoto K, Koezuka Y: **Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge Agelas mauritianus**. *Tetrahedron* 1994, **50**(9):2771-2784.
144. Bendelac A, Savage PB, Teyton L: **The biology of NKT cells**. *Annual review of immunology* 2007, **25**:297-336.
145. Yoshimoto T, Paul WE: **CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3**. *The Journal of experimental medicine* 1994, **179**(4):1285-1295.
146. Arase H, Arase N, Kobayashi Y, Nishimura Y, Yonehara S, Onoe K: **Cytotoxicity of fresh NK1.1+ T cell receptor alpha/beta+ thymocytes against a CD4+8+ thymocyte population associated with intact Fas antigen expression on the target**. *The Journal of experimental medicine* 1994, **180**(2):423-432.
147. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI: **Differential tumor surveillance by natural killer (NK) and NKT cells**. *The Journal of experimental medicine* 2000, **191**(4):661-668.
148. Gumperz JE, Miyake S, Yamamura T, Brenner MB: **Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining**. *The Journal of experimental medicine* 2002, **195**(5):625-636.
149. Lee PT, Benlagha K, Teyton L, Bendelac A: **Distinct functional lineages of human V(alpha)24 natural killer T cells**. *The Journal of experimental medicine* 2002, **195**(5):637-641.
150. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A: **A thymic precursor to the NK T cell lineage**. *Science (New York, NY)* 2002, **296**(5567):553-555.
151. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI: **A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage**. *The Journal of experimental medicine* 2002, **195**(7):835-844.
152. Hameg A, Gouarin C, Gombert JM, Hong S, Van Kaer L, Bach JF, Herbelin A: **IL-7 up-regulates IL-4 production by splenic NK1.1+ and NK1.1- MHC class I-like/CD1-dependent CD4+ T cells**. *Journal of immunology (Baltimore, Md : 1950)* 1999, **162**(12):7067-7074.

153. Hayakawa Y, Berzins SP, Crowe NY, Godfrey DI, Smyth MJ: **Antigen-induced tolerance by intrathymic modulation of self-recognizing inhibitory receptors**. *Nature immunology* 2004, **5**(6):590-596.
154. Hammond KJ, Pelikan SB, Crowe NY, Randle-Barrett E, Nakayama T, Taniguchi M, Smyth MJ, van Driel IR, Scollay R, Baxter AG *et al*: **NKT cells are phenotypically and functionally diverse**. *European journal of immunology* 1999, **29**(11):3768-3781.
155. Kronenberg M: **Toward an understanding of NKT cell biology: progress and paradoxes**. *Annual review of immunology* 2005, **23**:877-900.
156. Ogasawara K, Takeda K, Hashimoto W, Satoh M, Okuyama R, Yanai N, Obinata M, Kumagai K, Takada H, Hiraide H *et al*: **Involvement of NK1+ T cells and their IFN-gamma production in the generalized Shwartzman reaction**. *Journal of immunology (Baltimore, Md : 1950)* 1998, **160**(7):3522-3527.
157. Dieli F, Sireci G, Russo D, Taniguchi M, Ivanyi J, Fernandez C, Troye-Blomberg M, De Leo G, Salerno A: **Resistance of natural killer T cell-deficient mice to systemic Shwartzman reaction**. *The Journal of experimental medicine* 2000, **192**(11):1645-1652.
158. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, Schmidt R, Harris AL, Old L, Cerundolo V: **NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells**. *Journal of immunology (Baltimore, Md : 1950)* 2003, **171**(10):5140-5147.
159. Hu CK, Venet F, Heffernan DS, Wang YL, Horner B, Huang X, Chung CS, Gregory SH, Ayala A: **The role of hepatic invariant NKT cells in systemic/local inflammation and mortality during polymicrobial septic shock**. *Journal of immunology (Baltimore, Md : 1950)* 2009, **182**(4):2467-2475.
160. Nagarajan NA, Kronenberg M: **Invariant NKT cells amplify the innate immune response to lipopolysaccharide**. *Journal of immunology (Baltimore, Md : 1950)* 2007, **178**(5):2706-2713.
161. Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue LW, 3rd, Bland KI, Chaudry IH: **Cecal ligation and puncture**. *Shock (Augusta, Ga)* 2005, **24 Suppl 1**:52-57.
162. Kawamura T, Takeda K, Mendiratta SK, Kawamura H, Van Kaer L, Yagita H, Abo T, Okumura K: **Critical role of NK1+ T cells in IL-12-induced immune responses in vivo**. *Journal of immunology (Baltimore, Md : 1950)* 1998, **160**(1):16-19.
163. Wysocka M, Kubin M, Vieira LQ, Ozmen L, Garotta G, Scott P, Trinchieri G: **Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice**. *European journal of immunology* 1995, **25**(3):672-676.
164. Takahashi M, Ogasawara K, Takeda K, Hashimoto W, Sakihara H, Kumagai K, Anzai R, Satoh M, Seki S: **LPS induces NK1.1+ alpha beta T cells with potent cytotoxicity in the liver of mice via production of IL-12 from Kupffer cells**. *Journal of immunology (Baltimore, Md : 1950)* 1996, **156**(7):2436-2442.
165. Yu KO, Porcelli SA: **The diverse functions of CD1d-restricted NKT cells and their potential for immunotherapy**. *Immunology letters* 2005, **100**(1):42-55.
166. Rhee RJ, Carlton S, Lomas JL, Lane C, Brossay L, Cioffi WG, Ayala A: **Inhibition of CD1d activation suppresses septic mortality: a role for NK-T cells in septic immune dysfunction**. *The Journal of surgical research* 2003, **115**(1):74-81.

167. Teng MW, Yue S, Sharkey J, Exley MA, Smyth MJ: **CD1d activation and blockade: a new antitumor strategy**. *Journal of immunology (Baltimore, Md : 1950)* 2009, **182**(6):3366-3371.
168. Yue SC, Nowak M, Shaulov-Kask A, Wang R, Yue D, Balk SP, Exley MA: **Direct CD1d-mediated stimulation of APC IL-12 production and protective immune response to virus infection in vivo**. *Journal of immunology (Baltimore, Md : 1950)* 2010, **184**(1):268-276.
169. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, Li Y, Imamura M, Kaneko Y, Okawara A *et al*: **Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria**. *Nature immunology* 2011, **12**(10):966-974.
170. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N *et al*: **Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections**. *Nature* 2005, **434**(7032):525-529.
171. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB: **Mechanism of CD1d-restricted natural killer T cell activation during microbial infection**. *Nature immunology* 2003, **4**(12):1230-1237.
172. Taniguchi M, Seino K, Nakayama T: **The NKT cell system: bridging innate and acquired immunity**. *Nature immunology* 2003, **4**(12):1164-1165.
173. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, Locksley RM, Kronenberg M: **Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(14):8395-8400.
174. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, Kronenberg M, Locksley RM: **Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function**. *The Journal of experimental medicine* 2003, **198**(7):1069-1076.
175. Gadue P, Morton N, Stein PL: **The Src family tyrosine kinase Fyn regulates natural killer T cell development**. *The Journal of experimental medicine* 1999, **190**(8):1189-1196.
176. Etogo AO, Nunez J, Lin CY, Toliver-Kinsky TE, Sherwood ER: **NK but not CD1-restricted NKT cells facilitate systemic inflammation during polymicrobial intra-abdominal sepsis**. *Journal of immunology (Baltimore, Md : 1950)* 2008, **180**(9):6334-6345.
177. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, Bendelac A: **Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells**. *Journal of immunology (Baltimore, Md : 1950)* 1999, **163**(9):4647-4650.
178. Gapin L, Matsuda JL, Surh CD, Kronenberg M: **NKT cells derive from double-positive thymocytes that are positively selected by CD1d**. *Nature immunology* 2001, **2**(10):971-978.
179. Baev DV, Peng XH, Song L, Barnhart JR, Crooks GM, Weinberg KI, Metelitsa LS: **Distinct homeostatic requirements of CD4+ and CD4- subsets of Valpha24-invariant natural killer T cells in humans**. *Blood* 2004, **104**(13):4150-4156.
180. Berzins SP, Kyparissoudis K, Pellicci DG, Hammond KJ, Sidobre S, Baxter A, Smyth MJ, Kronenberg M, Godfrey DI: **Systemic NKT cell deficiency in NOD mice is not**

detected in peripheral blood: implications for human studies. *Immunology and cell biology* 2004, **82**(3):247-252.
181. Kenna T, Golden-Mason L, Porcelli SA, Koezuka Y, Hegarty JE, O'Farrelly C, Doherty DG: **NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells**. *Journal of immunology (Baltimore, Md : 1950)* 2003, **171**(4):1775-1779.
182. Dellabona P, Casorati G, Friedli B, Angman L, Sallusto F, Tunnacliffe A, Roosneek E, Lanzavecchia A: **In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor alpha/beta CD4-8- subset**. *The Journal of experimental medicine* 1993, **177**(6):1763-1771.
183. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, Balk SP, Exley MA: **Loss of IFN-gamma production by invariant NK T cells in advanced cancer**. *Journal of immunology (Baltimore, Md : 1950)* 2001, **167**(7):4046-4050.
184. Exley MA, Koziel MJ: **To be or not to be NKT: natural killer T cells in the liver**. *Hepatology (Baltimore, Md)* 2004, **40**(5):1033-1040.
185. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C: **Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity**. *European journal of immunology* 2009, **39**(7):1893-1901.
186. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E *et al*: **CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides**. *Science (New York, NY)* 1997, **278**(5343):1626-1629.
187. Hong C, Park SH: **Application of natural killer T cells in antitumor immunotherapy**. *Critical reviews in immunology* 2007, **27**(6):511-525.
188. Terabe M, Berzofsky JA: **The role of NKT cells in tumor immunity**. *Advances in cancer research* 2008, **101**:277-348.
189. Veldt BJ, van der Vliet HJ, von Blomberg BM, van Vlierberghe H, Gerken G, Nishi N, Hayashi K, Scheper RJ, de Knegt RJ, van den Eertwegh AJ *et al*: **Randomized placebo controlled phase I/II trial of alpha-galactosylceramide for the treatment of chronic hepatitis C**. *Journal of hepatology* 2007, **47**(3):356-365.
190. Woltman AM, Ter Borg MJ, Binda RS, Sprengers D, von Blomberg BM, Scheper RJ, Hayashi K, Nishi N, Boonstra A, van der Molen R *et al*: **Alpha-galactosylceramide in chronic hepatitis B infection: results from a randomized placebo-controlled Phase I/II trial**. *Antiviral therapy* 2009, **14**(6):809-818.
191. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM: **Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein**. *The Journal of experimental medicine* 2003, **198**(2):267-279.
192. Burdin N, Brossay L, Kronenberg M: **Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis**. *European journal of immunology* 1999, **29**(6):2014-2025.
193. Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, Kronenberg M, Koezuka Y, Van Kaer L: **Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype**. *Journal of immunology (Baltimore, Md : 1950)* 1999, **163**(5):2373-2377.

194. Fujii S, Shimizu K, Kronenberg M, Steinman RM: **Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs**. *Nature immunology* 2002, **3**(9):867-874.
195. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, Joyce S, Van Kaer L: **Glycolipid antigen induces long-term natural killer T cell anergy in mice**. *The Journal of clinical investigation* 2005, **115**(9):2572-2583.
196. Sireci G, La Manna MP, Di Liberto D, Lo Dico M, Taniguchi M, Dieli F, Salerno A: **Prophylaxis of lipopolysaccharide-induced shock by alpha-galactosylceramide**. *Journal of leukocyte biology* 2008, **84**(2):550-560.
197. Sireci G, La Manna MP, Di Sano C, Di Liberto D, Porcelli SA, Kronenberg M, Dieli F, Salerno A: **Pivotal advance: alpha-galactosylceramide induces protection against lipopolysaccharide-induced shock**. *Journal of leukocyte biology* 2007, **81**(3):607-622.
198. Morecki S, Panigrahi S, Pizov G, Yacovlev E, Gelfand Y, Eizik O, Slavin S: **Effect of KRN7000 on induced graft-vs-host disease**. *Experimental hematology* 2004, **32**(7):630-637.
199. Hashimoto D, Asakura S, Miyake S, Yamamura T, Van Kaer L, Liu C, Tanimoto M, Teshima T: **Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells**. *Journal of immunology (Baltimore, Md : 1950)* 2005, **174**(1):551-556.
200. Seino K, Yanagisawa K, Taniguchi H, Takada Y, Yuzawa K, Otsuka M, Fukao K: **The effect of alpha-galactosylceramide upon allogenic rejection**. *Transplantation proceedings* 2001, **33**(1-2):437-438.
201. Berkers CR, Ovaa H: **Immunotherapeutic potential for ceramide-based activators of iNKT cells**. *Trends in pharmacological sciences* 2005, **26**(5):252-257.
202. Miyamoto K, Miyake S, Yamamura T: **A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells**. *Nature* 2001, **413**(6855):531-534.
203. Yamamura T, Miyamoto K, Illes Z, Pal E, Araki M, Miyake S: **NKT cell-stimulating synthetic glycolipids as potential therapeutics for autoimmune disease**. *Current topics in medicinal chemistry* 2004, **4**(5):561-567.
204. Mizuno M, Masumura M, Tomi C, Chiba A, Oki S, Yamamura T, Miyake S: **Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice**. *Journal of autoimmunity* 2004, **23**(4):293-300.
205. Chiba A, Oki S, Miyamoto K, Hashimoto H, Yamamura T, Miyake S: **Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide**. *Arthritis and rheumatism* 2004, **50**(1):305-313.
206. Miellot-Gafsou A, Biton J, Bourgeois E, Herbelin A, Boissier MC, Bessis N: **Early activation of invariant natural killer T cells in a rheumatoid arthritis model and application to disease treatment**. *Immunology* 2010, **130**(2):296-306.
207. Walker KM, Rytelewski M, Mazzuca DM, Meilleur SA, Mannik LA, Yue D, Brintnell WC, Welch I, Cairns E, Haeryfar SM: **Preventing and curing citrulline-induced autoimmune arthritis in a humanized mouse model using a Th2-polarizing iNKT cell agonist**. *Immunology and cell biology* 2012, **90**(6):630-639.

208. Haeryfar SM, Lan Z, Leon-Ponte M, Duffy KR, Ge W, Liu W, Mele T, Garcia B, Wang H: **Prolongation of cardiac allograft survival by rapamycin and the invariant natural killer T cell glycolipid agonist OCH**. *Transplantation* 2008, **86**(3):460-468.
209. Ly D, Tohn R, Rubin B, Blumenfeld H, Besra GS, Veerapen N, Porcelli SA, Delovitch TL: **An alpha-galactosylceramide C20:2 N-acyl variant enhances anti-inflammatory and regulatory T cell-independent responses that prevent type 1 diabetes**. *Clinical and experimental immunology* 2010, **160**(2):185-198.
210. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, Bossi G, Salio M, Denkberg G, Reddington F *et al*: **The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation**. *The Journal of experimental medicine* 2007, **204**(5):1131-1144.
211. Forestier C, Takaki T, Molano A, Im JS, Baine I, Jerud ES, Illarionov P, Ndonye R, Howell AR, Santamaria P *et al*: **Improved outcomes in NOD mice treated with a novel Th2 cytokine-biasing NKT cell activator**. *Journal of immunology (Baltimore, Md : 1950)* 2007, **178**(3):1415-1425.
212. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, Yamamura T, Chayama K: **Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice**. *Inflammatory bowel diseases* 2005, **11**(1):35-41.
213. Parekh VV, Singh AK, Wilson MT, Olivares-Villagomez D, Bezbradica JS, Inazawa H, Ehara H, Sakai T, Serizawa I, Wu L *et al*: **Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids**. *Journal of immunology (Baltimore, Md : 1950)* 2004, **173**(6):3693-3706.
214. Wilkosz S, Ireland G, Khwaja N, Walker M, Butt R, de Giorgio-Miller A, Herrick SE: **A comparative study of the structure of human and murine greater omentum**. *Anatomy and embryology* 2005, **209**(3):251-261.
215. Carlow DA, Gold MR, Ziltener HJ: **Lymphocytes in the peritoneum home to the omentum and are activated by resident dendritic cells**. *Journal of immunology (Baltimore, Md : 1950)* 2009, **183**(2):1155-1165.
216. Cai J, Tang H, Xu L, Wang X, Yang C, Ruan S, Guo J, Hu S, Wang Z: **Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness**. *Carcinogenesis* 2012, **33**(1):20-29.
217. Krist LF, Eestermans IL, Steenbergen JJ, Hoefsmit EC, Cuesta MA, Meyer S, Beelen RH: **Cellular composition of milky spots in the human greater omentum: an immunochemical and ultrastructural study**. *The Anatomical record* 1995, **241**(2):163-174.
218. Mulholland MW: **Surgical Treatment of Perforated Duodenal Ulcer**, 5 edn. New York, NY: Lippincott Williams & Wilkins; 2010.
219. Cannaday JE: **Some uses of undetached omentum in surgery**. *American journal of surgery* 1948, **76**(5):502-505.
220. Cranshaw ML, Leak LV: **Milky spots of the omentum: a source of peritoneal cells in the normal and stimulated animal**. *Archives of histology and cytology* 1990, **53 Suppl**:165-177.
221. Ansel KM, Harris RB, Cyster JG: **CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity**. *Immunity* 2002, **16**(1):67-76.

222. Berberich S, Dahne S, Schippers A, Peters T, Muller W, Kremmer E, Forster R, Pabst O: **Differential molecular and anatomical basis for B cell migration into the peritoneal cavity and omental milky spots**. *Journal of immunology (Baltimore, Md : 1950)* 2008, **180**(4):2196-2203.
223. Dux K, Rouse RV, Kyewski B: **Composition of the lymphoid cell populations from omental milky spots during the immune response in C57BL/Ka mice**. *European journal of immunology* 1986, **16**(8):1029-1032.
224. Beelen RH: **The greater omentum: physiology and immunological concepts**. *The Netherlands journal of surgery* 1991, **43**(5):145-149.
225. Van Vugt E, Van Rijthoven EA, Kamperdijk EW, Beelen RH: **Omental milky spots in the local immune response in the peritoneal cavity of rats**. *The Anatomical record* 1996, **244**(2):235-245.
226. Rangel-Moreno J, Moyron-Quiroz JE, Carragher DM, Kusser K, Hartson L, Moquin A, Randall TD: **Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens**. *Immunity* 2009, **30**(5):731-743.
227. Kearney JF, Bartels J, Hamilton AM, Lehuen A, Solvason N, Vakil M: **Development and function of the early B cell repertoire**. *International reviews of immunology* 1992, **8**(2-3):247-257.
228. Solvason N, Chen X, Shu F, Kearney JF: **The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development**. *Annals of the New York Academy of Sciences* 1992, **651**:10-20.
229. Shah S, Lowery E, Braun RK, Martin A, Huang N, Medina M, Sethupathi P, Seki Y, Takami M, Byrne K *et al*: **Cellular basis of tissue regeneration by omentum**. *PloS one* 2012, **7**(6):e38368.
230. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M: **Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors**. *Science (New York, NY)* 1997, **278**(5343):1623-1626.
231. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML *et al*: **Coding of facial expressions of pain in the laboratory mouse**. *Nature methods* 2010, **7**(6):447-449.
232. Nemzek JA, Hugunin KM, Opp MR: **Modeling sepsis in the laboratory: merging sound science with animal well-being**. *Comparative medicine* 2008, **58**(2):120-128.
233. Huet O, Ramsey D, Miljavec S, Jenney A, Aubron C, Aprico A, Stefanovic N, Balkau B, Head GA, de Haan JB *et al*: **Ensuring animal welfare while meeting scientific aims using a murine pneumonia model of septic shock**. *Shock (Augusta, Ga)* 2013, **39**(6):488-494.
234. Goff RD, Gao Y, Mattner J, Zhou D, Yin N, Cantu C, 3rd, Teyton L, Bendelac A, Savage PB: **Effects of lipid chain lengths in alpha-galactosylceramides on cytokine release by natural killer T cells**. *Journal of the American Chemical Society* 2004, **126**(42):13602-13603.
235. Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T *et al*: **Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(9):3383-3388.

236. Pavlidis P, Noble WS: **Matrix2png: a utility for visualizing matrix data**. *Bioinformatics (Oxford, England)* 2003, **19**(2):295-296.
237. Gavrieli Y, Sherman Y, Ben-Sasson SA: **Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation**. *The Journal of cell biology* 1992, **119**(3):493-501.
238. Knaus WA, Draper EA, Wagner DP, Zimmerman JE: **APACHE II: a severity of disease classification system**. *Critical care medicine* 1985, **13**(10):818-829.
239. Knaus WA, Zimmerman JE, Wagner DP, Draper EA, Lawrence DE: **APACHE-acute physiology and chronic health evaluation: a physiologically based classification system**. *Critical care medicine* 1981, **9**(8):591-597.
240. Linder MM, Wacha H, Feldmann U, Wesch G, Streifensand RA, Gundlach E: **[The Mannheim peritonitis index. An instrument for the intraoperative prognosis of peritonitis]**. *Der Chirurg; Zeitschrift fur alle Gebiete der operativen Medizen* 1987, **58**(2):84-92.
241. Hanley JA, McNeil BJ: **The meaning and use of the area under a receiver operating characteristic (ROC) curve**. *Radiology* 1982, **143**(1):29-36.
242. Youden WJ: **Index for rating diagnostic tests**. *Cancer* 1950, **3**(1):32-35.
243. Martin GS, Mannino DM, Eaton S, Moss M: **The epidemiology of sepsis in the United States from 1979 through 2000**. *The New England journal of medicine* 2003, **348**(16):1546-1554.
244. Vincent JL, Sakr Y, Sprung CL, Ranieri VM, Reinhart K, Gerlach H, Moreno R, Carlet J, Le Gall JR, Payen D: **Sepsis in European intensive care units: results of the SOAP study**. *Critical care medicine* 2006, **34**(2):344-353.
245. Wilson MT, Johansson C, Olivares-Villagomez D, Singh AK, Stanic AK, Wang CR, Joyce S, Wick MJ, Van Kaer L: **The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(19):10913-10918.
246. Harada M, Seino K, Wakao H, Sakata S, Ishizuka Y, Ito T, Kojo S, Nakayama T, Taniguchi M: **Down-regulation of the invariant Valpha14 antigen receptor in NKT cells upon activation**. *International immunology* 2004, **16**(2):241-247.
247. Hayworth JL, Mazzuca DM, Maleki Vareki S, Welch I, McCormick JK, Haeryfar SM: **CD1d-independent activation of mouse and human iNKT cells by bacterial superantigens**. *Immunology and cell biology* 2012, **90**(7):699-709.
248. Esperatti M, Ferrer M, Giunta V, Ranzani OT, Saucedo LM, Li Bassi G, Blasi F, Rello J, Niederman MS, Torres A: **Validation of predictors of adverse outcomes in hospital-acquired pneumonia in the ICU**. *Critical care medicine* 2013, **41**(9):2151-2161.
249. Stanic AK, Shashidharamurthy R, Bezbradica JS, Matsuki N, Yoshimura Y, Miyake S, Choi EY, Schell TD, Van Kaer L, Tevethia SS *et al*: **Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Va14Ja18 natural T(iNKT) cell receptor [corrected]**. *Journal of immunology (Baltimore, Md : 1950)* 2003, **171**(9):4539-4551.
250. Marshall JC, Reinhart K: **Biomarkers of sepsis**. *Critical care medicine* 2009, **37**(7):2290-2298.
251. Buras JA, Holzmann B, Sitkovsky M: **Animal models of sepsis: setting the stage**. *Nature reviews Drug discovery* 2005, **4**(10):854-865.

252. Esmon CT: **Why do animal models (sometimes) fail to mimic human sepsis?** *Critical care medicine* 2004, **32**(5 Suppl):S219-222.
253. Marshall JC: **Such stuff as dreams are made on: mediator-directed therapy in sepsis**. *Nature reviews Drug discovery* 2003, **2**(5):391-405.
254. Dyson A, Singer M: **Animal models of sepsis: why does preclinical efficacy fail to translate to the clinical setting?** *Critical care medicine* 2009, **37**(1 Suppl):S30-37.
255. Maier S, Traeger T, Entleutner M, Westerholt A, Kleist B, Huser N, Holzmann B, Stier A, Pfeffer K, Heidecke CD: **Cecal ligation and puncture versus colon ascendens stent peritonitis: two distinct animal models for polymicrobial sepsis**. *Shock (Augusta, Ga)* 2004, **21**(6):505-511.
256. Wichterman KA, Baue AE, Chaudry IH: **Sepsis and septic shock: A review of laboratory models and a proposal**. *Journal of Surgical Research* 1980, **29**(2):189-201.
257. Dejager L, Pinheiro I, Dejonckheere E, Libert C: **Cecal ligation and puncture: the gold standard model for polymicrobial sepsis?** *Trends in Microbiology* 2011, **19**(4):198-208.
258. Rittirsch D, Hoesel LM, Ward PA: **The disconnect between animal models of sepsis and human sepsis**. *Journal of leukocyte biology* 2007, **81**(1):137-143.
259. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA: **Immunodesign of experimental sepsis by cecal ligation and puncture**. *Nature protocols* 2009, **4**(1):31-36.
260. Lee SW, Feingold DL, Carter JJ, Zhai C, Stapleton G, Gleason N, Whelan RL: **Peritoneal macrophage and blood monocyte functions after open and laparoscopic-assisted cecectomy in rats**. *Surgical endoscopy* 2003, **17**(12):1996-2002.
261. Huang SG, Li YP, Zhang Q, Redmond HP, Wang JH, Wang J: **Laparotomy and laparoscopy diversely affect macrophage-associated antimicrobial activity in a murine model**. *BMC immunology* 2013, **14**(1):27.
262. Zingarelli B, Piraino G, Hake PW, O'Connor M, Denenberg A, Fan H, Cook JA: **Peroxisome proliferator-activated receptor {delta} regulates inflammation via NF-{kappa}B signaling in polymicrobial sepsis**. *The American journal of pathology* 2010, **177**(4):1834-1847.
263. Doi K, Leelahavanichkul A, Hu X, Sidransky KL, Zhou H, Qin Y, Eisner C, Schnermann J, Yuen PS, Star RA: **Pre-existing renal disease promotes sepsis-induced acute kidney injury and worsens outcome**. *Kidney international* 2008, **74**(8):1017-1025.
264. Miyaji T, Hu X, Yuen PS, Muramatsu Y, Iyer S, Hewitt SM, Star RA: **Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice**. *Kidney international* 2003, **64**(5):1620-1631.
265. Yasuda H, Yuen PS, Hu X, Zhou H, Star RA: **Simvastatin improves sepsis-induced mortality and acute kidney injury via renal vascular effects**. *Kidney international* 2006, **69**(9):1535-1542.
266. Liu C, Li A, Weng YB, Duan ML, Wang BE, Zhang SW: **Changes in intestinal mucosal immune barrier in rats with endotoxemia**. *World journal of gastroenterology : WJG* 2009, **15**(46):5843-5850.
267. Zhang Y, Li M, Meng M, Qin C: **Effect of ethyl pyruvate on physical and immunological barriers of the small intestine in a rat model of sepsis**. *The Journal of trauma* 2009, **66**(5):1355-1364.

268. Otto GP, Busch M, Sossdorf M, Claus RA: **Impact of sepsis-associated cytokine storm on plasma NGAL during acute kidney injury in a model of polymicrobial sepsis**. *Critical care (London, England)* 2013, **17**(2):419.
269. Hotchkiss RS, Opal S: **Immunotherapy for sepsis--a new approach against an ancient foe**. *The New England journal of medicine* 2010, **363**(1):87-89.
270. Linch SN, Danielson ET, Kelly AM, Tamakawa RA, Lee JJ, Gold JA: **Interleukin 5 is protective during sepsis in an eosinophil-independent manner**. *American journal of respiratory and critical care medicine* 2012, **186**(3):246-254.
271. Cheng SS, Lukacs NW, Kunkel SL: **Eotaxin/CCL11 is a negative regulator of neutrophil recruitment in a murine model of endotoxemia**. *Experimental and molecular pathology* 2002, **73**(1):1-8.
272. Castillo EF, Schluns KS: **Regulating the immune system via IL-15 transpresentation**. *Cytokine* 2012, **59**(3):479-490.
273. Jinquan T, Jing C, Jacobi HH, Reimert CM, Millner A, Quan S, Hansen JB, Dissing S, Malling HJ, Skov PS *et al*: **CXCR3 expression and activation of eosinophils: role of IFN-gamma-inducible protein-10 and monokine induced by IFN-gamma**. *Journal of immunology (Baltimore, Md : 1950)* 2000, **165**(3):1548-1556.
274. Driscoll KE: **Macrophage inflammatory proteins: biology and role in pulmonary inflammation**. *Experimental lung research* 1994, **20**(6):473-490.
275. Barral P, Sanchez-Nino MD, van Rooijen N, Cerundolo V, Batista FD: **The location of splenic NKT cells favours their rapid activation by blood-borne antigen**. *The EMBO journal* 2012, **31**(10):2378-2390.
276. Nakano Y, Onozuka K, Terada Y, Shinomiya H, Nakano M: **Protective effect of recombinant tumor necrosis factor-alpha in murine salmonellosis**. *Journal of immunology (Baltimore, Md : 1950)* 1990, **144**(5):1935-1941.
277. Doherty GM, Lange JR, Langstein HN, Alexander HR, Buresh CM, Norton JA: **Evidence for IFN-gamma as a mediator of the lethality of endotoxin and tumor necrosis factor-alpha**. *Journal of immunology (Baltimore, Md : 1950)* 1992, **149**(5):1666-1670.
278. Pinsky MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E: **Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality**. *Chest* 1993, **103**(2):565-575.
279. Hayworth JL, Kasper KJ, Leon-Ponte M, Herfst CA, Yue D, Brintnell WC, Mazzuca DM, Heinrichs DE, Cairns E, Madrenas J *et al*: **Attenuation of massive cytokine response to the staphylococcal enterotoxin B superantigen by the innate immunomodulatory protein lactoferrin**. *Clinical and experimental immunology* 2009, **157**(1):60-70.
280. Matsukawa A, Hogaboam CM, Lukacs NW, Lincoln PM, Evanoff HL, Strieter RM, Kunkel SL: **Expression and contribution of endogenous IL-13 in an experimental model of sepsis**. *Journal of immunology (Baltimore, Md : 1950)* 2000, **164**(5):2738-2744.
281. Wynn TA: **IL-13 effector functions**. *Annual review of immunology* 2003, **21**:425-456.
282. Muchamuel T, Menon S, Pisacane P, Howard MC, Cockayne DA: **IL-13 protects mice from lipopolysaccharide-induced lethal endotoxemia: correlation with down-modulation of TNF-alpha, IFN-gamma, and IL-12 production**. *Journal of immunology (Baltimore, Md : 1950)* 1997, **158**(6):2898-2903.

283. Van Kaer L: **alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles**. *Nature reviews Immunology* 2005, **5**(1):31-42.
284. Silva AT, Cohen J: **Role of interferon-gamma in experimental gram-negative sepsis**. *The Journal of infectious diseases* 1992, **166**(2):331-335.
285. Kohler J, Heumann D, Garotta G, LeRoy D, Bailat S, Barras C, Baumgartner JD, Glauser MP: **IFN-gamma involvement in the severity of gram-negative infections in mice**. *Journal of immunology (Baltimore, Md : 1950)* 1993, **151**(2):916-921.
286. Girardin E, Grau GE, Dayer JM, Roux-Lombard P, Lambert PH: **Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura**. *The New England journal of medicine* 1988, **319**(7):397-400.
287. Weigent DA, Huff TL, Peterson JW, Stanton GJ, Baron S: **Role of interferon in streptococcal infection in the mouse**. *Microbial pathogenesis* 1986, **1**(4):399-407.
288. Tohn R, Blumenfeld H, Haeryfar SM, Veerapen N, Besra GS, Porcelli SA, Delovitch TL: **Stimulation of a shorter duration in the state of anergy by an invariant natural killer T cell agonist enhances its efficiency of protection from type 1 diabetes**. *Clinical and experimental immunology* 2011, **164**(1):26-41.
289. Torchinsky MB, Garaude J, Martin AP, Blander JM: **Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation**. *Nature* 2009, **458**(7234):78-82.
290. Flierl MA, Rittirsch D, Gao H, Hoesel LM, Nadeau BA, Day DE, Zetoune FS, Sarma JV, Huber-Lang MS, Ferrara JL *et al*: **Adverse functions of IL-17A in experimental sepsis**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2008, **22**(7):2198-2205.
291. Rittirsch D, Flierl MA, Ward PA: **Harmful molecular mechanisms in sepsis**. *Nature reviews Immunology* 2008, **8**(10):776-787.
292. Zizzo G, Cohen PL: **IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids**. *Journal of immunology (Baltimore, Md : 1950)* 2013, **190**(10):5237-5246.
293. Liao YH, Xia N, Zhou SF, Tang TT, Yan XX, Lv BJ, Nie SF, Wang J, Iwakura Y, Xiao H *et al*: **Interleukin-17A contributes to myocardial ischemia/reperfusion injury by regulating cardiomyocyte apoptosis and neutrophil infiltration**. *Journal of the American College of Cardiology* 2012, **59**(4):420-429.
294. Heffernan DS, Monaghan SF, Chung CS, Cioffi WG, Gravenstein S, Ayala A: **A divergent response of innate regulatory T-cells to sepsis in humans: Circulating invariant natural killer T-cells are preserved**. *Human immunology* 2013.
295. Benjamim CF, Hogaboam CM, Lukacs NW, Kunkel SL: **Septic mice are susceptible to pulmonary aspergillosis**. *The American journal of pathology* 2003, **163**(6):2605-2617.

Appendix

Western

Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. John McCormick
File Number:103036
Review Level: Delegated
Approved Local Adult Participants:240
Approved Local Minor Participants:0
Protocol Title: The Immunobiology of Human Omentum: Detection and Analysis of invariant Natural Killer T Cell (iNKT Cell) Populations in Human Omental Tissue
Department & Institution: Schulich School of Medicine and Dentistry\Microbiology & Immunology,Western University
Sponsor: London Health Sciences Centre

Ethics Approval Date: November 20, 2012 **Expiry Date:**June 30, 2014
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Other	Data Abstraction Sheet for Collection of Patient Physiological Variables	2012/08/10
Western University Protocol		
Revised Letter of Information & Consent	This is the reformatted Letter of Information and Consent as requested in the review of our proposal. Tracking changes has been enabled to show the changes we have made.	2012/10/15

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Curriculum Vitae

Ram Venkatesh Anantha, M.D., M.Sc.

UNDERGRADUATE EDUCATION

2006-2010 **Doctor of Medicine**, University of Ottawa, Ottawa, ON

2002-2006 **Bachelor of Science** with Honours in Biopharmaceutical Sciences, University of Ottawa, Ottawa, ON

POSTGRADUATE TRAINING

2012-2014 **Master of Science in Microbiology and Immunology (with Distinction)**, Western University, London, ON

2012-2014 **Clinical Investigator Program**, Royal College of Physicians and Surgeons of Canada, London, ON

2010-2016 **General Surgery Residency**, Western University, London, ON

LICENSURE

2012 Surgical Foundations Examination, Royal College of Physicians and Surgeons of Canada (Pass)

2011 Licentiate of the Medical Council of Canada Part II Exam (Pass)

2010 Licentiate for Resident Practice (College of Physicians and Surgeons of Ontario)

2010 Licentiate, Advanced Trauma Life Support (ATLS) Course

2010 Licentiate of the Medical Council of Canada Part I Exam (Pass)

2009 Licentiate, Advanced Cardiac Life Support (ACLS) Provider Course

AWARDS, HONOURS

2014 **Resident Research Presentation Award**, General Surgery Residents' Research Day, London, Ontario

2014 **Presentation Award (1st place)**, Western Research Forum, Western University

2013 **Resident Travel Reimbursement Award**, Postgraduate Medical Education, Schulich School of Medicine and Dentistry, Western University

2013 **Travel Award**, Advanced Course in Basic and Clinical Immunology, Federation of Clinical Immunology Societies

2013 **Poster Award**, Infection and Immunity Research Forum, Western University

2013 **Poster Award**, Young Investigator's Forum- Clinical Investigator Training Association of Canada Annual Conference, Ottawa

2013 **Danone Science Award**, Department of Microbiology and Immunology, Western University

2013	**Western Graduate Research Scholarship**, Faculty of Science, Western University
2013	**Frederick Banting and Charles Best Canada Graduate Scholarship**, Canadian Institutes of Health Research
2013	**CSCI/CIHR Resident Research Award**, Canadian Society for Clinical Investigation/ Canadian Institutes of Health Research, Royal College of Physicians and Surgeons of Canada
2013	**Resident Travel Grant**, Division of General Surgery, Western University
2013	**Graduate Student Travel Award**, Department of Microbiology and Immunology, Western University
2013	**Ontario Graduate Scholarship (declined),** Western University
2013	**Class of Meds'49 Award for Excellence in Teaching by Resident (Nominated)**, Western University
2012	**Novus Biologicals Award for Excellence in Oral Presentation**, Infection and Immunity Research Forum, Western University
2012	**Western Graduate Entrance Scholarship**, Department of Microbiology and Immunology, Western University
2012	**Western Graduate Research Scholarship**, Faculty of Science, Western University
2012	**Resident Research Presentation Award**, General Surgery Residents' Research Day, London, Ontario
2011	**Resident Research Poster Award**, Canadian Association for General Surgeons, London, Ontario
2011	**Highest standing**, Canadian Association of General Surgeons In-Training Exam, London, Ontario
2010-2012	**Nominated for Outstanding Resident Teaching Award**, Division of General Surgery, London, Ontario
2006-2010	**Honours standing, Years I, II, III**, Faculty of Medicine, University of Ottawa *Nominated for an award for outstanding clinical performance in psychiatry* *Nominated for an award for outstanding clinical performance in family medicine* *Nominated for an award for outstanding clinical performance in internal medicine* *Nominated for an award for outstanding clinical performance in emergency medicine*
2007	**Student Research Poster Award**, Faculty of Medicine, University of Ottawa
2007	**Summer Student Scholarship**, Faculty of Medicine, University of Ottawa
2006	**Professional Training Scholarship**, Faculty of Medicine, University of Ottawa
2006	**International Post-graduate Scholarship (declined)**, NSERC
2006	**Canada Graduate Scholarship (declined)**, NSERC
2006	**Student Research Poster Award**, Faculty of Science, University of Ottawa
2005	**Summer Research Fellowship**, Ontario Genomics Institute
2005	**NSERC Undergraduate Summer Research Award**, NSERC
2004	**NSERC Undergraduate Summer Research Award**, NSERC
2002-2003	**Undergraduate Research Scholarship**, University of Ottawa
2002-2006	**High Academic Distinction (*Summa Cum Laude*)**, University of Ottawa
2002-2006	**Dean's Honours List**, Faculty of Science, University of Ottawa
2002-2006	**Entrance Scholarship**, Faculty of Science, University of Ottawa

PUBLICATIONS

Peer-Reviewed Journal Articles:

1. Delport J, Wakabayashi AT*, **Anantha RV***, McCormick J, Lannigan R, John M. 2014. Case Report: *Cellulosimicrobium cellulans* Isolated from a Patient with Acute Renal Failure. *Journal of Medical Microbiology Case Reports, in press*. *- equal contribution.

2. Shrum B*, **Anantha RV***, Xu SX, McCormick JK, Mele T. 2014. A Robust Scoring System to Evaluate Sepsis Severity in an Animal Model. *BMC Research Notes,* 7:233. *- equal contribution.

3. **Anantha RV**, Parry N, Vogt KN, Jain V, Crawford S, Leslie K. 2014. The Implementation of an Acute Care Emergency Surgical Service: A Cost Analysis from the Surgeon's Perspective. *Canadian Journal of Surgery*, 57(2):e9-e14.

4. **Anantha RV**, Paskar D, Parry N, Vogt KN, Crawford S, Leslie K. 2014. Allocation of Resources Towards an Acute Surgical Care Service Does not Affect Wait-times for Elective Cancer Operations: A Retrospective Cohort Study. *World Journal of Emergency Surgery*, 9:21.

5. **Anantha RV,** Brackstone M, Parry N, Leslie K. 2014. ACCESS to Surgery: An Acute Care Surgery Service Expedites the Treatment of Patients with Emergency Colorectal Cancer: A Retrospective Case-Control Study. *World Journal of Emergency Surgery*, 9:19.

6. **Anantha RV**, Lim R, Merritt, NH. 2014. Laparoscopic Removal of an Occult Foreign Body Causing Penetrating Abdominal Trauma in a Child. *Canadian Medical Association Journal (CMAJ)* 186(4):285-288.

7. **Anantha RV,** Stewart TC, Rajagopalan A, Walsh J, Merritt NH. 2014. Analgesia in the Management of Pediatric Trauma during the Resuscitative Phase: The Role of the Pediatric Trauma Centre. *Injury,* 45(5):845-849.

8. Ranger AM, Patel YK, Chaudhary N, **Anantha RV**. 2014. Familial Syndromes Associated with Intracranial Tumours: A Review. *Child's Nervous System,* 30(1):47-64.

9. **Anantha RV**, Kasper K, Zeppa JJ, Patterson K, Delport J, McCormick JK. 2013. Fournier's Gangrene of the Penis Caused by *Streptococcus dysgalactiae* subspecies *equisimilis*: Case Report and Incidence Review in a Tertiary-Care Centre. *BMC Infectious Diseases* 13(1):381.

10. **Anantha RV,** Chadi SA, Merritt NH. 2013. Trichobezoar Causing Intussusception: Youngest Case of Rapunzel Syndrome in a Boy in North America. *Journal of Pediatric Surgery Case Reports* 1(1): e11-e13.

11. Celo V, **Ananth RV**, Scott SL, Lean DRS. 2004. Methylmercury Artefact Formation During Solid-Phase Extraction of Water Samples Using Sulfhydryl Cotton Fiber Adsorbent. *Analytica Chemica Acta* 516: 171-177.

Peer-Reviewed Abstract Publications:

1. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. 2013 Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. *Can J Surg* 56 (4 Suppl 3): S85.

2. **Anantha RV**, Parry N, Leslie K. 2013. An Acute Care Surgery Service Facilitates the Timely Treatment of Emergency Colorectal Cancer Patients. *Can J Surg* 56 (4 Suppl 3): S91.

3. **Anantha RV**, Bottoni D, Fortin, D, Inculet, R, Malthaner, R. Closed Video-Assisted Thoracoscopic Surgery (VATS) is a Viable Alternative to Thoracotomy to Resect Pulmonary Metastases: Nine Years of Experience at a Single Institution. *Can J Surg* 56 (4 Suppl 3): S112.

4. Choi J, **Anantha RV**, Bottoni D, Fortin, D, Inculet, R, Malthaner, R. 2013. A completely closed VATS technique appears to be superior to thoracotomy for the resection of primary lung cancers: a single-institution experience. *Can J Surg* 56 (4 Suppl 3): S116.

5. Rieder S, **Anantha RV**, Leslie K. 2013. From colonoscopy to colectomy: wait-times for colorectal cancer resection are reduced when diagnostic colonoscopies are performed by general surgeons. 2013. *Can J Surg* 56 (4 Suppl 3): S137.

6. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. 2012. Implementation of an acute care surgery service does not affect wait-times for elective cancer surgeries: an institutional experience. *Can J Surg* 55(4 Suppl 2): S88-S89.

7. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. 2011. Impact of an acute surgical care team on the management of general surgical emergencies: an institutional experience. *Can J Surg* 54 (Suppl): S63-S64.

8. **Anantha RV**, Jain V, Vogt KN, Crawford S, Parry N, Leslie K. 2011. The Implementation of an Acute Care Emergency Surgical Service: What Does It Cost Surgeons? *Can J Surg* 54 (Suppl): S69.

Submitted Manuscripts:

1. **Anantha RV**, Jegatheswaran J*, Pepe DL*, Delport J, Haeryfar SMM, McCormick JK, Mele T. Staphylococcus aureus bloodstream infections among intravenous drug users in Southwestern Ontario: a five-year retrospective cohort study. *Submitted to the Canadian Journal of Infectious Diseases and Medical Microbiology*.

2. **Anantha RV**, Salvadori M, Hussein M, Merritt N. Clinical Image: Abdominal Cocoon Syndrome Caused by *Mycobacterium bovis* from Unpasteurized Cow's Milk. *Submitted to Lancet Infectious Diseases.*

3. **Anantha RV**, Mazzuca D, Xu S, Porcelli S, Fraser D, Martin C, Welch I, Mele T, Haeryfar SM, McCormick J. Th2-polarized invariant natural killer T cells reduce disease severity in intra-abdominal sepsis. *Submitted to Infection and Immunity.*

4. **Anantha RV**, Jegatheswaran J*, Pepe DL*, Delport J, Haeryfar SMM, McCormick JK, Mele T. Risk factors for mortality among Canadian patients with Staphylococcus aureus bacteremia: a retrospective cohort study. *Submitted to CMAJ Open.* *- equal contribution.

Manuscripts In Preparation:

1. **Anantha RV**, Bottoni DA, Fortin F, Inculet RI, Malthaner RA. 2013. Closed CO_2VATS is a Viable Alternative to Thoracotomy to Resect Pulmonary Metastases: Nine Years of Experience at a Single Institution. *Submitted to the European Journal of Cardiothoracic Surgery.*

2. **Anantha RV***, Choi J*, Bottoni DA, Fortin F, Inculet RI, Malthaner RA. 2013. Closed CO_2VATS is a Viable Alternative to Thoracotomy to Resect Lung Cancer. *In preparation for submission to the Annals of Thoracic Surgery.* *- equal contribution

PRESENTATIONS

National and International Meetings:

1. Jegatheswaran J, **Anantha RV**, Pepe DL, Delport J, McCormick JK, Mele T. Infective Endocarditis Among Intravenous Drug Users with *Staphylococcus aureus* Bacteremia. Poster presentation at the 2014 American College of Physicians Annual Meeting, Orlando, FL **(Finalist)**.

2. Pepe DL, **Anantha RV**, Jegatheswaran J, Delport J, McCormick JK, Mele T. Recurrent prosthetic valve endocarditis following valve replacement and aortic root reconstruction for infective endocarditis. Podium presentation at the 2014 American College of Physicians Annual Meeting, Orlando, FL **(Finalist)**.

3. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Mitigation of splenocyte apoptosis by Th2-polarized invariant natural killer T cells reduces disease severity in intra-abdominal sepsis. Podium presentation at the 2014 Surgical Infection Society Annual Meeting, Baltimore, MD.

4. **Anantha RV**, Jegatheswaran J, Pepe DL, Delport J, McCormick JK, Mele T. Predictors of Mortality Among Intravenous Drug Users with *Staphylococcus aureus* Bacteremia. Podium presentation at the 2014 Surgical Infection Society Annual Meeting, Baltimore, MD.

5. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. Poster presentation at the 2013 American College of Surgeons Clinical Congress, Washington, D.C.

6. **Anantha RV**, Parry N, Leslie K. An Acute Care Surgery Service Facilitates the Timely Treatment of Emergency Colorectal Cancer Patients. Poster presentation at the Canadian Surgical Forum, Ottawa, ON.

7. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. Podium presentation at the Canadian Surgical Forum, Ottawa, ON.

8. Rieder S, **Anantha RV**, Leslie K. From colonoscopy to colectomy: wait-times for colorectal cancer resection are reduced when diagnostic colonoscopies are performed by general surgeons. Podium presentation at the Canadian Surgical Forum, London, ON.

9. **Anantha RV**, Bottoni D, Fortin, D, Inculet, R, Malthaner, R. Closed CO_2VATS is a Viable Alternative to Thoracotomy to Resect Pulmonary Metastases: Nine Years of Experience at a Single Institution. Poster presentation at the Canadian Association of Thoracic Surgeons Meeting, Ottawa, ON.

10. Choi J, **Anantha RV**, Bottoni D, Fortin, D, Inculet, R, Malthaner, R. A completely closed VATS technique appears to be superior to thoracotomy for the resection of primary lung cancers: a single-institution experience. Poster presentation at the Canadian Association of Thoracic Surgeons Meeting, Ottawa, ON.

11. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Th2-polarized invariant natural killer T cells reduce disease severity in intra-abdominal sepsis. Poster presentation at the 2013 Clinician Investigator Trainee Association of Canada/Canadian Society for Clinical Investigation Annual Meeting, Ottawa, ON (**Second Place Winner**).

12. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. Podium presentation at the 2013 Clinician Investigator Training Association of Canada Young Investigators Forum, Ottawa, ON.

13. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. Evaluation of Elective Cancer Surgery Wait-times Following the Implementation of an Acute Care Service at a Tertiary-Care Centre. Poster at the Canadian Surgical Forum: TAC/ACS Canadian Resident Papers Competition, Calgary, Alberta, September 2012.

14. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. Implementation of an acute care surgery service does not affect wait-times for elective cancer surgeries: an institutional experience. Poster at the Canadian Surgical Forum, Calgary, Alberta, September 2012.

15. **Anantha RV**, Jain V, Vogt KN, Crawford S, Parry N, Leslie K. The Implementation of an Acute Care Emergency Surgical Service: What Does It Cost Surgeons? Poster presentation at the Canadian Surgery Forum, London, Ontario, September 2011 **(Winner)**.

16. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. Assessment of the Impact of an Acute Surgical Care Service in London Ontario. Podium presentation at the Canadian Surgical Forum: TAC/ACS Canadian Resident Papers Competition, London, Ontario, May 2011.

Local and Regional Meetings:

1. **Anantha RV**, Jegatheswaran J, Pepe DL, Delport J, McCormick JK, Mele T. Predictors of Mortality Among Intravenous Drug Users with *Staphylococcus aureus* Bacteremia. Podium presentation at the 2014 Western Research Forum, Western University, London, Ontario **(Winner)**.

2. Jegatheswaran J, **Anantha RV**, Pepe DL, Delport J, McCormick JK, Mele T. Infective Endocarditis Among Intravenous Drug Users with *Staphylococcus aureus* Bacteremia. Poster presentation at the 2014 London Health Research Day, Western University, London, Ontario.

3. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Mitigation of splenocyte apoptosis by Th2-polarized invariant natural killer T cells reduces disease severity in intra-abdominal sepsis. Poster presentation at the 2014 London Health Research Day, Western University, London, Ontario.

4. Pepe DL, **Anantha RV**, Jegatheswaran J, Delport J, McCormick JK, Mele T. Assessment of Catheter-Related Staphylococcus aureus bloodstream infections in a Canadian cohort. Poster presentation at the 2014 London Health Research Day, Western University, London, Ontario.

5. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Th2-polarized invariant natural killer T cells reduce disease severity in intra-abdominal sepsis. Poster presentation at the 2013 Infection and Immunity Research Forum, Western University, London, Ontario.

6. **Anantha RV**, Jegatheswaran J, Pepe DL, Delport J, McCormick JK, Mele T. Predictors of Mortality Among Intravenous Drug Users with *Staphylococcus aureus* Bacteremia. Poster presentation at the 2013 Infection and Immunity Research Forum, Western University, London, Ontario (**Second Place Winner**).

7. Jegatheswaran J, **Anantha RV**, Jegatheswaran J, Pepe DL, Delport J, McCormick JK, Mele T. Infective Endocarditis among Patients with *Staphylococcus aureus* Bacteremia: A Tale of Two

Populations. Poster presentation at the 2013 Infection and Immunity Research Forum, Western University, London, Ontario.

8. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. Poster presentation at the Annual Robert Zhong Department of Surgery Research Day, London, ON.

9. **Anantha RV**, Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Polarizing invariant natural killer T cells towards a Th2 phenotype reduces disease severity in intra-abdominal sepsis. Oral presentation at the General Surgery Residents Research Day, Division of General Surgery, London, ON.

10. Choi J, **Anantha RV**, Bottoni D, Fortin, D, Inculet, R, Malthaner, R. COVATS in the treatment of lung cancers compared to conventional thoracotomy. Oral presentation at the General Surgery Residents Research Day, Division of General Surgery, London, ON.

11. Rieder S, **Anantha RV**, Leslie K. From colonoscopy to colectomy: wait-times for colorectal cancer resection are reduced when diagnostic colonoscopies are performed by general surgeons. Oral presentation at the General Surgery Residents Research Day, Division of General Surgery, London, ON.

12. Howe B, Ott M, **Anantha RV**. Chest Imaging in Colorectal Cancer. Oral presentation at the General Surgery Residents Research Day, Division of General Surgery, London, ON

13. **Anantha RV,** Mazzuca D, Xu SX, Shrum B, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. The Role of Invariant Natural Killer T Cells in Intra-abdominal Sepsis. Poster at the London Health Research Day, Western University, London, Ontario, March 2013.

14. **Anantha RV,** Mazzuca D, Mele T, Fraser D, Martin C, Haeryfar SM, McCormick J. Elevated Frequency of invariant Natural Killer T Cells in Septic Patients. Podium presentation at the Infection and Immunity Research Forum, Western University, London, Ontario, November 2012 **(Winner)**.

15. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. Evaluation of Elective General Surgery Wait-times Following the Implementation of an Acute Care Service at a Tertiary-Care Centre. Podium presentation at the General Surgery Residents Research Day, Division of General Surgery, London, Ontario, May 2011 **(Winner)**.

16. **Anantha RV**, Vogt KN, Crawford S, Parry N, Leslie K. Distribution of General Surgery Cases Before and After the Implementation of ACCESS at Victoria Hospital. Podium presentation at the General Surgery Residents Research Day, Division of General Surgery, London, Canada, May 2011.

17. Perron LBC, **Anantha RV**, Nagpal S, Brandys T, Goeree R. Comparison of Open Repair and Fenestrated Endografting in the Treatment of Type IV Thoracoabdominal Aortic Aneurysms— A Systematic Review. Poster presentation at the Collins Surgical Research Day, The Civic Hospital, Ottawa, Ontario, April 2010 **(Honourable Mention)**.

18. **Anantha RV**, Huang A, Wehbe H, Nokhbeh R, Dimock K. Knockdown of sialyltransferase ST3Gal4 reduces susceptibility to Enterovirus 70 in human corneal epithelial cells. Podium presentation at the Summer Research Fellows meeting, University of Ottawa, Ottawa, Ontario, October 2007 **(Winner)**.

19. **Anantha RV**, Nokhbeh R, Dimock K. Detection of α-2,3 Sialyltransferases in different cell lines by RT-PCR. Podium presentation at the Research Fellows meeting, Ontario Genomics Institute, Toronto, Ontario, August 2005.

SCHOLARLY AND PROFESSIONAL ACTIVITIES

Professional Memberships:

2013-present	**Resident Member**, American Physician Scientists Association
2013-2015	**Member**, Canadian Institutes for Health Research
2013-2015	**Resident Member**, Canadian Society for Clinical Investigation
2013-2014	**Resident Member**, Clinician Investigator Trainee Association of Canada
2012-2014	**Student Member**, Canadian Society of Immunology
2012-2014	**Student Member**, American Society of Microbiology
2010-present	**Resident Member**, American College of Surgeons
2010-present	**Resident Member**, Royal College of Physicians and Surgeons of Canada
2010-present	**Resident Member**, College of Physicians and Surgeons of Ontario
2006-present	**Resident Member**, Canadian Medical Association
2006-present	**Resident Member**, Ontario Medical Association
2006-2010	**Student Member**, Canadian Federation of Medical Students

Journal Reviewer:

2014	**Ad-hoc Journal Reviewer**, *Canadian Journal of Surgery*
2014	**Ad-hoc Journal Reviewer,** *Journal of Medical Microbiology Case Reports*
2013	**Ad-hoc Journal Reviewer,** *Pathogens and Disease*
2013	**Ad-hoc Journal Reviewer,** *Journal of Medical Microbiology*
2013	**Ad-hoc Journal Reviewer,** *African Journal of Microbiology Research*

RESEARCH

Research Interests:

Acute care surgery (ACS) services, comprised of dedicated teams that provide around-the-clock coverage for general surgery emergencies are emerging as an effective model for the delivery of emergency surgical care. I am interested in optimizing clinical outcomes for emergency general surgery patients, from the development and refinement of acute care surgery (ACS) services, to the evaluation and management of these patients using clinical and basic science approaches. In particular, I am interested in applying immunological techniques (flow cytometry, multiplex cytokine analysis) to provide diagnostic and prognostic clarity for patients presenting with intra-abdominal sepsis (IAS), and to develop immunomodulatory therapies to complement current treatments for IAS.

Basic Science Research:

2013 **Analysis of immune determinants involved in liver hypertrophy following Associated Liver Partition Prior to Staged Hepatectomy (ALPPS) procedures**
Dr. Mansour Haeryfar, Dr. Jeremy Parfitt, Dr. Ian Welch, Dr. Roberto Hernandez-Alejandro, Division of General Surgery and Department of Microbiology and Immunology, Western University, London, Ontario

2013 **Assessment of circulating tumour cells in colorectal cancer with liver metastases**
Dr. Barbara Fisher, Dr. Gavin Beck, Dr. Kristopher P. Croome, Dr. Stephen Welch, Dr. Douglas Quan, Dr. Bertha Garcia, Dr. Roberto Hernandez-Alejandro, Division of General Surgery, Division of Surgical Oncology, Western University, London, Ontario

2012-present **Analysis of invariant Natural Killer T cell populations in human omentum**
Dr. John McCormick, Dr. Mansour Haeryfar, Dr. Tina Mele, Dr. Ken Leslie, Division of General Surgery and Department of Microbiology and Immunology, Western University, London, Ontario

2005-2007 **Targeting Enterovirus 70 susceptibility in human corneal epithelial cells by modulation of sialyltransferase ST3GAL4 activity**
Dr. Ken Dimock, Department of Microbiology, University of Ottawa, Ottawa, Ontario

2004 **Synthesis of a linker between a second-generation Grubbs' catalyst and a polystyrene bead**
Dr. William Ogilvie, Department of Chemistry, University of Ottawa, Ottawa, Ontario

2003 **Abiotic formation of methylmercury in wetland and aquatic ecosystems**
Dr. David Lean, Department of Biology, University of Ottawa, Ottawa, Ontario

2002 **Methylation of mercury by organic matter in aquatic ecosystems**
Dr. Susannah Scott, Department of Chemistry, University of Ottawa, Ottawa, Ontario

Clinical Research:

2012-present **Risk factors associated with mortality in *Staphylococcus aureus* bacteremia**
Januvi Jegatheswaran, Daniel L. Pepe, Dr. Johan Delport, Dr. John McCormick and Dr. Tina Mele, Department of Surgery and Department of Microbiology and Immunology, Western University, London, Ontario

2012-present **From colonoscopy to colectomy: assessment of time differences among general surgeons and gastroenterologists in the operative management of patients with colorectal cancer in Southwestern Ontario**
Dr. Scott Rieder and Dr. Ken Leslie, London Health Sciences Centre, London, Ontario

2012 **Analysis of the epidemiology of Non Group A, Group B Streptococci (NABS) in a tertiary-care centre in Southern Ontario**
Dr. John McCormick, Dr. Johan Delport, Department of Microbiology and Immunology, Western University, London, Ontario

2011-2012 **Assessment of surgical wait-times following implementation of an acute surgical care service in London, Ontario**
Dr. Kelly Vogt, Dr. Neil Parry and Dr. Ken Leslie, London Health Sciences Centre, London, Ontario

2012-present **Comparison of Carbon Dioxide Video-Assisted Thoracoscopic Surgery (COVATS) and thoracotomy in the resection of pulmonary metastases**
Dr. Richard Malthaner, London Health Sciences Centre, London, Ontario

2011-present **Assessment of a Carbon Dioxide Video-Assisted Thoracoscopic Surgery (COVATS) technique in resection of lung cancer**
Dr. James Choi, Dr. David Bottoni and Dr. Richard Malthaner, London Health Sciences Centre, London, Ontario

2010-2011 **Evaluation of Clinical and Economic Outcomes of An Acute Surgical Care Service in London, Ontario**
Dr. Kelly Vogt, Dr. Neil Parry and Dr. Ken Leslie, London Health Sciences Centre, London, Ontario

2009-2010 **Comparison of open repair and fenestrated endografting in the treatment of Type IV thoracoabdominal aortic aneurysms**
Dr. Lygia Perron, Dr. Sudhir Nagpal and Dr. Tim Brandys, The Ottawa Hospital, Ottawa, Ontario

2008-2010 **Cost comparison of open surgical repair versus endovascular graft repair of juxtarenal aortic aneurysms**
Dr. Lygia Perron, Dr. Sudhir Nagpal, The Ottawa Hospital, Ottawa, Ontario

Students and Residents Supervised for Research:

2013-2014	Januvi Jegatheswaran (BSc, MD Candidate): Assessment of Risk Factors Contributing to Mortality in Patients with *Staphylococcus aureus* Bacteremia and Infective Endocarditis.
2013-2014	Daniel Pepe (BSc, MD Candidate): Predictors of Mortality in Patients with *Staphylococcus aureus* Bacteremia.
2012-2014	James Choi (MD): Comparison of COVATS and Conventional Thoracotomy for Resection of Lung Cancer at the London Health Sciences Centre.
2012-2014	Scott Rieder (MD): Evaluation of Wait-times for Surgical Resection of Colorectal Cancer Following Diagnostic Colonoscopy at the London Health Sciences Centre.

GRANT SUPPORT

Current Funding Support:

Academic Medical Organization of Southwestern Ontario (AMOSO) Innovation Fund (INN14-006), May 1, 2014 – April 30, 2016. Evidence-based medicine for the enhanced management of complicated *Staphylococcus aureus* infections: Can we do better? Co-investigator, $97 400.

Pending Funding Support:

Department of Surgery (Western University, London, Ontario) Internal Research Fund, July 1, 2014 to June 30, 2016. Analysis of immune determinants involved in liver hypertrophy following Associated Liver Partition Prior to Staged Hepatectomy (ALPPS). Co-investigator, $20 000 (*pending*).

PATENTS AND PATENT APPLICATIONS

1. **Anantha RV**, McCormick JK, Haeryfar SMM. 2014. "Modulation of invariant Natural Killer T Cells in the Treatment of Sepsis" U.S. Provisional Patent No. 61/978,467

ACADEMIC ACTIVITIES

Teaching:

2013	**Instructor**, Clinical Skills Methods for Clinical Clerks, Schulich School of Medicine and Dentistry, Western University
2012-2014	**Instructor**, Advanced Trauma Life Support Course, Western University
2010-present	**Resident Teacher for Clinical Clerks**, Division of General Surgery, Western University

Invited Lectures:

2013 "Why is Immunology Important in Medicine?" Invited talk for first-year medical students, Schulich School of Medicine and Dentistry, Western University

Committees:

2013 **Attendee**, Western Research Symposium, Western University
2012-2013 **Resident Member**, Resident Training Committee (General Surgery), Western University
2008-2009 **Class Representative**, Clerkship Committee, Faculty of Medicine, University of Ottawa
2008 **Secretary**, ORBIS Vision 2020 East Africa Conference, Dar Es Salaam, Tanzania

Leadership:

2010-present **Attendee**, General Surgery Journal Club, Western University, London, Ontario
2013 **Resident Judge**, Medical Student Poster Sessions, American College of Surgeons Clinical Congress, Washington D.C.
2008 **Participant**, Canadian Medical Association Leadership Training Workshop, Ottawa
2007-2008 **Organizer**, Mentorship Group, Faculty of Medicine, University of Ottawa, Ottawa
2007-2008 **Coordinator**, Seminars on Manuscripts Revolutionizing Therapies, Faculty of Medicine
2011 **Instructor Candidate,** Advanced Trauma Life Support Course, University of Western Ontario, London, Ontario

COMMUNITY ACTIVITIES

2007 **Performer**, Faculty of Medicine MedShow, University of Ottawa
2003-2008 **Guitar performer**, Ottawa
2002-2005 **English and Math tutor**, Kumon Education Centre, Ottawa
Volunteered as a tutor in English and Math for underprivileged children in a south Ottawa neighborhood