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Exploiting the immunomodulatory potentials of iNKT cells in sepsis and cancer.

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

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Abstract

Invariant natural killer T (iNKT) cells are a unique unconventional T cell subset that recognize glycolipids presented by CD1d expressing cells. The prototypical glycolipid agonist of iNKT cells, α -Galactosylceramide (α -GalCer), can induce the rapid release of an arsenal of cytotoxic effector molecules and enormous amounts of immunomodulatory cytokines as early as two hours after activation. In addition to α -GalCer, various glycolipid agonists are available that allow for specific, *in vivo* targeting of iNKT cells, and can exert divergent T-helper (T_H)1 and/or T_H2 immune responses. Therefore, the type of response instigated by iNKT cells can profoundly influence the nature of downstream immune response pathways. Sepsis and cancer are two distinct, detrimental pathologies where dysregulated immune responses play a key role in the pathogenesis and disease progression. The extent to which iNKT cells contribute to the pathology of sepsis and cancer has not been fully explored. Furthermore, whether iNKT cells can be targeted by glycolipid immunotherapy to mitigate disease progression has yet to be fully elucidated. In this thesis, the immunomodulatory capacity of iNKT cells were manipulated to skew the host immune response towards a protective phenotype. Firstly, using the surgical cecal ligation and puncture model on C57BL/6 (B6) mice to induce polymicrobial sepsis, iNKT cells were activated with a two-pronged glycolipid immunotherapy. I found that glycolipid treatment conferred significant improvements in sepsis morbidity and mortality. Moreover, glycolipid treatments induced an alteration in the cytokine milieu, restored immunocompetence and NK cell cytotoxicity in septic survivors when compared to

vehicle treated controls. Secondly, I discovered a tumoricidal population known as, precursors to mature NK (pre-mNK) cells, that robustly expanded in the liver of naïve B6 mice, upon α -GalCer injection. Notably, *in situ* expansion of resident hepatic pre-mNK cells was found to be dependent on IL-12 and IL-18 signaling. Moreover, α -GalCer-expanded pre-mNK cells were found to mediate cytotoxicity via the granzyme/perforin pathway and significantly contributed to the anti-metastatic activity of NK cells *in vivo*. Collectively, the findings reported in this thesis show novel mechanisms by which glycolipid therapies can exploit the immunomodulatory potentials of iNKT cells to ameliorate immunopathologies in sepsis and cancer.

Keywords

Sepsis, cytokine storm, cecal ligation and puncture, immunosuppression, cancer, metastasis, invariant natural killer T cells, precursors to mature NK cells, α -GalCer, immunotherapy.

Summary for Lay Audience

Invariant natural killer T (iNKT) cells are a special group of immune cells that recognize sugar coated fat compounds known as glycolipids. The most commonly used glycolipid to study iNKT cells is α -Galactosylceramide (α -GalCer). Upon activation with α -GalCer, iNKT cells rapidly produce numerous small proteins that can have significant impact on shaping the course of the overall immune response. Although sepsis and cancer are two distinct diseases, they are both affected by a severe dysfunctional immune response that can exacerbate disease progression. The functional role of iNKT cells in sepsis and cancer is not fully known. Considering the dysfunctional immune response invariably found in sepsis and cancer, whether iNKT cells can be targeted by glycolipids, like α -GalCer, to steer the overall immune response towards normalcy is unknown. In this thesis, glycolipids were used to target iNKT cells in live mice to improve disease outcomes in sepsis and cancer. First, using a surgical mouse model of sepsis, I found that glycolipid treated mice had greater survival than untreated mice. In addition, I found that glycolipid activated iNKT cells were able to steer the immune response to produce vastly different proteins in the blood and restore immune cell function in septic mice. Second, using a different mouse model, I discovered another population known as precursors to mature NK (pre-mNK) cells that vastly increased in number in the liver when injected with α -GalCer. This expansion was a result of cell division of pre-existing pre-mNK cells in the liver instead of newly recruited pre-mNK cells. I uncovered that pre-mNK cell expansion was dependent on two soluble proteins, IL-12 and IL-18, which was caused by α -GalCer

injection. In terms of function, pre-mNK cells were found to efficiently kill cancer cells and contribute to the overall anti-cancer response in live mice. Taken together, these findings reveal new ways glycolipids can be used to target iNKT cells to impact the immune response to improve outcomes in sepsis and cancer.

Co-Authorship Statement

The investigations reported in this thesis were predominantly executed by Joshua Choi under the supervision and guidance of Dr. S. M. Mansour Haeryfar. Information regarding the relative contributions of the authors are detailed below:

Chapter 3: Choi J, Mele TS, Porcelli SA, Savage PB, Haeryfar SMM. Harnessing the versatility of iNKT cells in a novel step-wise approach to sepsis immunotherapy. Submitted to *The Journal of Immunology*. Manuscript # 20-00220-FL

Choi J, contributed to the designing and execution of the experiments, in addition to the analysis and interpretation of the data. Mele TS provided creative and intellectual contributions. Savage PB and Porcelli SA, provided intellectual input and material support. Haeryfar SMM, conceived the project, secured funding for the project, provided guidance, supervised all aspects of the study, and wrote the manuscript.

Chapter 4: Choi J, Rudak PT, Lesage S, Haeryfar SMM. 2019. Glycolipid stimulation of iNKT cells expands a unique population of pre-mNK cells endowed with oncolytic and anti-metastatic properties. *The Journal of Immunology*. 203(7): 1808-19

Choi J, helped conceive the project, designed, and executed experimental work, analyzed, and interpreted the data and wrote the first draft of the manuscript. Rudak PT, contributed in performing experiments. Lesage S, provided creative and intellectual contributions. Haeryfar SMM, conceived the project, secured the funding for the project, supervised all aspects of the study, and wrote the final version of the manuscript.

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“Some trust in chariots, and some in horse, but we will trust in the name of the Lord our God.” – Ps 20:7

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List of Abbreviations

Ags	antigens
aPC	activated protein C
APCs	antigen presenting cells
BCL-2	B cell lymphoma 2
BFA	brefeldin A
CLP	cecal ligation and puncture
CMA	concanamycin A
CMV	cytomegalovirus
CTLA-4	cytotoxic T-lymphocyte antigen-4
DCs	dendritic cells
DN	double negative
DTH	delayed-type hypersensitivity
FasL	fas ligand
FDA	food and drug administration
FIP	feces-induced peritonitis

G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
GZM(s)	granzyme(s)
HLA	human leukocyte antigen
HSV	herpes simplex virus
IDO	indoleamine 2,3-dioxygenase
IFN- γ	interferon gamma
iGb3	isoglobotrihexosylceramide
IKDCs	IFN-producing killer dendritic cells
iNK	intermediate NK
iNKT	invariant natural killer T
ITAM	tyrosine-based activation motif
LPS	lipopolysaccharide
MDSCs	myeloid derived suppressor cells
MHC	major histocompatibility complex
mNK	mature NK

MSS	mature NK
NKT	natural killer T
ONPG	ortho-nitrophenyl- β -D-galactopyranoside
PD-1	programmed cell death-1
pDC	plasmacytoid DC
pre-mNK	precursors to mature NK
S1PR1	sphingosine-1-phosphate receptor 1
SIRS	systemic inflammatory response syndrome
SOFA	sequential [Sepsis-related] Organ Failure Assessment
SP	single positive
TCR	T cell receptor
T _H	T-helper
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells
α -GalCer	α -galactosylceramide

β 2M β 2 microglobulin

γ c common γ chain

Preface

The following thesis describes my PhD journey in exploring the immunomodulatory prowess of invariant natural killer T (iNKT) cells when appropriately targeted with their glycolipid agonists. In truth, I started out my PhD with an outlined plan to exclusively study iNKT cells in sepsis. However, within a year into my studies, I serendipitously discovered a secondary phenotype in one of my experiments purposed for optimization. It was this latter discovery that threw me deep into the rabbit hole of cancer immunology. Over the next few years, two distinct projects emerged, and the following thesis describes my pursuit in applying the steps of scientific inquiry in delineating the unknown. At first glance, the two projects, namely sepsis and cancer, may seem to be in disjunction with each other. However, the emphasis of this dissertation is neither sepsis nor cancer, but the mechanism of therapeutic intervention. The common thread that flows throughout is the targeting of iNKT cells via their glycolipid agonists to galvanize the host immune response to respond to two distinct and devastating pathologies.

This dissertation describes detailed methodology on using surgical, wildtype, and transgenic mouse models to determine the truth. Flow cytometry and many *in vitro* based assays were extensively used for endpoint analysis. The following may be of interest for researchers that are inclined to study sepsis, tumor immunology, and other immune based pathologies.

Chapter 1

1. Introduction

1.1 Sepsis

The immune response to infection is a robust network of finely tuned interactions with the unifying goal of controlling disease. Typically, many infectious diseases caused by pathogens, are a result of successful evasion and subversion of the immune response. In contrast, sepsis is a life-threatening disease where the immune response is not only directly involved, but the problem. Although sepsis etiology is invariably initiated by an infection, whether it be bacterial, fungal or viral, the underlying pathology is caused by an overwhelming dysregulated systemic host response to the infection that may lead to multiple organ failure and in many cases death. The sheer magnitude of the toll on human health is strikingly clear as sepsis kills more people worldwide than prostate cancer, breast cancer and AIDS combined (1). Despite major advancements in healthcare and medical technology, sepsis remains the leading cause of death in critically ill patients, with mortality rates of 25-50%, and alarmingly, the incidence rates have risen 71% in the United States, from 2003-2007, with a concurrent increase in healthcare cost of 57% (2-5). The conspicuous absence of a cure for sepsis however, is not due to a lack of effort from researchers by any means; in the last three decades over 35 clinical trials have been conducted without avail. This is due to sundry reasons; the traditional definition of sepsis has low sensitivity in catching severe septic events which may have translated into inconsistencies in sampling during the clinical trials. Additionally, sepsis was historically categorized as predominately a hyperinflammatory syndrome. This dogma had spurred the majority of the clinical trials to treat with some type of therapy to block inflammation. It is now appreciated that sepsis is a biphasic response with an initial hyperinflammatory phase immediately followed by immunosuppression (6, 7).

Understanding the pathogenesis of sepsis and its causative role on immune impairment is a challenging task. Despite decades of research, the exact mechanisms underlying the immunopathogenesis of sepsis which can lead to organ failure and death remain elusive. Although sepsis is still an area of intense research, there is no mechanism-based drug available with current treatment options limited to antibiotics and largely supportive care. Currently, sepsis immunopathology is now recognized as a biphasic syndrome with a distinct protracted immunosuppressive phenotype, however its pathogenesis is multifactorial which adds to the complexity of the disease. Moreover, a major limitation in human studies is the high degree of difficulty to address mechanism-based questions. Mouse models of sepsis are typically used to elucidate the undergirding mechanisms but, these models have a high degree of variability between them, which may result in inconsistent findings between models. Nevertheless, growing evidence suggests that therapies that target to boost the immune response may be a promising approach to treat sepsis.

1.1.1 Sepsis Definitions

Historically, a patient was diagnosed with sepsis when they exhibited symptoms of systemic inflammatory response syndrome (SIRS) in the presence of infection. The diagnosis of SIRS requires at least 2 of the following: tachycardia ($\geq 90/\text{min}$), hypothermia ($\leq 36^\circ\text{C}$) or hyperthermia ($\geq 38^\circ\text{C}$), tachypnea (≥ 20 breathes/min) or $\text{PaCO}_2 \leq 32$ mm Hg, and leukocytosis ($\geq 12\,000/\mu\text{L}$) or leukopenia ($\leq 4000/\mu\text{L}$). Patients were considered to have “severe sepsis” when their condition was complicated by hypotension (< 90 mm Hg) and/or organ failure. Finally, a severe septic patient was further classified to have “septic shock”

when, in their hypotensive state, became unresponsive to fluid resuscitation. These criteria have been largely unchanged since 1991, but researchers have criticized these definitions to be misleading, not comprehensive of the complexity of sepsis pathobiology, and resulted in inconsistency for epidemiologic studies and clinical trials (8-10). In 2016, a consensus panel of experts have revised the definition of sepsis for the first time in 25 years (8). The definition of sepsis has now been revised to include organ dysfunction in the presence of infection. Keeping the clinical scenario and early disease management at the forefront of discussion, a new bedside clinical score to determine organ failure was established. Early organ dysfunction was characterized with a Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score of 2 points or more (Table 1). The criteria for septic shock was also revised to be a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone. This new definition will allow for faster and more accurate diagnosis of sepsis, while providing more rigorous patient inclusion criteria for future clinical trials.

Table 1: Sequential [Sepsis-related] Organ Failure Assessment

System	Score				
	0	1	2	3	4
Respiration					
PaO ₂ /FIO ₂ , mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, ×10 ³ /μL	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular					
	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose) ^b	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1 ^b	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1 ^b
Central nervous system					
Glasgow Coma Scale score ^c	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL (μmol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
Urine output, mL/d				<500	<200

Abbreviations: FIO₂, fraction of inspired oxygen; MAP, mean arterial pressure; PaO₂, partial pressure of oxygen.

^a Adapted from Vincent et al.²⁷

^b Catecholamine doses are given as μg/kg/min for at least 1 hour.

^c Glasgow Coma Scale scores range from 3-15; higher score indicates better neurological function.

Adopted from Singer et al. (8)

1.1.2 Protracted sepsis and immunosuppression

Early deaths due to sepsis are typically due to the initial hyperinflammatory phase that overwhelms the patient leading to cardiovascular collapse, metabolic abnormalities and multiple organ failures. Fortunately, accessibility to modern medical healthcare, especially in developed countries, have improved patient prognosis which has led to decreasing mortality rates in the USA (3). However, these patients that survive the initial inflammatory insult invariably succumb to the protracted immunosuppressive phase of sepsis. In fact, more than 60% of deaths due to sepsis occur 7-17 days after admission during the protracted phase (11). The protracted sepsis pathology can manifest in a multitude of ways including: persistent inflammation and catabolism (12), immune dysfunction (13), decrease in human leukocyte antigen (HLA)-DR expression on circulating peripheral

monocytes(14), and increased susceptibility to nosocomial infections (6). Of note, HLA-DR expression on peripheral monocytes has been widely used as a surrogate biomarker indicative of systemic immunosuppression and is a strong predictor of nosocomial infections (15) and mortality (16) in septic patients. Another piece of evidence for sepsis-induced immunosuppression is the reactivation of otherwise latent viruses, cytomegalovirus (CMV) (17) and herpes simplex virus (HSV) (18) in septic and critically ill patients . Taken together, these manifestations suggest a culmination of diverse pathways that ultimately cause a functionally impaired immune response even after clearance of the initial infection.

Although there are several cellular mechanisms that undergird sepsis-induced immunosuppression, it is unclear whether these are acting independently or co-dependently with each other. Two main pathways that have been implicated are regulatory immune cells and apoptosis (19). Previous studies have highlighted the potential role that regulatory subsets, such as T regulatory cells (Tregs) (20) and myeloid derived suppressor cells (MDSCs) (21) may have in sepsis. Perhaps as expected, Tregs were found to expanded in septic mice and contribute to long term immune dysfunction. Depletion of Tregs during sepsis increased T cell proliferation, reduced bacterial burden, as well as improved survival to a secondary bacterial challenge (20). Similarly, MDSCs were found to dramatically expanded in polymicrobial sepsis, suppressed T cells, and contributed to a T-helper (T_H)₂ polarizing immune response. Alternatively, a significant phenotype associated with both early and protracted sepsis pathology is the drastic apoptotic depletion of both immune (13, 22)and gastric parenchymal cells (23). Previous investigations found that T and B cells of transgenic mice that overexpressed the anti-apoptotic protein, B cell lymphoma 2 (BCL-

2), exhibited complete resistance to apoptosis during sepsis leading to improved overall survival (24). Furthermore, BCL-2 overexpression in dendritic cells were also conferred a survival advantage and led to a reversal of an immunosuppressive phenotype in an endotoxic shock model of sepsis (25).

Two main mechanisms by which apoptosis in sepsis can cause immunosuppression have been delineated. The first mechanism is the direct effect of apoptosis which leads to the severe loss of immune cells and antigen presenting cells (26). Notably, autopsies of septic patients, immediately performed after death, revealed a marked depletion of B cells, CD4 T cells, and follicular dendritic cells in the spleen compared to control patients (27). The loss of these subsets would have a profound impact on both the adaptive, and innate arms of immunity. The loss of CD4 T cells and B cells would significantly perturb both cell-mediated and humoral immune responses which would be critical in clearing persistent infections as well as protecting against potential nosocomial infections. Moreover, the loss of follicular dendritic cells may also dramatically contribute to sepsis-induced immunosuppression as there would be fewer antigen presenting cells (APCs) to activate the adaptive immune response. The second mechanism by which apoptosis attributes to the impairment of the immune response during protracted sepsis is through the induction of anergy and the polarization towards a T_H2 response (26). Specifically, the engulfment of apoptotic bodies by dendritic cells and macrophages fails to induce the upregulation of co-stimulatory molecules (28). Consequently, T cells that come into contact with these APCs may become anergized or even undergo apoptosis themselves (28). Furthermore, engulfment of these apoptotic bodies by APCs has a secondary effect of inducing the production of anti-inflammatory cytokines such as IL-10 and TGF- β (29). Not only are

these anti-inflammatory cytokines a strong predictor for mortality in sepsis (30), they can contribute to the induction of tolerance (31), further exacerbating an immunosuppressive and dysfunctional immune response. Whether apoptosis is the only mediator of immunosuppression during protracted sepsis is unclear, many research efforts have been focused on targeting this phenomenon for potential therapies.

1.1.3 **Lasting impairments in sepsis survivors**

Sepsis is a highly complex, intricately pathologic with a myriad of systemic responses that can act in concert and/or in opposition to lead to hypotension and organ failure in response to infection. Although, sepsis is now largely characterized as biphasic with acute inflammatory syndrome which is invariably followed by a compensatory immunosuppressive phase, there is mounting evidence that the physiological implications of sepsis-induced immunosuppression reach much further than a patient's stay in the ICU (32). Iwashyna *et al.* reported, in a long-term study, a whopping 119% increase in sepsis survivors in the elderly; however, they found within 3 years of the septic event, 75% of the survivors exhibited long term morbidity such as, functional disabilities, and moderate to severe cognitive impairment (33, 34). Additionally, symptoms of depression have been shown to be a major risk for both septic survivors (35) and their spouses (36). Sepsis-induced immunosuppression has also been linked with a "global" depression of cytokine production and severe depletion of immune effector cells in all age groups (37, 38). Researchers are now attributing sepsis-induced immunosuppression to be a key factor in contributing to the long-term morbidity associated with septic survivors (32). Although the precise mechanism underlying long-term sepsis morbidity and mortality remain uncertain,

it is becoming clear that long-term implications of sepsis should be considered as an additional phase of sepsis pathology when developing potential therapeutic interventions.

1.1.4 **Clinical management and prospective immunotherapies**

The historical track record of failed clinical trials notwithstanding, tremendous strides have been made in elucidating the important factors for managing sepsis in the clinic (39). Unfortunately, there is yet to be a cure for sepsis and current treatments in the ICU are largely supportive care. The prevailing strategy for emergency management in the clinic is early, goal-directed therapy, which involves rapid diagnosis (within 6 hours) and immediate deployment of “resuscitation bundles” which are tailored to address cardiorespiratory issues during early sepsis (40). Following the initiation of early, goal-directed therapy, lung-supportive ventilation is also supplied (39). During this time, blood cultures are obtained, and broad-spectrum antibiotics are administered intravenously for infection source control(39).

Other slightly controversial treatment strategies that can be considered are the administration of activated protein C (aPC) and corticosteroids. Activated protein C works as an anticoagulant which has had some mixed success in clinical trials; treatments were shown to be effective and decrease mortality rates by 13% in patients at high risk of death (41). In contrast, aPC conferred no beneficial outcomes in severe sepsis patients with low death risk (42). Corticosteroid treatments may also be considered as a treatment for septic patients in need of critical care, however similar to aPC, their effectiveness when tested in clinical trials have proven to be capricious, highlighting the need to perhaps focus research efforts on the dose, timing and duration of corticosteroid administration (39).

With the advancement of our understanding of the immunopathology of sepsis as a syndrome that is initiated by infection but exacerbated by a dysregulated host immune response, immunotherapies have taken the forefront of prospective candidates for novel treatments. Considering the drastic depletion of lymphocyte populations in septic patients, recombinant IL-7 has emerged as an attractive potential therapeutic intervention (43, 44). Alternatively, with the recent success of checkpoint inhibitors to enable the immune response to overcome a hyporesponsive environment in various cancers (45, 46), it is curious to see whether this type of intervention would be effective in treating sepsis. Interestingly, blockade of inhibitory markers, programmed cell death protein (PD)-1 and PD-L1, were found to improve survival in mice induced with polymicrobial sepsis (47). Clinical trials are underway of other potential therapies such as, administration of recombinant interferon gamma (IFN- γ), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), to address the profound defects observed in septic patients in their monocyte and neutrophil, and macrophage populations, respectively (19). Sepsis is a life-threatening syndrome that is incredibly complex and finding a cure has proven to be profoundly difficult. Due the diverse range of etiologies that are linked with sepsis, it is unlikely that any one specific therapy will be the cure. Instead, a combinational approach which includes intensive supportive care, source control, and rigorous immunotherapies to target the invariable immunosuppressive nature of sepsis, may indeed provide the best chance of success in managing this devastating disease.

1.1.5 Mouse Models of Sepsis

Mouse models are widely used as preclinical systems for studying and testing novel therapies in sepsis. Moreover, mouse models have been essential in discovering novel biomarkers as both diagnostic and prognostic indicators. There are several sepsis models, each with their own distinct strengths and limitations, and researchers have taken advantage of these systems to successfully test a plethora of interventions and therapies to improve survival outcomes (48). Despite the relative success of these studies, many of these findings failed to effectively translate to the clinic, highlighting the complexity of clinical sepsis and the paramount importance that these preclinical findings need to be validated in human systems as well. Nevertheless, mouse models are still an invaluable tool in elucidating the underlying mechanisms of sepsis pathology.

The differences between mouse sepsis models can be described to follow 1 of 3 variations: i) systemic administration of exogenous toxins, such as lipopolysaccharide (LPS); ii) systemic administration of pathogens or feces; and iii) surgical models that disrupts the endogenous host barrier. LPS is the most commonly used endotoxin to recapitulate septic shock (49, 50). LPS is an integral endotoxin found in Gram-negative bacteria and rapidly provokes a systemic inflammatory response in a Toll-like receptor (TLR)4-dependent fashion, and thus is appropriately named, endotoxic shock. This model is fast, simple, and highly reproducible, however there are several caveats. First, this model lacks an infectious component which is an essential part of sepsis. Second, although this model induces rapid production of pro-inflammatory cytokines, it is absent of any sustained or prolonged pathology such as increasing levels of cytokines or immunosuppression, as observed in the

clinic (51). While it is true that LPS-challenged mice exhibit increased IL-10 production by macrophages (52) and Tregs (53), this fails to translate to global immunosuppression (54). Lastly, mice are endotoxin resistant, whereas humans are highly sensitive (48). Suffice to say, conclusions drawn from endotoxic shock models need to be taken with a healthy dose of skepticism when translating these findings to clinical sepsis. Administration of systemic pathogens or feces is another model which is also simple and highly reproducible. Systemic injection of individual bacterial pathogens can be done either i.v. to induce bacteremia (55), or i.p. to induce peritonitis (56). These models have an infectious component adding another layer of complexity which more closely resembles clinical sepsis. However, single bacterium models fail to represent the microbial diversity that is often seen in polymicrobial sepsis. Furthermore, depending on the strain and species of the bacterial strain, a high degree of variability in the immunopathology can be observed (51). Many of these limitations are addressed in the Feces-Induced Peritonitis (FIP) model. The FIP procedure consists of collecting the contents of the cecum from isogenic donor mice to produce purified cecal slurry, which can be kept at -80°C for longer term storage (57). The cecal slurry is injected i.p. into recipients to induce polymicrobial peritonitis and causes a rapid systemic inflammatory response. The advantage of this model is that there is minimal variability between recipients, as all mice receive a determined amount of pooled slurry. Moreover, the pathology and phenotype of recipients are consistent with acute sepsis, and a scoring system allows for standardized monitoring (58, 59). The disadvantages are few but important; FIP is still highly artificial as a large bolus of slurry is injected at one time rather than prolonged exposure over time. In addition, the FIP model induces an acute sepsis immunopathology and the protracted immunosuppressive phase

has not been characterized; therefore, whether mice that receive FIP exhibit the biphasic sepsis is still yet to be determined. Finally, surgical models that disrupt the host barrier to causes fecal leakage into the peritoneum are an attractive alternative that addresses many of the aforementioned limitations observed in other models. Of these surgical models, the cecal ligation and puncture (CLP) is the most routinely used and will be discussed further below.

1.1.5.1 **Cecal Ligation and Puncture**

The cecal ligation and puncture model is a surgical procedure which effectively recapitulates the clinical manifestations of polymicrobial peritonitis (48). Amongst the various sepsis mouse models, CLP is considered the gold standard as it is the only model where the subject exhibits both an acute hyperinflammatory response followed by immunosuppression (48). The CLP procedure consists of ligation of the distal region of the cecum, followed by the perforation of the ligated portion, providing a constant source of fecal bacteria leaking into the peritoneum. The length of the ligation, number of perforations, and the gauge of the needle, all affect the severity of the septic insult. As a result, variability is inherent in this model as slight changes made by the operator, albeit unintentional, may affect the prognosis of the mouse.

This model is ideal in addressing the protracted phase of sepsis and at the same time, delineates the confounding effects contributed by the acute inflammatory response that is responsible for early mortality. Immunosuppression in CLP has been well characterized and is typified by an impaired Delayed-Type Hypersensitivity (DTH) response (47). The DTH response is a classical measurement of the adaptive immune response to a contact

happen. CLP addresses many of limitations of different models previously outlined and is considered by researchers to be an essential preclinical test for any potential new therapeutics in human sepsis (60). Specifically, CLP induced pathology is mediated by an infectious component that is not limited to a singular species. Moreover, due to the slow leakage of feces it provides a distinct and sustained host response that can be categorized, and it is the only model to date that clearly exhibits an immunosuppressive phase following sepsis. As such, researchers have described CLP to be one of the most clinically relevant models available to study sepsis (61, 62).

1.2 Immunopathogenesis of cancer

Significant strides have been made in the perennial fight against cancer and in the concerted effort in finding a cure. Despite this, cancer is still one of the leading causes of death worldwide (63, 64) and incidences rates are projected to climb even higher by 2030 (65). Weinberg and Hanahan first described, twenty years ago, the underlying pathogenesis of cancer and codified them into 6 hallmarks: persistent growth signals, evasion of apoptosis, insensitivity to anti-growth signals, unlimited replicative potential, angiogenesis and tissue invasion/metastasis (66). Numerous efforts have been employed to target these oncogenic events with varied success. Although these hallmarks describe the invariable stages of tumorigenesis of all cancer types, the mechanisms by which these outcomes are achieved are highly diverse and thus difficult to target (67). The incredible heterogeneity that exists within different cancers provide a spectrum of morphological and physiological phenotypes (68). The corollary to this is the formation of unique tumor microenvironments which select for increased diversity within gene expression, morphology, metabolism and

metastatic potential (69, 70). Ultimately the culmination of these factors work to establish a tumor ‘friendly’ niche by subverting, evading, and overwhelming the immune response.

Since the original definition of the hallmarks of cancer, we have come to appreciate the essential role of the immune response, or more specifically, the evasion of the immune response by the tumor, as another bona fide hallmark of cancer pathogenesis (66) . Of note, this section will discuss several of the key mechanisms that cancers employ in evading the immune response and the various immunotherapies and mouse models that researchers have utilized to test and target these perturbations of immune surveillance by cancer cells. However, this is not meant to be an exhaustive description, but rather, a brief overview of the content that is relevant within the scope of this thesis.

1.2.1 **Evasion of the immune response by tumors**

The immune response, classically categorized as innate and adaptive responses that comprise the two arms of immunity, is well equipped to recognize and combat virtually a limitless pool of antigens and/or epitopes that can be harbored by foreign invaders or neoplastic formations. However, cancer cells have a vast number of strategies to avoid detection from the immune response. These strategies can be loosely categorized under exogenous and/or endogenous pathways. Exogenous pathways would describe mechanisms where cancer cells influence other cell types to facilitate tolerance and evasion. Conversely, endogenous pathways would describe various mechanisms by which cancer cells prevent their own destruction and induce tolerance through the direct interaction with immune effector cells.

Tumors can exercise exogenous pathways to suppress the immune response thereby creating a milieu that effectively causes the effector cells to be functionally inert. This is largely mediated by the recruitment of Tregs (71) to the tumor microenvironment or through the induction of peripheral CD4⁺ T cells into Tregs through the production of tumor-derived TGF- β (72). In either case, Tregs produce high levels of anti-inflammatory cytokine IL-10 which inhibit the function and proliferation of cytotoxic effector cells. Another cell population that is often recruited by tumors are MDSCs which act to suppress CD8⁺ T cells as well as promote angiogenesis (73).

Tumors utilize a number of different endogenous pathways to induce tolerance or promote evasion of the immune response. For example, tumors can express the enzyme Indoleamine 2,3-dioxygenase (IDO), which acts to directly suppress local effector immune cells (74). Another critical process of immunosurveillance evasion is immunoediting by tumor cells, where surviving colonies are selected to modify their surface proteins, ultimately decreasing its immunogenicity, thereby promoting escape (75). Additionally, tumor cells have the ability to “switch off” cytotoxic T and NK cells by targeting the intrinsic checkpoints molecules that immune cells possess. The most notable of these checkpoints have undoubtedly been the discovery of PD-1 (76) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (77). Incidentally, these discoveries have garnered the 2018 Nobel Prize in Physiology or Medicine (78) and are the focus of a tremendous concerted research effort for various cancer immunotherapies.

1.2.2 **Immunotherapies in cancer**

Among the varied treatment regimen for cancer which include chemotherapy, radiation therapy, and surgical procedures, immunotherapies have been the focus of many new developments for cancer treatments. Consequently, a wide range of cancer immunotherapies have already been approved by the Food and Drug Administration (FDA), and these can be described as, but are not limited to, the following: i) cytokine therapies (79, 80) ii) cancer vaccines (81, 82) and iii) monoclonal antibody-based therapies (83).

Current research efforts have largely been focused on targeting checkpoint molecules, such as PD-1 and CTLA-4, using monoclonal based-therapies. Defined as checkpoint blockade/inhibitors, these monoclonal therapies have found success in treating against melanoma (84, 85) , and non-small cell lung cancers (86). These discoveries have led to alternative co-inhibitory receptor such as, Tim-3, Lag-3, and TIGIT to be investigated as prospective candidates for monoclonal-based checkpoint blockades (87). Moreover, the success of checkpoint blockade therapies have spurred on the search for novel co-inhibitory targets to be treated in combination with or without current treatment protocols (88).

1.2.3 **Mouse models of cancer**

The use of mouse models to study cancer have proved to be an invaluable tool in determining the various factors that are involved in oncogenesis to cancer progression. There is a plethora of models with varying oncogenic events that researchers can choose from, each with its own strengths and weaknesses (89). For instance, ectopic or orthotopic

injection of syn or xeno-grafts can be performed for the study of tumor growth. These collective models are highly reproducible and can recapitulate the tumor microenvironment, however, are limited due to their divergent histological phenotypes compared to primary cancers (90). For inquiries into the physiology of metastasis, intravenous injection of a cancer cell line is routinely used by many, such as the prototypical B16 metastatic melanoma model (91). Metastatic models, although widely employed, fail to capture the effect of tumor heterogeneity that significantly contributes to evasion of the immune response (89). Transgenic or carcinogen-induced tumor models alternatively, recapitulate tumor heterogeneity and have predictive power for the development of human cancers (89). They are also useful for the study of spontaneous formation of cancer (75). Transgenic and carcinogen-induced tumor models however, are highly time and labour intensive, and require large sample sizes. Therefore, researchers can choose between a range of diverse mouse models with the understanding that any single model does not provide a complete translational corollary with human cancer. Importantly, mouse models also provide the opportunity for drug discovery in an *in vivo* setting. Moreover, the effect of a given drug on specific signaling pathways and molecular targets in relationship with its anti-tumor efficacy can be readily defined and delineated (89). As such, the ability of these models to eloquently answer these questions is the prerequisite to making it into the human clinical trials and eventually the bedside.

1.3 Invariant Natural Killer T cells

Natural Killer T (NKT) cells are a subpopulation of T lymphocytes that have the phenotypic properties of both T and NK cells, that recognize lipids and glycolipids instead

of peptides. Similar to conventional T cells, they are selected for a functional $\alpha\beta$ T cell receptor (TCR) during thymic development, and can be further classified into single positive (SP) $CD4^+CD8^-$ or $CD4^-CD8^+$ or double negative (DN) $CD4^-CD8^-$ subtypes (92). They constitutively express NK cell surface markers, such as NK1.1 and DX5 in mice or CD161 in humans. NKT cells distinguish themselves further from conventional T cells with their exception to the rule of classical major histocompatibility complex “(MHC) restriction.” NKT cells recognize glycolipids in the context of CD1d, a conserved MHC class I-related glycoprotein with a deep hydrophobic antigen-binding groove that allow lipids to be presented (93).

NKT cells can be further characterized by their TCR repertoire. A subset of NKT cells express a unique α chain rearrangement ($V\alpha14-J\alpha18$ and $V\alpha24-J\alpha18$ in mice and humans, respectively) paired with a limited number of β chains ($V\beta8.2$, $V\beta2$ or $V\beta7$ and $V\beta11$ in mice and humans respectively) (92). Due to their distinct TCR, these cells have been aptly named as invariant natural killer T (iNKT) cells. A more varied subset of NKT cells includes variant NKT cells that have a more diverse $TCR\alpha\beta$ repertoire and have been relatively poorly studied and will not be discussed in this thesis.

The activation of iNKT cells can occur either in a TCR-dependent or -independent manner. There are a few but important distinctions between TCR-dependent activation of iNKT cells and conventional T cells. Although, conventional effector T cells are essential and effective in controlling infection, they are limited by their long, arduous process of activation, which requires TCR and MHC engagement (signal 1) and costimulatory molecule interactions (signal 2). After priming, an effective immune response still requires clonal expansion and migration into the peripheries which can take as long as 10 days (94).

iNKT cells however, circumvent this process by leaving the thymus in a partially activated state before antigen encounter (95). This allows them to be activated and respond as early as 2 hours after infection (96). Alternatively, iNKT cells can be activated independent of CD1d engagement by multiple mechanisms. Our lab has previously reported that group II bacterial superantigens induced iNKT cell activation by crosslinking specific iNKT cell V β TCR chains with MHC II (97). Another mechanism, that arguably may be more prevalent, is activation via IL-12 and IL-18 signaling (98). Typically, IL-12 and IL-18 are secreted by dendritic cells and other APCs during infection, upon engagement of their TLRs. Interestingly, Leite-De-Moraes *et al.* showed that IL-12 and IL-18-mediated activation of iNKT cells induced a 10-fold higher cytokine response compared to TCR cross-linking (98). In either case, upon activation iNKT cells secrete copious amounts of cytokines, notably IFN- γ and/or IL-4. In addition, they have been shown to produce IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF α , TGF β and GM-CSF (96, 99). Furthermore, since iNKT cells are activated so early, they have significant influence on the downstream activation on other immune effector cells including, dendritic cells, macrophages, neutrophils, NK cells and lymphocytes (96). In addition to cytokines, iNKT cells boast an impressive arsenal of cytotoxic effector molecules such as perforin, granzymes, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (100, 101). For this reason, iNKT cells have been referred to as “the swiss army knife of the immune system (96).”

1.3.1 **iNKT cells in sepsis**

The role of iNKT cells in the context of sepsis immunopathology is not completely understood. However, there is evidence to suggest that iNKT activation during the

hyperinflammatory phase may exacerbate the disease leading to increased mortality. This was apparent when $J\alpha 18^{-/-}$ mice, which lack iNKT cells, had reduced mortality due to sepsis as well as decreased levels of inflammatory cytokines compared to wild-type mice (102). This and other studies strongly indicated that iNKT cells had a pathogenic role, at least in acute sepsis (58). However, it was recently revealed that the $J\alpha 18^{-/-}$ mice did not only lack iNKT cells but had a ~60% reduction in their TCR α repertoire, calling into question the validity of the previous findings that used this transgenic model (103). Nonetheless, there is still evidence that suggests a pathogenic role for iNKT in sepsis. Mice that were given anti-CD1d blocking antibody prior to sepsis had increased survival compared to isotype, however an important caveat is that this antibody neutralizes all NKT cells and not exclusively the *invariant* subtype (104). Therefore, whether iNKT cells are having synergistic or antagonistic effects with different NKT subtypes during sepsis, remains to be delineated. Our laboratory had investigated the therapeutic application of iNKT glycolipid agonists to ameliorate sepsis pathology in mice. Mice that were given the T_H2-skewing glycolipid OCH shortly after FIP were found to have enhanced survival when compared to vehicle treated mice (58). However, the FIP model effectively recapitulates the acute inflammatory pathology of sepsis, not the protracted immunosuppressive phase. Although the protective effects of T_H2 polarization by other subsets, such as MDSCs, in sepsis is already known (21), the extent to which T_H2-skewing of iNKT cells via OCH activation mitigates mortality and sepsis-induced immunosuppression has never been explored. With advancements in our understanding of human sepsis as a biphasic disease, it is necessary to use a sepsis model that encapsulates both phases in order to test effective immunotherapies that can be translatable to the clinic.

1.3.2 Glycolipid agonists of iNKT cells

iNKT cells can recognize a range of exogenous microbially-derived glycolipid agonists (105). An important question however, is whether iNKT cells are thymically selected by exogenous agonists or if there exists an endogenous ligand that is essential in the development and maintenance of this population. The unaltered presence of iNKT cells in germ-free mice suggests the importance of the latter (106), although recent reports suggest that the gut microbiota is important for iNKT cell function (107, 108). Zhou *et al.* first described a lysosomal glycosphingolipid with previously unknown function, isoglobotrihexosylceramide (iGb3), as an endogenous iNKT cell ligand (109). Mice with impaired generation of iGb3 resulted in a severe deficiency of iNKT cells thus lending credence to the critical function of endogenous glycolipids in iNKT cell development (109). A caveat to this is that humans lack iGb3 synthase and therefore cannot endogenously produce iGb3 (110). Interestingly, putative endogenous iNKT cell ligands in humans were recently identified by Kain *et al.* as α -linked glycosylceramides, however whether human iNKT cell development is solely dependent on such ligands is yet to be determined (111). Nevertheless, for the study of iNKT cells, α -Galactosylceramide (α -GalCer), which was initially isolated from an extract of a marine sponge species during a screening for novel anti-cancer therapeutics, has been most extensively employed for its ability to potently induce a robust response (112). The immunomodulatory properties of iNKT cells can be potentiated with the use of commercially synthesized homologs of α -GalCer which is known to induce a robust T_H1/T_H2 response (113). α -GalCer is composed of a galactose head covalently attached to sphingosine and fatty acyl chains (Fig. 1.1). Modifications to the sphingosine and/or fatty acyl chain lengths have been shown to be

alter the affinity of the iNKT cell TCR to CD1d as well as the threshold of activation (114). For instance, OCH, a truncated analog of α -GalCer, has been shown to skew iNKT cells towards a T_H2 response (115). Moreover, alternative glycolipids with varying acyl chain lengths, such as C20:2, PBS-25 and PBS-128 have also been reported to have polarize iNKT cells towards a T_H2 bias, and be protective against type 1 diabetes in mice (116, 117) (Fig. 1.1). Importantly, due to the impressively conserved homology of the CD1d molecule, α -GalCer presented by CD1d is recognized by both mice and humans, which has implications for the translation from mice to human studies (118). More recently, numerous microbial agonists that directly activate iNKT cells, via CD1d presentation, have been identified in *Sphingomas* species, *Borrelia burgdorferi*, and *Streptococcus pneumoniae* (93, 119). Indeed, these T_H1/T_H2 skewing glycolipids provide vital tools for manipulating iNKT cells which in turn can shape the course and nature of the downstream immune response (Fig. 1.2). Several studies have reported the benefits of this type of immunotherapy in autoimmune disease, cancer and allergy (115, 120, 121).

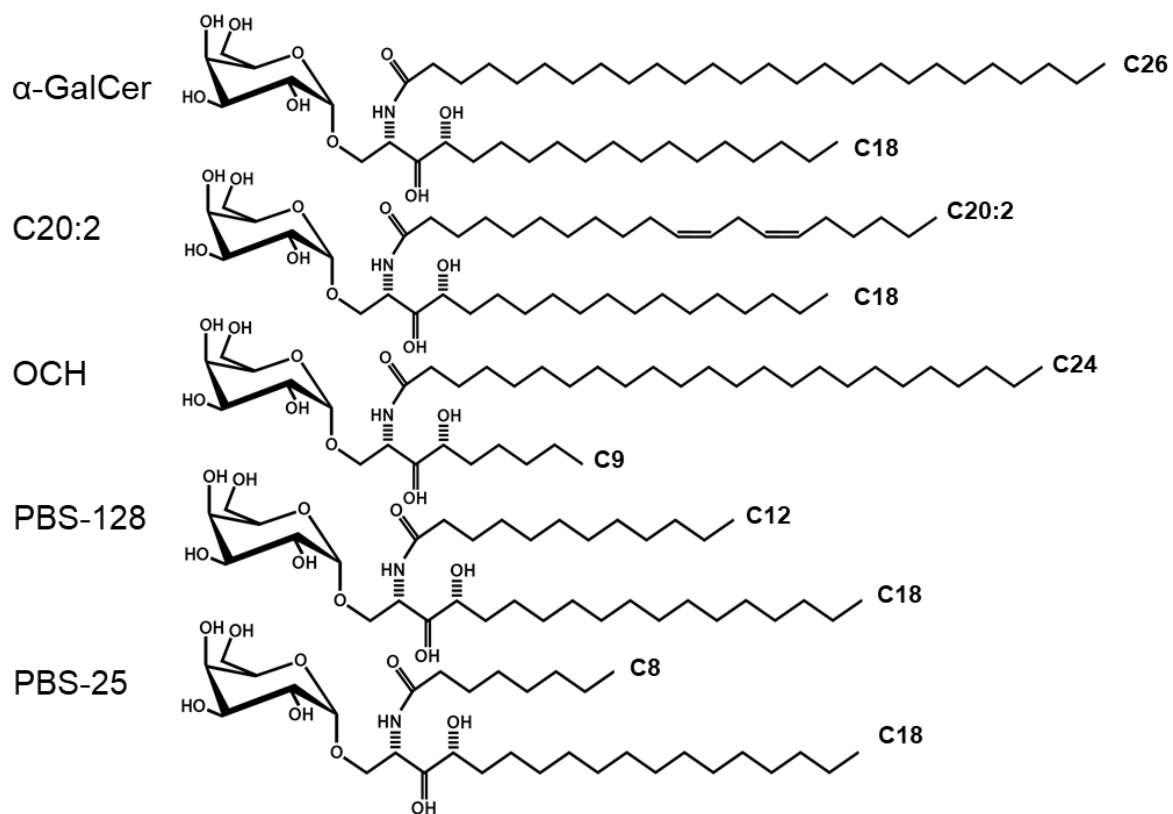


Figure 1.1: Structural depiction of various *i*NKT cell glycolipid agonists.

Different *i*NKT cell glycolipid agonists, that are used as potential therapeutic interventions in chapters 3 and 4, are depicted. Specific alterations to the sphingosine and acyl chains have been shown to polarize *i*NKT cells towards a T_H2 bias.

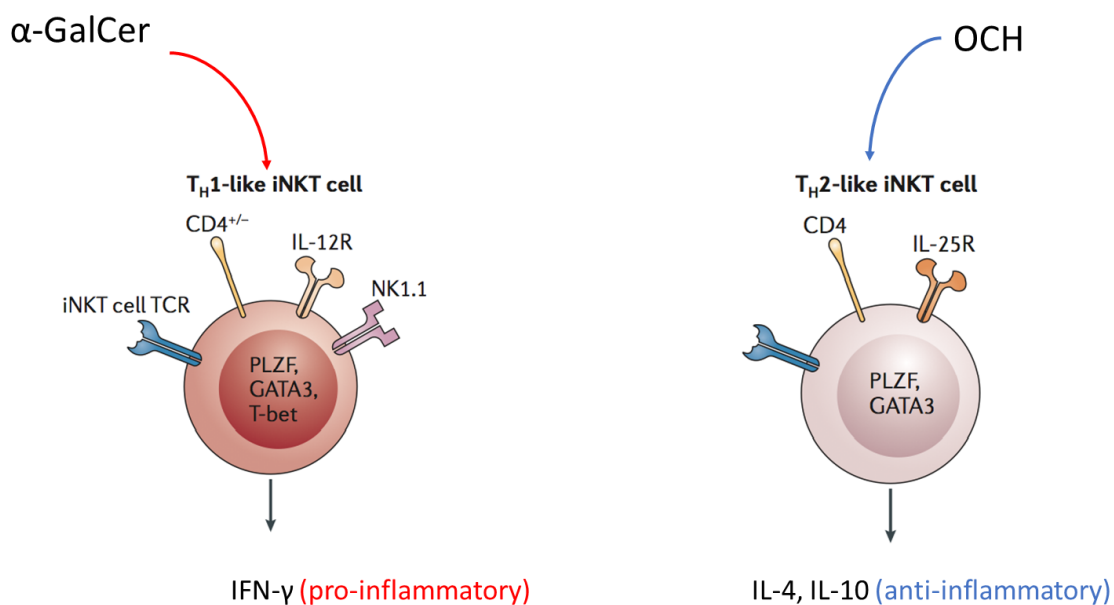


Figure 1.2: The immunomodulatory potentials of iNKT cells.

α -GalCer or OCH glycolipid agonists produce divergent responses by iNKT cells polarizing them towards either a T_H1 or T_H2 like phenotype, respectively.

1.3.3 iNKT cells in cancer

Due to their “pre-activated state” and their ability to readily produce copious amounts of pro- and/or anti-inflammatory cytokines (122), iNKT cells have been shown to mobilize immune responses against viral (123) and/or bacterial invaders (124, 125), and arguably most notably against cancer (126). Mouse iNKT cells express the canonical $V\alpha14$ - $J\alpha18$ rearrangement in their TCR α chain, which is coupled with one of a limited number of $V\beta$ chains. In contrast with their conventional T cell counterparts, their specific TCR repertoire limits iNKT cell reactivity to select agonists which allows targeting for immunotherapies to be more precise. In mice, α -GalCer has been used to promote the anti-tumor activity of

iNKT cells against pancreatic cancer (127), liver metastasis of melanoma (128) and thymoma (129) cells. In humans, iNKT cells have also been the target of immunotherapy in clinical studies against prostate (130) and colorectal carcinomas (131) as well as in several clinical trials for multiple solid tumors (126, 132-135). Moreover, the mobilization of downstream effector cells via iNKT cell activation is arguably one of the more profound impacts of α -GalCer-mediated anti-tumor immunity (126). Several studies have already highlighted how iNKT cell activation results in the recruitment and transactivation of CD8⁺ T cells(136) , NK cells (137-139) , and $\gamma\delta$ T cells(140) in various *in vivo* cancer models.

1.4 Precursor to mature NK cells

Precursors to mature NK (pre-mNK) cells are typified by a unique B220⁺ NK1.1⁺ CD11c⁺ phenotype that shares functional similarities with mature NK (mNK) and conventional dendritic cells (DCs). Interestingly, pre-mNK cells were initially defined as interferon-producing killer dendritic cells (IKDCs), ostensibly due to their capacity to migrate to lymph nodes to present antigens, in addition to producing IFN- γ and exhibiting tumoricidal activity *in vivo* (141, 142). However, subsequent studies provided new evidence to suggest that IKDCs indeed belonged to the NK cell lineage due to their strict dependence on the Id-2 transcription factor and IL-15 signaling for their development (143-145). Furthermore, pre-mNK cells were also determined to be upstream of the mNK cells and did not simply bear a unique phenotype of an activated mNK population (146). Specifically, Guitmont-Desrocher *et al.* showed upon *in vivo* activation with either anti-CD40 or poly I:C, mNK cells did not upregulate the unique phenotypic markers precluded to pre-mNK cells; on the other hand, pre-mNK cells exhibited a propensity to acquire a mNK cell phenotype upon

adoptive transfer (146). These findings suggest that pre-mNK cells lie upstream in the NK cell developmental pathway with little to no chance of reversion.

There is a paucity of research on the fundamental developmental processes of pre-mNK cells and thus, many questions remain to be answered. For instance, whether mature NK cell populations arise strictly from pre-mNK pools or whether there are independent, mutually exclusive pathways that give rise to mNK cells is unclear. The various stages of NK cell development have been previously outlined into 4 stages of progressive maturation and found to be associated with the differential expression of 2 key surface markers, CD27 and CD11b (147). Stage 1 comprises of CD27^{low}CD11b^{low}, stage 2 is CD27^{high}CD11b^{low}, stage 3 is CD27^{high}CD11b^{high}, and stage 4 is CD27^{low}CD11b^{high}. Each stage corresponds with changes in the cytokine production and cytotoxic ability of NK cells. A previous study has suggested that an intermediate NK (iNK) pool are responsible for producing the stage 1 population of NK cells and that pre-mNK cells differentiate directly into the stage 2 population (146). However, whether there are functional discrepancies of mNK cells derived from pre-mNK cells or from conventional iNK pools remain unclear, and whether these pathways intersect or are mutually exclusive are unknown.

Another key distinction from conventional mNK cells is the capacity of pre-mNK cells to present antigens to both CD4 and CD8 T cells (141). Furthermore, pre-mNK cells can be licensed by tumor cells following killing to cross-present antigens to neighbouring T cells, thereby engaging tumor-specific cytotoxic T cells in the host immune response (148, 149). Collectively, these findings show the diverse range of effector functions of pre-mNK cells, combining the features of conventional NK and DCs which provide a link between the innate and adaptive arms of immunity.

There are several outstanding questions regarding pre-mNK cell biology which will be briefly discussed however not exhaustively. The unique expression of B220 on pre-mNK cells and not mNK cells is arguably the most distinguishing phenotypic feature between the populations. Notably, mice deficient for B220 had higher numbers of NK cells (150), but exhibited an impairment in cytokine production in immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors (151). These results suggest that B220 expression has deeper implications than solely a biomarker in distinguishing pre-mNK and NK cell populations, however further investigations are required to elucidate the nature of the relationship between B220 and pre-mNK cells and its potential contribution on NK cell function. In addition, pre-mNK cells are present in relatively low abundance compared to mNK cells and have, to our knowledge, been exclusively isolated and/or studied in either the bone marrow or spleen; thus, the relative distribution of pre-mNK cells among other organs and whether these cells actively participate during a systemic immune response remain unexplored. Although extensive research is required to elucidate these outstanding questions, the most important contribution of pre-mNK cells and indeed the reason behind their serendipitous discovery is their role in cancer.

1.4.1 **Pre-mNK cells in cancer**

Following the initial discovery of pre-mNK cells, controversy over its lineage ensued which caused the research focus to shift towards this point of contention. However, prior to the controversy over its lineage, pre-mNK cells were discovered for their prolific cytotoxicity against B16-F10 melanoma cells (142). Indeed, the combination immunotherapy of imatinib mesylate and IL-2 potentiated their tumoricidal activity against

metastatic melanoma, leading to prominent influxes of pre-mNK cells into tumor beds and subsequent tumor clearance (142). Notably, pre-mNK cell cytotoxic activity was found to be mediated by TRAIL in a NKG2D-dependent manner (142). This phenomenon was not limited to melanoma but was subsequently observed in the context of mouse thymoma and lung cancers (152). TRAIL-mediated killing was a surprising finding since NK cell cytotoxic activity is largely mediated by the perforin/granzyme pathway, however this was evidence that perhaps pre-mNK cells utilizes alternate pathways to exert its tumoricidal activity. Indeed, when compared to mNK cells, pre-mNK cells exhibited a similar cytotoxic capacity but produced more IFN- γ and TNF- α (143). Some evidence for the underlying mechanism for increased cytokine production suggested NKG2D signaling; since various tumor cell lines that expressed NKG2D ligands induced production of IFN- γ from pre-mNK cells and treatment with blocking anti-NKG2D mAb attenuated cytokine production (152). It is important to note that IFN- γ production can indirectly promote tumor apoptosis as well. Specifically, IFN- γ signaling induces the upregulation of CD95 (Fas) on tumor cells (153) as well as sensitize cancers to TNF- α mediated apoptosis (154).

IL-15 is another key cytokine that is critical for effective pre-mNK cell function. IL-15 receptor is comprised of the IL-15R α subunit and share IL-2R β and the common γ chain receptor to make a heterotrimer. Interestingly, pre-mNK cells do not express IL15R α , but express the β and γ receptor subunits, which confers the ability to receive IL-15 signaling through trans-presentation by neighbouring bone marrow derived cells (155). The essential nature of IL-15 for pre-mNK cells is illustrated in studies where mice deficient for IL-15 resulted in functional inert pre-mNK cells (156), whereas exogenous injection of recombinant IL-15 in mice potentiated their anti-tumor activity (157).

To discuss the role of pre-mNK cells in cancer without addressing their antigen presenting ability would provide an incomplete picture of their total contribution to the anti-tumor response. A specific subset of CD11b⁺ pre-mNK cells are able to process tumor antigens after killing and cross-present these to cognate CD8 T cells (148). Moreover, pre-mNK cells migrate from the tumor beds into the draining lymph nodes while upregulating MHC II expression and costimulatory molecules (158). The significance of cross-presentation was displayed in the finding that adoptive transfer of pre-mNK cells were protective in immunocompetent tumor-bearing mice but not immunodeficient mice (152).

Pre-mNK cells are an incredibly dynamic population with a broad range of effector functions that provide a link between the innate and adaptive immune systems. Although they were first discovered for their prolific anti-tumor activity, a lot of the research focus has shifted from their cytolytic ability towards questions of their lineage. Their relatively low abundance in various tissues is a challenging obstacle with respect to potential therapies targeting pre-mNK cells. Nevertheless, pre-mNK cells remain an attractive candidate for therapeutic intervention if these challenges can be overcome.

1.5 References

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Chapter 2

2. Rationale and Objectives

2.1 Rationale

Invariant natural killer T (iNKT) cells are a unique population of T cells that recognize glycolipid antigens in the context of CD1d molecules instead of peptide antigens on major histocompatibility complex (MHC) molecules (1). Various glycolipid agonists are available to target iNKT cells *in vivo* to effectively modulate the immune response. They have a diverse array of functions which include exerting direct cytotoxic activity against CD1d bearing target cells through the upregulation of surface FasL expression and the production of soluble mediators such as IFN- γ , TNF- α , granzymes and perforin (2). Unlike their conventional naïve T cell counterparts, iNKT cells rapidly produce copious amounts of cytokines and chemokines as early as 2 hours after activation (3). The corollary to this is that iNKT cells possess the ability to shape the course of the ensuing immune response towards a proinflammatory or regulatory nature.

Sepsis and cancer are two distinct, devastating pathologies where a dysregulated immune response contributes to the pathogenesis and progression of the disease. Sepsis is caused from dysregulated immune response to systemic infection that may result in organ failure and death. Whereas, cancer is formed by the culmination of several factors, including evasion of the immune response, that cause host cells to become immortalized and malignant. The role of iNKT cells in the contribution of both of these diseases has not been fully elucidated. Furthermore, whether iNKT cells can be targeted by glycolipid immunotherapy to mitigate disease progression has yet to be fully explored. Therefore, I outlined 2 objectives that would delineate the contribution of iNKT cells in these pathologies, using two distinct mouse models of sepsis and cancer.

2.2 Objective 1

2.2.1 Elucidation of the efficacy of iNKT-cell targeted glycolipid immunotherapy in polymicrobial sepsis.

Sepsis is a catastrophic syndrome which is biphasic in nature. Early death is associated with the proinflammatory phase which is typified by a cytokine storm, cell apoptosis, and tissue damage (4). Survivors invariably succumb to the latter phase of immunosuppression where patients are plagued with persistent organ failure and nosocomial infections (5). Previous findings from our lab have shown that T_H2 polarizing iNKT cell glycolipid, OCH, effectively mitigated morbidity and mortality in an acute mouse model of sepsis (6). However, this mouse model did not recapitulate the immunosuppressive phase of sepsis, therefore, I investigated whether a novel phase-tailored glycolipid immunotherapy was a viable option in curbing both the proinflammatory and immunosuppressive pathologies, using the cecal ligation and puncture (CLP) sepsis model (7). Specifically, we proposed to administer OCH to dampen the early inflammatory phase of sepsis followed by α -Galactosylceramide (α -GalCer) injections during the immunosuppressive phase to bolster the immune response (Fig. 2.1). I hypothesized that this phase-tailored immunotherapy would ameliorate early morbidity and mortality associated with sepsis, as well as restore immunocompetence in survivors. In Chapter 3, I show that our proposed immunotherapy effectively circumvents iNKT cell anergy and induces a robust cytokine response that ameliorates early morbidity and overall sepsis-associated mortality. Moreover, I show that the immunotherapy alters the cytokine milieu to be more proinflammatory during protracted sepsis which is manifested in a rescuing of immunosuppression in both innate

and adaptive immune responses. Finally, I show that OCH can be substituted with select alternative T_H2 polarizing glycolipid agonists to improve overall mortality. Taken together, we show that for the first time that iNKT cells can be targeted to reduce sepsis morbidity, mortality, and reverse immunosuppression.

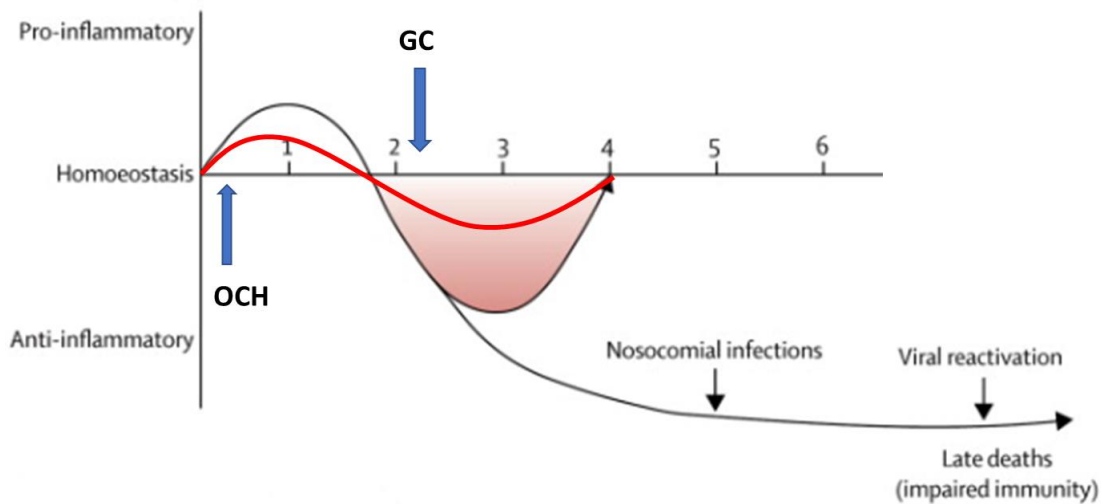


Figure 2.1: Proposed iNKT cell specific two-pronged glycolipid immunotherapy.

Schematic representation of our proposed immunotherapy. Black lines represent the progression of sepsis pathology, where the red line indicates the desired outcome from our therapeutic intervention in curbing the biphasic pathology of sepsis.

2.3 Objective 2

2.3.1 Determination of the contribution of iNKT cell activation in shaping the anti-cancer immune response.

One of the definitive hallmarks of cancer progression is the evasion of immune system that allow malignant cells to proliferate unchecked (8). As a result, there has been intensive research focus on immunotherapies that help arm the host immune response to recognize and clear cancers. Activation of iNKT cells by α -GalCer can induce the release of an impressive arsenal of cytotoxic effector molecules which has shown promise in controlling tumor growth (9). Indeed, iNKT cells can be exploited via α -GalCer to directly kill cancer cells, as well as indirectly induce anti-cancer immunity through transactivating many downstream effector cells (10). Here, we describe another unique tumoricidal population known as precursors to mature NK (pre-mNK) cells (11, 12). Whether iNKT cells can be targeted by α -GalCer to transactivate pre-mNK cells to contribute to the anti-cancer immune response is unknown and has never been shown. In Chapter 4, I show for the first time α -GalCer-mediated activation of iNKT cell induce massive pre-mNK cells expansion in several tissue compartments, but most prominently in the liver within a few days. I also determined that pre-mNK cell expansion occurs *in situ* rather than recruitment from peripheral lymphoid tissues. Using adoptive serum transfer experiments, I delineated the mechanism by which pre-mNK cells expand in the liver was dependent on IL-12 and IL-18 signaling. Finally, I investigated the function of pre-mNK cells and their contribution to anti-cancer immunity and found that they were highly cytotoxic, in both *in vitro* and *in vivo* settings, and they contributed significantly in controlling metastatic tumor burden.

Together, we have shown for the first time that the therapeutic benefits of targeting iNKT cells via α -GalCer injection, also extend towards the transactivation pre-mNK cells and involve them in contributing to the overall anti-cancer response.

2.4 References

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Chapter 3

3. Harnessing the versatility of iNKT cells in a novel step-wise approach to sepsis immunotherapy

This chapter is currently under review:

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3.1 Introduction

Sepsis is a catastrophic syndrome triggered by a dysregulated host response to infection leading to hyperinflammation and an early cytokine storm, which could culminate in vital organ failure. Although sepsis takes many lives in its early phase, improved critical care has resulted in a shift in the mortality pattern of sepsis. Accordingly, most deaths from sepsis now occur due to immunosuppression in the protracted phase of the syndrome, in which patients succumb to secondary or opportunistic infections (1-3). In addition, sepsis may elevate the long-term risk of certain malignancies (4). However, many studies to date have focused on early sepsis, and numerous clinical trials targeting antigen-presenting cells (APCs), conventional T cells, or their products (e.g., inflammatory cytokines) have failed (5, 6). There is increasing appreciation that optimal immunotherapy of sepsis requires a two-pronged approach, namely to prevent exaggerated inflammation early on while boosting immunity in the protracted phase (2, 3, 6).

Invariant natural killer T (iNKT) cells are innate-like T lymphocytes with remarkable immunomodulatory properties and therapeutic potentials in a variety of disease models and settings. They secrete a wide array of inflammatory cytokines copiously and rapidly after they detect microbe-derived or synthetic glycolipid antigens (Ags), typified by α -galactosylceramide (α -GalCer), presented by CD1d (7-10). In doing so, iNKT cells activate or regulate the functions of APCs (11, 12) and multiple downstream effector cell types belonging to both innate and adaptive arms of immunity (13-17). Importantly, iNKT cells are highly versatile entities and can be polarized to produce predominantly T helper 1

(T_H1)- or T_H2-type cytokines (18), a characteristic that has been explored in preclinical studies and exploited in clinical trials for cancer and infectious diseases.

Several studies have suggested a pathogenic role for iNKT cells in murine sepsis (6, 19-21). We reported increased frequencies of iNKT cells among circulating T cells of septic patients and also demonstrated the benefit of skewing iNKT cell responses towards a T_H2-like phenotype in a mouse model of acute intraabdominal sepsis (21). Whether iNKT cell polarization can be achieved in a septic phase-tailored fashion has not been addressed before. This is perhaps because iNKT cells undergo long-lasting anergy following exposure to α -GalCer, which renders them unresponsive to subsequent treatments with this glycolipid (22). This represents a major impediment to the success of iNKT cell-based immunotherapies for various conditions, including but not limited to sepsis. To address this limitation, we devised a two-step treatment regimen that prevented iNKT cell anergy through sequential administration of different glycolipid agonists. This approach was efficacious in a clinically relevant mouse model of biphasic sepsis in which hyperinflammation is followed by immunosuppression.

3.2 Materials and Methods

3.2.1 Mice

B6 and BALB/c mice were purchased from Charles River Canada Inc. (St. Constant, Quebec). We also maintained a breeding colony of B6 mice in our institutional barrier facility. $\beta 2M^{-/-}$ mice on a B6 background were provided by Dr. Anthony Jevnikar (Western University). Age-matched cohorts of adult male mice were used in this study. Our animal use protocol (AUPs 2010-241) was approved by the Western University Animal Use Subcommittee.

3.2.2 Glycolipids

KRN7000 from Funakoshi Co. Ltd was prepared in a vehicle containing 0.5% Tween 20, 56 mg/mL sucrose and 7.5 mg/mL histidine, heated at 80°C, aliquoted and stored at -80°C. Shortly before use, KRN7000 aliquots were thawed, reheated at 80°C for 10 minutes, and diluted in sterile PBS for injection. Lyophilized OCH was supplied by the NIH Tetramer Core Facility (Atlanta, GA), reconstituted with sterile water and stored at 4°C until use. Dry, solvent-free C20:2 was solubilized in a PBS solution containing 1% DMSO and 0.5% Tween 20 and stored at -20°C. Aliquots were thawed, sonicated at 37°C for 5 minutes, heated at 80°C for 1 minute, and then diluted in PBS for injections. Lyophilized PBS-25 and PBS-128 were formulated for direct dissolution in water and subsequent injections.

3.2.3 Polymicrobial sepsis models

In the vast majority of experiments, we employed a sublethal version of CLP with slight modifications (23). Ten-twelve-week-old male B6 mice were placed in a plastic chamber containing 2% vaporized isoflurane. Once stable, mice were transported into a makeshift surgical station where anesthesia was maintained by 1% isoflurane applied through a nose cone. The abdominal skin was disinfected using sterile gauze pads presoaked in a 2% chlorhexidine solution, wiped with 70% ethanol, and subjected to additional disinfection with 0.5% chlorhexidine. Following a midline laparotomy, the distal end of the cecum was externalized and ligated at a 0.5-cm distance from the apex. Cecum was then perforated twice using a 27-gauge needle. The peritoneal cavity and abdominal skin were closed with sutures, and 1 mL of normal saline was administered s.c. behind the ear. Mice were left to recover in separate cages under a heat lamp for 30 minutes. For sham mice, the procedure was identical except that the cecum was neither ligated nor punctured. All animals were injected s.c. with 0.5 mg/kg of buprenorphine twice, once 20 minutes before and again 24 hours after the surgery.

To induce severe CLP in separate cohorts of mice, one-third of the cecum was ligated followed by 3 perforations made with a 25-gauge needle. In a limited number of experiments, we also employed the FIP model of polymicrobial sepsis. Briefly, a slurry containing 200 mg/mL of fecal material was prepared in PBS after pooling feces from 20 age- and sex-matched donors residing in the same barrier environment. To induce FIP, mice were injected i.p. with 50 or 100 μ L of the above slurry.

Animals were closely monitored, and their morbidity was scored in a blinded manner using a murine sepsis scoring (MSS) system we previously described (24). Four, 18, 24 and 48 hours after CLP, each mouse was assigned a score of 0 to 4 for each of several criteria, including coat and eye appearance, respiration rate, consciousness level, motility, and response to various stimuli. A weight loss of $\geq 20\%$ and/or unresponsiveness to physical provocation were considered experimental endpoints.

3.2.4 *In vivo* treatments

Four μg of each glycolipid were administered i.p. in a final volume of 200 μL to naïve or septic mice as indicated. Primary and secondary glycolipid injections were separated by 4 days. Control animals received an equal volume of a corresponding vehicle solution.

In several experiments, 100 μg of a low-endotoxin, azide-free rat anti-mouse PD-1 mAb (clone RMP1-14 from Bio X Cell, West Lebanon, NH) were administered i.p. on day 4 post-priming with α -GalCer or OCH and 20 minutes before a secondary glycolipid challenge. Control mice were given 100 μg of a rat IgG2a isotype control (clone 2A3 from Bio X Cell).

Phase-tailored glycolipid immunotherapy of septic mice involved two injections. Mice received 4 μg of either OCH or PBS-25 i.p. 4 hours after CLP, which was followed by a 4- μg i.p. injection of α -GalCer on day 4 post-CLP. Control mice were given the appropriate vehicles following the above schedule, and sham controls were left untreated.

3.2.5 Serum cytokine measurements

Saphenous blood was collected from non-septic mice 2, 8 and 24 hours after a sole glycolipid injection or following a secondary challenge. Septic animals that had been subjected to CLP before receiving successive injections of OCH and α -GalCer (or corresponding vehicles) were bled 12 hours after the secondary α -GalCer (or vehicle) treatment. Sera were isolated via centrifugation at 17×1000 g for 15 minutes at 4°C, and then stored at -80°C. Serum cytokine levels were quantified using ELISA kits from ThermoFisher Scientific (Waltham, MA) or a multiplexing platform employing Luminex xMAP technology by Eve Technologies (Calgary, AB).

3.2.6 Customized gene expression analysis

Five days after CLP, glycolipid- and vehicle-treated survivors were sacrificed for their liver. Hepatic CD3⁻NK1.1⁺ cells were stained and sorted, after dead cell and doublet exclusion, by a BD FACSAria III cytometer achieving a purity of >99%. RNA was extracted using a Purelink RNA Mini Kit (ThermoFisher) and converted to cDNA using the Invitrogen SuperScript VILO MasterMix. Samples were plated in singlets and quantitative PCR was performed using Custom TaqMan Array 96-Well Fast Plates (ThermoFisher) in a StepOnePlus Real-Time PCR System. Changes in gene expression were assessed by the $\Delta\Delta$ CT method.

3.2.7 Cytofluorimetric analyses

Mice were sacrificed for their spleen and/or liver, which were mechanically homogenized in sterile PBS. Liver tissue homogenates were subsequently placed in a 33.75% Percoll

PLUS solution (GE Healthcare, Chicago, IL) and spun at $700 \times g$, without brake, for 12 minutes at room temperature to remove parenchymal cells. Brief treatment with ACK lysis buffer and filtration through 70- μm pores of a cell strainer rid the resulting splenic and HMNC preparations of erythrocytes and debris, respectively. To block Fc γ receptors before staining, cells were briefly incubated on ice with 20 μL of culture supernatant from a hybridoma producing an anti-CD16/CD32 mAb (clone 2.4G2). Staining then followed using fluorophore-conjugated mAbs to surface CD3 ϵ (clone 145-2C11), CD107a (1D4B), F4/80 (BM8), I-A/Eb (AF6-120.1), NK1.1 (PK136), and/or intracellular GZM A (GzA-3G8.5). Labeled mAbs and isotype controls were all from ThermoFisher.

Cell surface staining was carried out at 4°C for 30 minutes. For intracellular staining, an eBioscience Intracellular Fixation & Permeabilization Buffer Set was utilized. To detect cytolytic molecules present in hepatic NK cells, bulk HMNCs were co-cultured at a 30:1 ratio with YAC-1 thymoma cells in the presence of 10 $\mu\text{g}/\text{mL}$ brefeldin A (BFA) (Sigma). After 4 hours at 37°C and 6% CO₂, cells were washed, co-stained with anti-CD3 and anti-NK1.1 mAbs, fixed and permeabilized, and stained for indicated effector molecules. For CD107a staining, anti-CD107a mAb was present at 1 $\mu\text{g}/\text{mL}$ in 4-hour cultures containing HMNCs, YAC-1 cells, 2 μM monensin (BioLegend, San Diego, CA) and BFA. Stained cells were washed and interrogated using a FACSCanto II flow cytometer, and data were analyzed using FlowJo software version 10.

3.2.8 ⁵¹Cr release assays

YAC-1 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 0.1 mM MEM nonessential amino acids, GlutaMAX-I, 1 mM sodium pyruvate, 10

mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin, which we simply refer to as medium. To prepare target cells for cytotoxicity assays, YAC-1 cells were labeled with 100 µCi of Na₂⁵¹CrO₄, with occasional shaking, inside an incubator set at 37°C and 6% CO₂. After 90 minutes, labeled cells were washed, resuspended in medium, and seeded at 10,000 cells/well in a U-bottom microtiter plate.

HMNCs from glycolipid- and vehicle-treated mice were prepared and employed as a source of effector cells against ⁵¹Cr-labeled YAC-1 cells at indicated ratios. After 4 hours at 37°C and 6% CO₂, microplates were spun, and a 100-µL co-culture supernatant sample was harvested from each well for reading in a γ-counter. Experimental release (ER) values were obtained from wells in which effector and ⁵¹Cr-labeled target cells were co-present. Spontaneous release (SR) and total release (TR) were determined in supernatant samples collected from wells in which target cells were suspended in medium or in 1% Triton X-100, respectively. Specific cytotoxicity against YAC-1 target cells was calculated using the following equation: % specific lysis = [(ER - SR) ÷ (TR - SR)] × 100.

3.2.9 *In vivo* killing assays

The *in vivo* lytic function of α-GalCer-transactivated NK cells was assayed using a method we described elsewhere (16). Erythrocyte-depleted, naïve target splenocytes from WT and β2M^{-/-} B6 mice were labeled with two different concentrations of CFSE, typically 0.2 µM and 2 µM respectively. Target cells were extensively washed, mixed in equal numbers, and co-injected at 1 × 10⁷ total cells in 200 µL PBS into the tail vein of glycolipid- and vehicle-treated CLP survivors. Three hours later, the recipients were euthanized for their spleen and liver in which CFSE-labeled target cells were traced by flow cytometry. Percent

specific lysis of $\beta 2M^{-/-}$ target cells was calculated using the following formula: % specific killing = $\{1 - [(CFSE^{high} \text{ event number in organ} \div CFSE^{low} \text{ event number in organ}) \div (CFSE^{high} \text{ event number within mixed target cells before injection} \div CFSE^{low} \text{ event number within mixed target cells before injection})]\} \times 100$.

3.2.10 Evaluation of delayed-type hypersensitivity (DTH)

On day 4 post-CLP, indicated cohorts of sepsis survivors were injected s.c., behind the ear, with 100 μ L of a 10-mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) solution (Sigma-Aldrich, St. Louis, MO). Four days later, mice were challenged via a 50- μ L injection of the same solution in the left footpad and also received 50 μ L of PBS in the right (control) foot. After 24 hours, footpad swelling was measured using a digital caliper after deducting the baseline thickness recorded prior to the TNBS challenge. In experiments in which septic mice received glycolipid immunotherapy, the sensitizing dose of TNBS was given on day 4 post-CLP and 20 minutes after α -GalCer injection. Sham mice were given an intrafootpad (i.f.p.) injection of PBS.

3.2.11 Statistical analyses

Graphpad Prism 5 software was employed to compare various datasets. We used unpaired Student t-tests and ANOVA as appropriate, and conducted log rank tests for survival analyses. *, ** and *** denote statistically significant differences with calculated p values equal to or less than 0.05, 0.01 and 0.001, respectively.

3.3 Results

3.3.1 OCH-primed iNKT cells retain *in vivo* responsiveness to α -GalCer

Sepsis is a complex syndrome consisting of both hyper- and anti-inflammatory phases. We previously proposed the possibility of using iNKT cell glycolipid agonists therapeutically for sepsis (6). iNKT cell ligands that either suppress or promote inflammatory responses do exist. However, administration of even a single dose of KRN7000 (α -GalCer), the prototypic example of such ligands, abrogates or attenuates iNKT cell responses to a recall challenge with the same molecule (22). Whether other glycolipid combinations follow the same pattern has remained largely unclear. We sought to determine if *in vivo* priming with OCH, a truncated T_H2-polarizing analog of α -GalCer (25), alters subsequent iNKT cell responses to α -GalCer. We chose to test the impact of sequential treatments with OCH and α -GalCer (OCH \rightarrow α -GalCer) because: i) we previously found OCH treatment alone to be protective in the context of feces-induced peritonitis (FIP), a well-established model of acute sepsis (21); ii) treatment with α -GalCer relieves immunosuppression in certain other models or conditions (26, 27). Therefore, we posited that if iNKT cells retain their full responsiveness to α -GalCer following an initial exposure to OCH, the OCH \rightarrow α -GalCer regimen should alleviate both sepsis-induced hyperinflammation and immunosuppression.

We first tested the above hypothesis in the absence of sepsis. C57BL/6 (B6) mice were treated with α -GalCer, OCH or vehicle followed, 4 days later, by a second injection of α -GalCer and quantitation of serum interleukin (IL)-4 and interferon (IFN)- γ levels (Fig. 3.1A). As anticipated, pretreatment with the glycolipid vehicle did not prevent subsequent

IL-4 and IFN- γ responses to α -GalCer, which reached their peak levels at 2 hours and between 8-24 hours, respectively (Fig. 3.1B-C). *In vivo* exposure to α -GalCer before a second treatment with this glycolipid (α -GalCer \rightarrow α -GalCer) dramatically reduced serum IL-4 levels (Fig. 3.1B) and abolished IFN- γ production (Fig. 3.1C). This is consistent with previous reports on iNKT cell anergy, which was judged by a hyporesponsive state after an *ex vivo* challenge with α -GalCer (22). In contrast, priming with OCH did not alter the magnitude of the recall IL-4 response to α -GalCer (Fig. 3.1B). In addition, the OCH \rightarrow α -GalCer treatment resulted in a sharp rise in serum IFN- γ levels, which were even higher than the levels detected in the vehicle \rightarrow α -GalCer cohort at the 2-hour time point (Fig. 3.1C). IFN- γ levels in the OCH \rightarrow α -GalCer cohort were maximal at 8 hours and returned to the baseline at 24 hours.

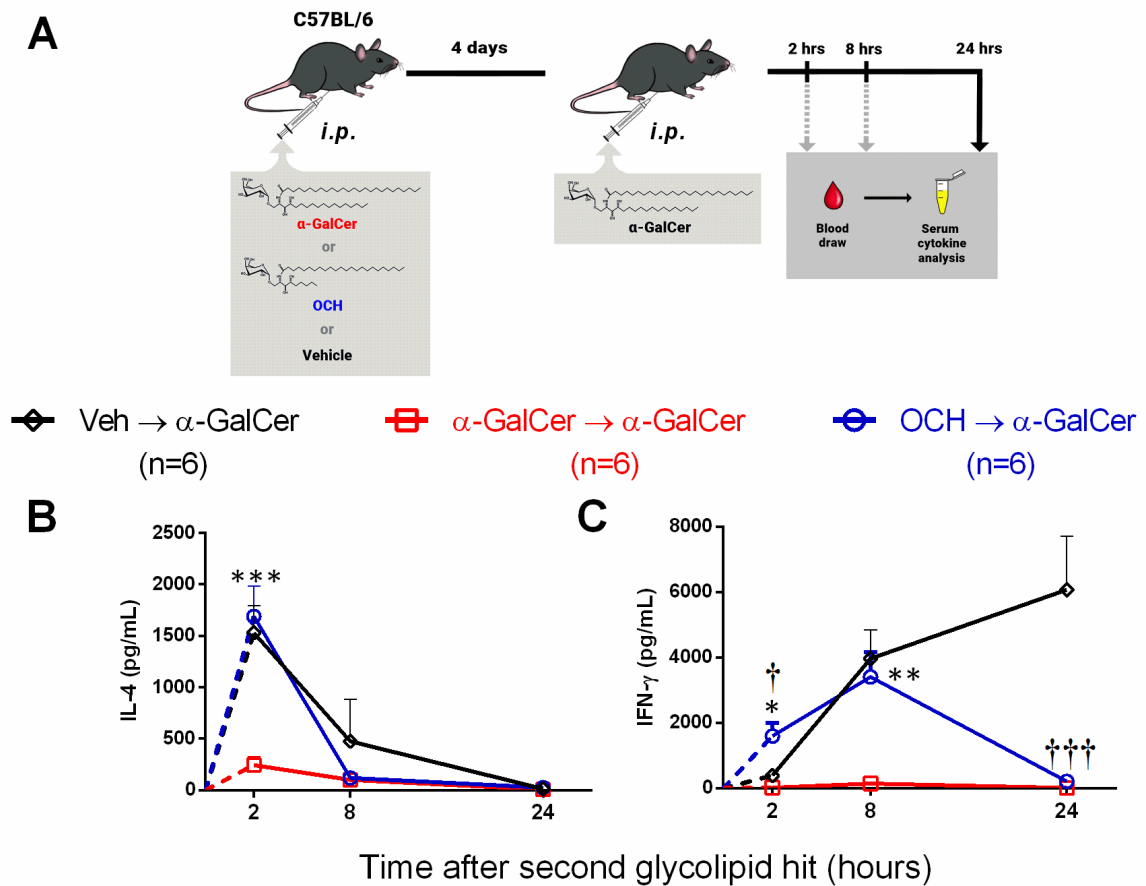


Figure 3.1: OCH-primed iNKT cells remain responsive to α -GalCer.

(A) B6 mice were injected i.p. with 4 μ g of OCH or α -GalCer, or with vehicle. Four days later, mice were challenged with 4 μ g of α -GalCer before they were bled via saphenous vein at indicated time points (A). Serum IL-4 (B) and IFN- γ (C) levels were quantified by ELISA. Error bars represent SEM. Statistical comparisons were carried out using unpaired Student's t-tests. *, ** and *** denote differences between mice treated with OCH \rightarrow α -GalCer and those receiving α -GalCer \rightarrow α -GalCer, with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. † and ††† indicate differences between OCH \rightarrow α -GalCer- and vehicle \rightarrow α -GalCer-treated cohorts with $p < 0.05$ and $p < 0.001$, respectively.

Next, we extended our findings to another standard mouse strain, namely BALB/c. These mice are traditionally considered to be T_H2 -dominant and are reportedly more vulnerable than B6 mice to cecal ligation and puncture (CLP), a surgical procedure that causes sepsis (28). Similar to T_H1 -dominant B6 mice, BALB/c mice that received two doses of α -GalCer exhibited severe iNKT cell anergy (Fig. 3.2). However, they were able to launch an augmented response to α -GalCer if they had been primed with OCH first (Fig. 3.2).

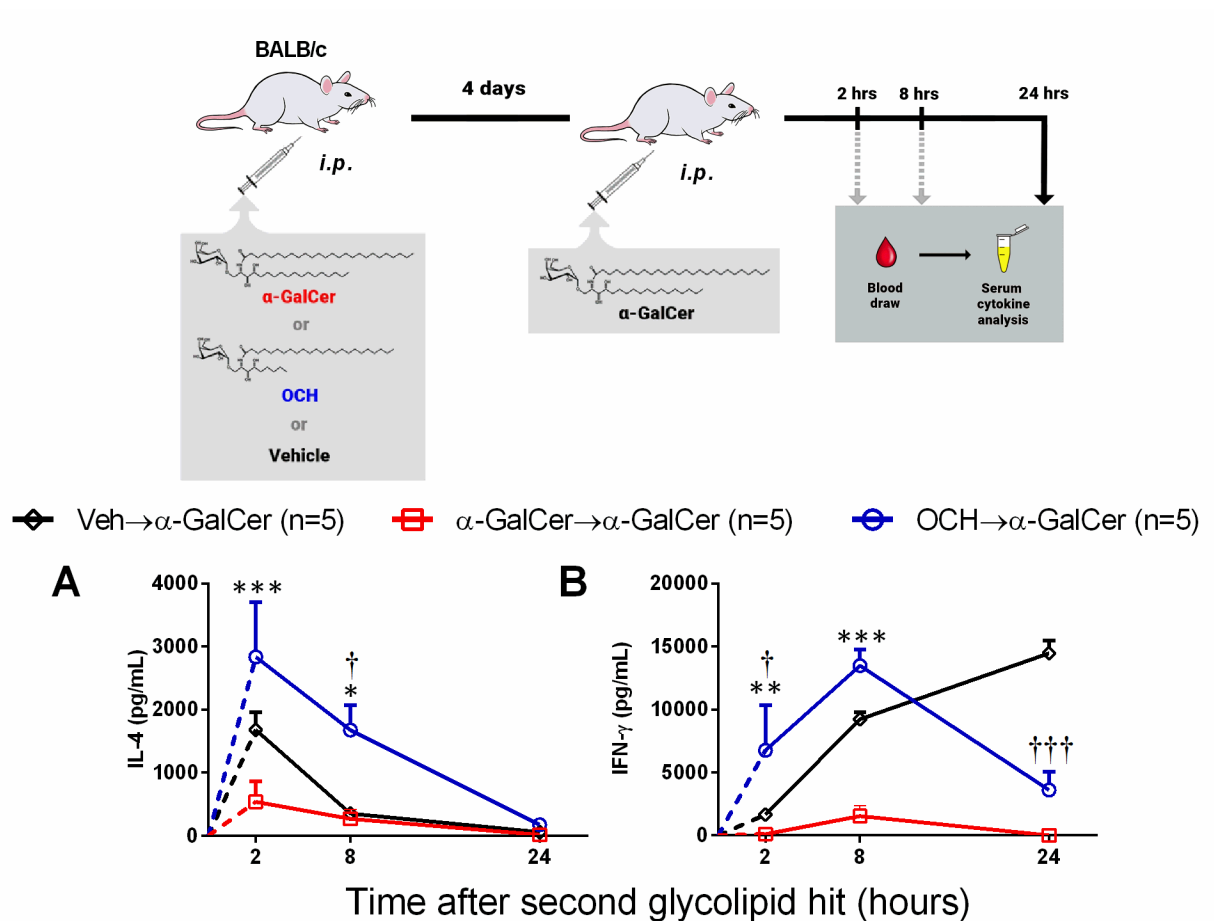


Figure 3.2: OCH-primed BALB/c mice retain their ability to respond to α -GalCer.

BALB/c mice were given 4 μ g of OCH or α -GalCer, or were injected with vehicle *i.p.* Four days later, they were challenged with 4 μ g of α -GalCer and subsequently bled as indicated for serum IL-4 (B) and IFN- γ (C) measurements. Error bars represent SEM. Statistical

comparisons were performed using Student's *t*-tests. *, ** and *** denote differences between animals treated with OCH → α -GalCer and those receiving α -GalCer → α -GalCer, with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. † and ††† indicate differences between OCH → α -GalCer- and vehicle → α -GalCer-treated mice with $p < 0.05$ and $p < 0.001$, respectively.

3.3.2 **The OCH → α -GalCer treatment reduces the severity and mortality of sepsis**

Since OCH did not anergize iNKT cells in our two-step stimulation regimen, we tested its efficacy as the initial component of a 'double-hit' immunotherapeutic protocol for biphasic sepsis. This required an *in vivo* model with low early mortality. Although very informative, the FIP model simulates only the acute phase of the syndrome (6). In a pilot experiment, intraperitoneal (i.p.) injection of B6 mice (n=3) with 100 μ L of a fecal slurry resulted in 100% mortality within 24 hours. Therefore, we resorted to the CLP model, the gold standard of sepsis models in rodents.

CLP involves laparotomy and ligation of the cecum, which is then perforated to allow fecal content to leak into the peritoneal cavity (29). A mild or sublethal form of CLP can be conducive to survival in a fraction of septic animals and their progression to an immunosuppressed state (6, 23). In our hands, ligating one-third of the cecum, which was then punctured thrice with a 25-gauge needle, led to severe sepsis that was highly lethal within a short period of time (Fig. 3.3). By comparison, ligating a smaller portion of the cecum followed by two perforations inflicted by a 27-gauge needle resulted in relatively

mild sepsis with a ~40-60% survival rate (Fig. 3.3). Therefore, we proceeded to test the efficacy of the OCH → α -GalCer treatment in this model.

Following a protocol that is schematically illustrated in Fig. 3.4A, mice were subjected to mild CLP before they received OCH (or vehicle), monitored for signs of morbidity using a scoring system we previously developed (24), re-injected with α -GalCer (or vehicle) and continued to be observed. As expected, unlike sham-operated controls, animals that underwent CLP showed signs of overt morbidity, which intensified over time before a maximum murine sepsis score (MSS) was reached 48 hours after the surgery (Fig. 3.4B). We found OCH to significantly minimize the severity of sepsis in this timeframe (Fig. 3.4B). Furthermore, treating septic mice with OCH → α -GalCer ameliorated their overall survival rate reproducibly (Fig. 3.4C). The observed survival advantage was evident early after OCH administration (Fig. 3.4C), consistent with low MSS scores recorded for animals that had been primed with OCH but not yet challenged with α -GalCer (Fig. 3.4B). Equally important, treatment with α -GalCer four days after OCH did not compromise the improved survival (Fig. 3.4C). In fact, even in the absence of the initial anti-inflammatory stimulation with OCH, α -GalCer on its own failed to worsen the mortality of CLP (Fig. 3.5).

Taken together, the above results demonstrate that OCH → α -GalCer reduces the morbidity and mortality of sublethal CLP, which is afforded by the early OCH hit and not reversed by subsequent α -GalCer treatment.

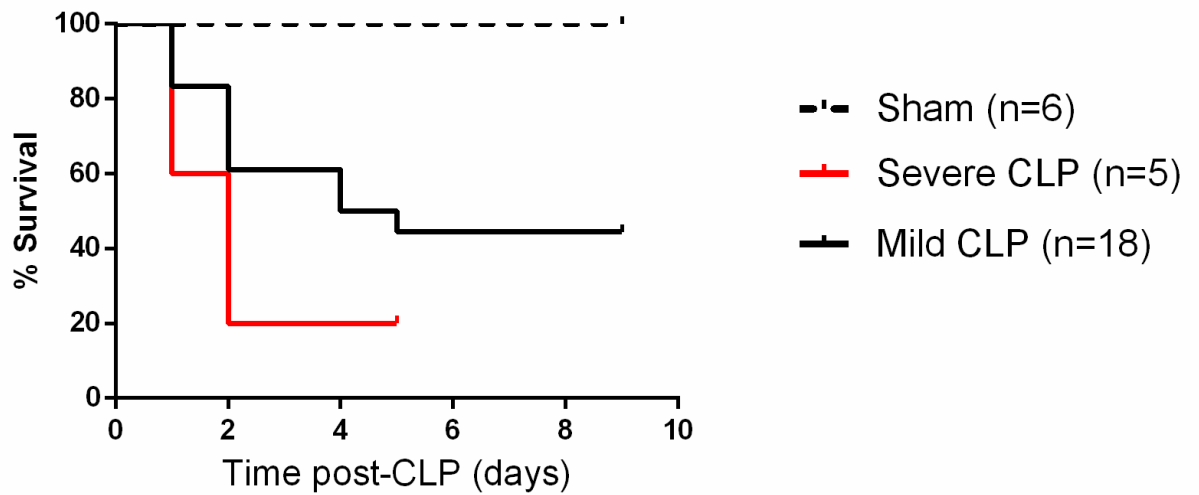


Figure 3.3: Procedural changes in CLP surgery results in distinct mortality patterns.

Severe CLP was induced by externalization of the cecum, the one-third distal region of which was ligated and perforated thrice with a 25-gauge needle. To induce a mild, sublethal form of CLP, 0.5 cm of the distal cecum was ligated and punctured twice with a 27-gauge needle. In sham mice, the cecum was neither ligated nor punctured. Kaplan-Meier survival curves for the three cohorts are depicted.

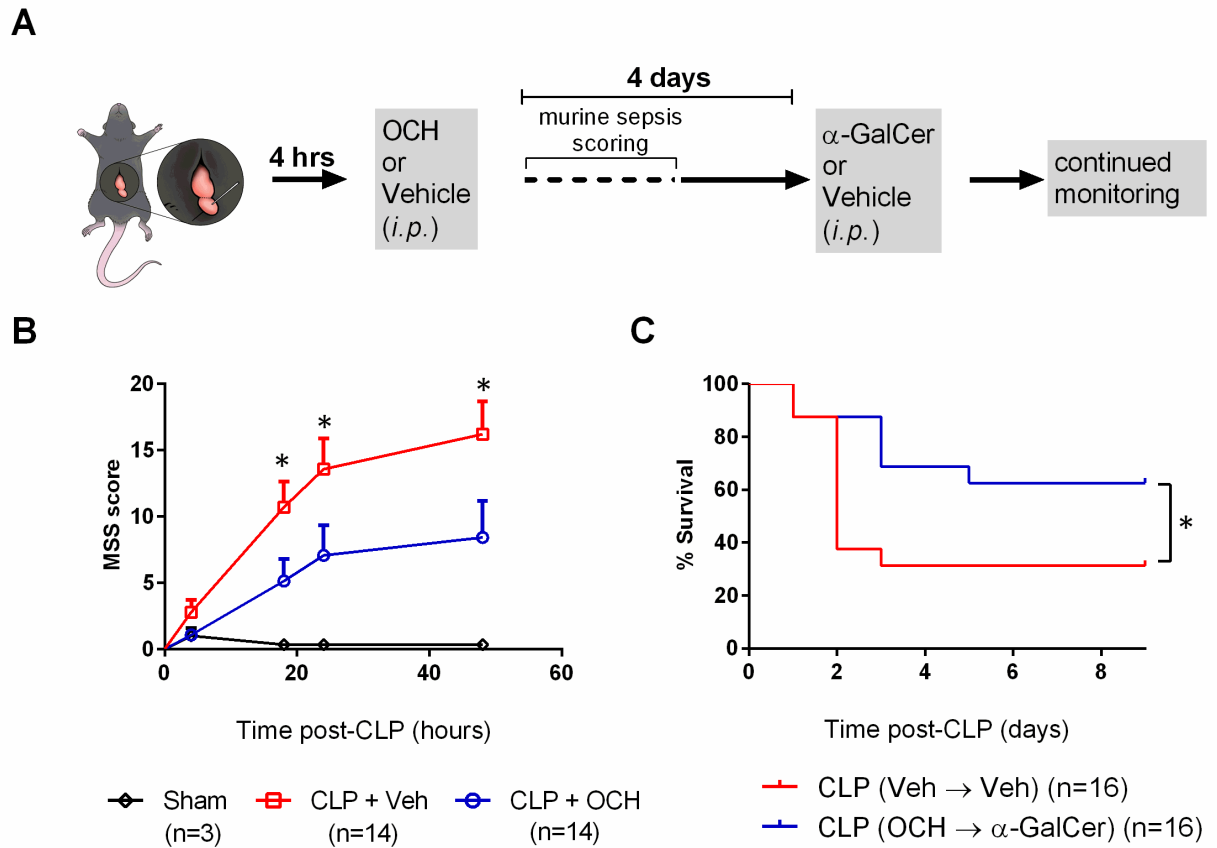


Figure 3.4: Treatment with OCH \rightarrow α -GalCer reduces CLP-induced morbidity and mortality.

B6 mice were subjected to sublethal CLP four hours before they received 4 μ g of OCH or vehicle i.p. according to a regimen schematically illustrated in A. Animals were monitored, and their 48-hour morbidity was recorded in a blinded fashion using a murine sepsis score (MSS) described in Materials and Methods. Error bars represent SEM, and * denotes $p < 0.05$ of CLP + Veh and CLP + OCH statistical comparison by unpaired Student's t-tests (B). Survivors were treated with 4 μ g of α -GalCer or vehicle i.p. (A.C), and continued to be monitored. Kaplan-Meier survival curves were generated with a weight loss of $\geq 20\%$

and/or unresponsiveness to provocation defining the endpoints. * indicates $p < 0.05$ based on a log-rank test (C).

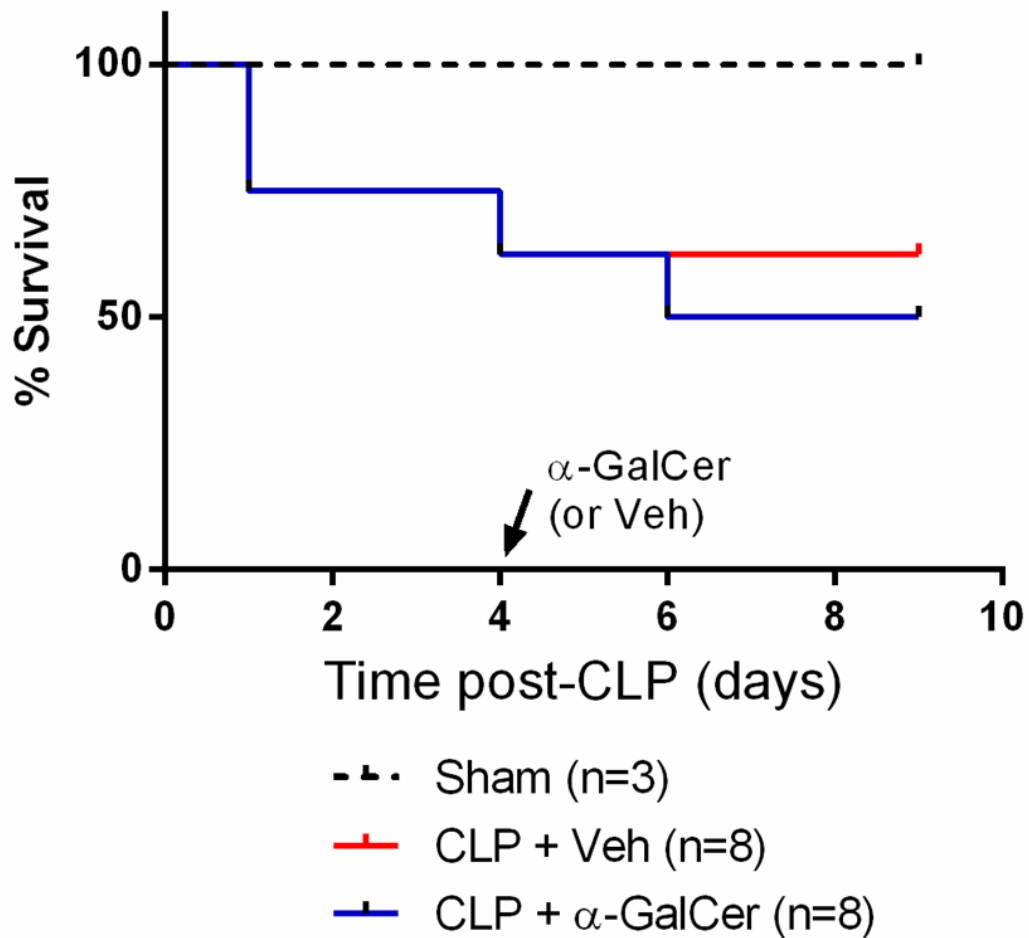


Figure 3.5: α -GalCer administration during protracted sepsis, in the absence of prior OCH priming, fails to reduce the mortality of CLP.

Four days after B6 mice had been subjected to sublethal CLP surgery, survivors were injected i.p. with 4 μ g of α -GalCer or with vehicle. The experimental endpoint was defined

as a weight loss of $\geq 20\%$ and/or a lack of response to physical stimuli. In sham controls, the cecum was neither ligated nor perforated. Kaplan-Meier survival curves are shown

3.3.3 Sequential treatment with OCH and α -GalCer yields a systemic pro-inflammatory cytokine picture

Given the clear benefit of OCH \rightarrow α -GalCer in our CLP model, it was pertinent to investigate the impact of this regimen on blood cytokine and chemokine levels in the face of an ongoing septic challenge. To this end, we used an extensive panel of cytokines/chemokines to quantify pro- and anti-inflammatory mediators in the serum of CLP survivors 12 hours after they were given the second hit with α -GalCer. This time point was chosen because of our interest in relieving sepsis-induced immunosuppression and also to closely mimic the timeline used in our iNKT cell anergy experiments (Fig. 3.1). Similar to non-septic mice (Fig. 3.1C), CLP survivors that had undergone treatment with OCH \rightarrow α -GalCer had significantly more IFN- γ in their blood (Fig. 3.6). Moreover, they had elevated levels of tumor necrosis factor (TNF)- α , IL-2, IL-5, eotaxin, CCL2, CXCL9 and CXCL10. In contrast, circulating levels of several T_H2-polarizing and/or anti-inflammatory mediators, such as IL-4, IL-10, IL-13 and transforming growth factor (TGF)- β , were comparable between vehicle \rightarrow vehicle and OCH \rightarrow α -GalCer cohorts (Fig. 3.6). These findings are consistent with an overall pro-inflammatory signature induced by the OCH \rightarrow α -GalCer treatment. In addition, they reinforce our conclusion that iNKT cells from OCH-primed animals maintain their ability to respond to α -GalCer even in a septic milieu.

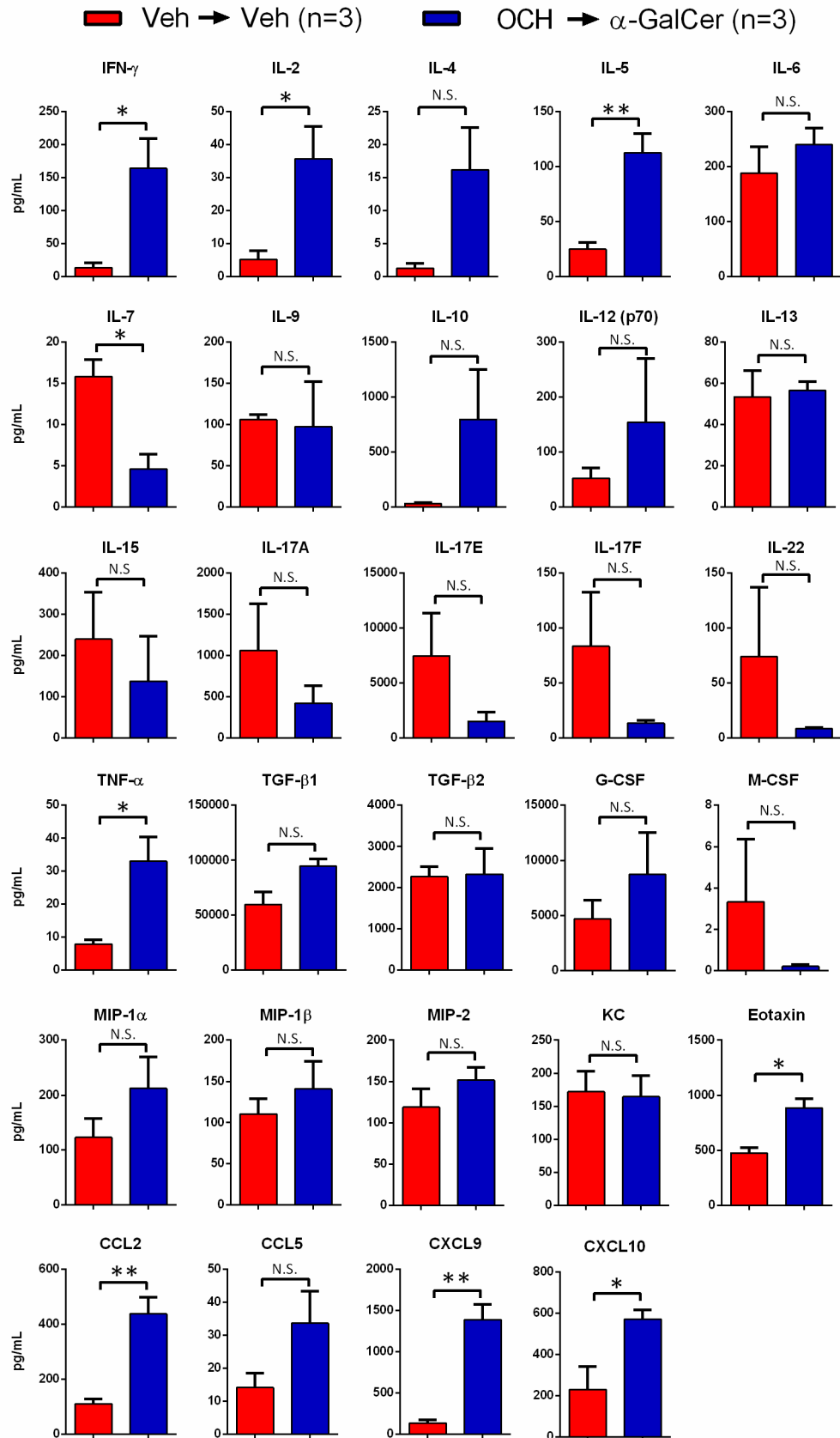


Figure 3.6: Treating septic mice with OCH→ α -GalCer gives rise to a pro-inflammatory blood cytokine pattern.

B6 mice underwent sublethal CLP surgery before they were treated with either OCH→ α -GalCer or vehicle→vehicle as described in Materials and Methods. Saphenous blood was collected 12 hours after the second hit, and serum levels of indicated cytokines and chemokines were determined using Luminex xMAP multiplexing technology. Error bars represent SEM. Statistical analyses were performed by unpaired Student's t-tests, and * and ** denote statistically significant differences with $p < 0.05$ and $p < 0.01$, respectively. N.S. indicates a non-significant difference.

3.3.4 The OCH → α -GalCer treatment results in NK cell transactivation

Glycolipid-stimulated iNKT cells are known to transactivate a multitude of downstream effector cell types, including NK cells that play critical roles in antipathogen immunity. This is largely owed to iNKT cells' capacity to secrete IFN- γ amply and swiftly (30). We found OCH → α -GalCer to induce a robust but transient rise in serum IFN- γ levels (Fig. 3.1C and Fig. 3.2B). Furthermore, iNKT cells harbored by septic mice were not anergized by this treatment and remained capable of triggering IFN- γ production (Fig. 3.6).

To begin to explore the functional significance of the above phenomenon, we examined gene expression by NK cells in CLP survivors (Fig. 3.7A). Transcriptomic analyses of hepatic NK cells from glycolipid-treated animals revealed elevated mRNA levels of Gzmb, Gzma and Perforin, suggesting enhanced cytolytic potentials (Fig. 3.7B). Consistent with this signature, non-parenchymal hepatic mononuclear cells (HMNCs) prepared from OCH

→ α -GalCer-treated survivors were much more potent than those harvested from vehicle-treated or sham-operated mice in destroying YAC-1 cells (Fig. 3.7C), the prototypic mouse NK cell targets. At the protein level, intracellular granzyme A (GZM A) was highly abundant in NK cells, albeit at comparable levels between glycolipid- and vehicle-treated mice ($93.8 \pm 1.9\%$ and $90.8 \pm 1.9\%$, respectively; $n=3/\text{group}$). We found increased cell surface expression of CD107a (LAMP-1) in NK cells from OCH → α -GalCer-treated animals, suggesting that more efficient degranulation was responsible, at least partially, for their augmented cytolytic effector function (Fig. 3.7D).

To validate our results in an *in vivo* setting, we took advantage of a CFSE-based killing assay, which we recently optimized for transactivated NK cell-mediated cytotoxicity (16). In this assay, naïve splenocytes from $\beta 2$ microglobulin ($\beta 2\text{M}$)-deficient B6 mice that are devoid of cell surface MHC class I molecules serve as NK targets. These targets were more efficiently removed from both the spleen and the liver of OCH → α -GalCer-treated mice (Fig. 3.7E-F), further supporting the conclusion that this therapeutic regimen results in greater NK cell-mediated cytotoxicity.

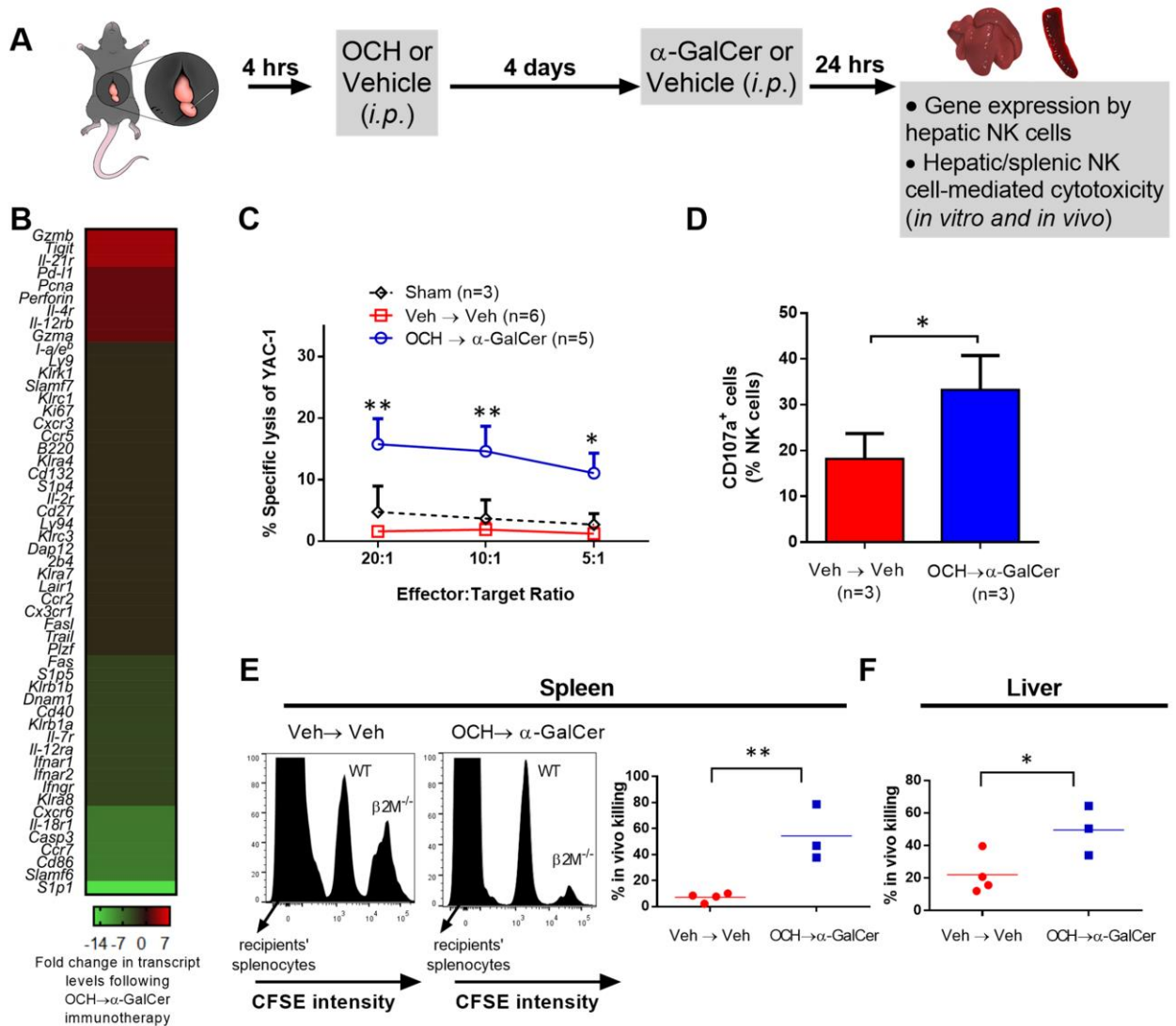


Figure 3.7: Sequential treatments of septic mice with OCH and α-GalCer augments the expression of cytotoxic effector molecules, degranulation and lytic function by NK cells.

B6 mice were subjected to sublethal CLP before they were injected *i.p.* with OCH (or vehicle) and α-GalCer (or vehicle) (A). Twenty-four hours after the second hit, hepatic CD3-NK1.1⁺ NK cells were stained, FACS-purified and pooled for RNA extraction and reverse transcriptase-quantitative PCR using a custom TaqMan gene array. Fold

increases/decreases in the expression of indicated genes, relative to the vehicle→vehicle control condition, were used to generate a heat map (B). Bulk non-parenchymal hepatic mononuclear cells (HMNCs) were co-incubated for 4 hours with ⁵¹Cr-labeled YAC-1 target cells. The ⁵¹Cr activity of culture supernatant samples was then determined by a γ counter, and the specific lysis of target cells was calculated using a formula that is detailed in Materials and Methods (C). In parallel, bulk HMNCs were co-cultured with YAC-1 cells in the presence of monensin and brefeldin A. After 4 hours, the surface expression of CD107a on NK cells was analyzed by flow cytometry (D). In separate experiments, 24 hours after the second hit, wild-type and $\beta 2M^{-/-}$ splenocytes, which were respectively labeled with a low and a high dose of CFSE, were mixed at a 1:1 ratio and injected via tail vein into glycolipid-treated and control CLP survivors. Three hours later, target cells in the spleens (E) and in the livers (F) were tracked and distinguished based on their differential CFSE labeling intensities, and their relative abundance was used to calculate % specific killing of $\beta 2M^{-/-}$ target cells as described in Materials and Methods. Error bars represent SEM. Statistical analyses were performed using a two-way ANOVA with Tukey's post-hoc test where treated and untreated septic mice were compared (C) or using unpaired Student's t-tests (D-F). * and ** denote differences with $p < 0.05$ and $p < 0.01$, respectively.

3.3.5 **The α -GalCer hit in OCH → α -GalCer-treated mice alleviates CLP-induced immunosuppression**

One of the primary goals of our step-wise treatment approach was to avert late immunosuppression. As predicted, CLP survivors in our model were immunosuppressed.

This was judged by the meager swelling of their footpad that had been injected with a recall dose of 2,4,6-trinitrobenzene sulfonic acid (TNBS) following subcutaneous (s.c.) priming with this hapten (Fig. 3.8A-B). This was indicative of a suboptimal delayed-type hypersensitivity (DTH) response. We also found the splenic F4/80+ macrophages of CLP survivors to express low levels of MHC class II molecules (Fig. 3.8C). The above readouts were used due to their clinical relevance. Many patients who survive sepsis's early hyperinflammatory phase do not exhibit normal DTH skin reactions to standard Ags (31). In addition, HLA-DR expression on CD14+ monocytes is routinely assessed in the clinic not only to identify septic patients in an immunosuppressed state but also to monitor the efficacy of the immunotherapies they receive (32).

To evaluate the efficacy of OCH \rightarrow α -GalCer in reversing immunosuppression, mice were given OCH (or vehicle) shortly after they underwent CLP surgery, followed 4 days later by TNBS sensitization and α -GalCer (or vehicle) injection. Four days later, they were challenged via an intrafootpad (i.f.p.) injection of TNBS and subsequently assessed for footpad swelling and MHC II expression (Fig. 3.9A). As with mice treated with OCH \rightarrow α -GalCer without TNBS priming/challenge (Fig. 3.4C), a clear survival advantage was manifest in this cohort (Fig. 3.9B). Importantly, OCH \rightarrow α -GalCer could significantly increase footpad swelling as an indication of a partially restored DTH response (Fig. 3.9C). We also detected increased expression of I-A/I-E on a per cell basis, as judged by the geometric mean fluorescence intensity (gMFI) of staining for these MHC class II molecules, on splenic macrophages (Fig. 3.9D-E).

Of note, in a control experiment in which α -GalCer was administered in the absence of prior treatment with OCH, neither the DTH response to TNBS nor the expression of I-A/I-

E could be recovered (Fig. 3.10A-B). Collectively, the above results demonstrate that $\text{OCH} \rightarrow \alpha\text{-GalCer}$ can partially but effectively relieve CLP-induced immunosuppression.

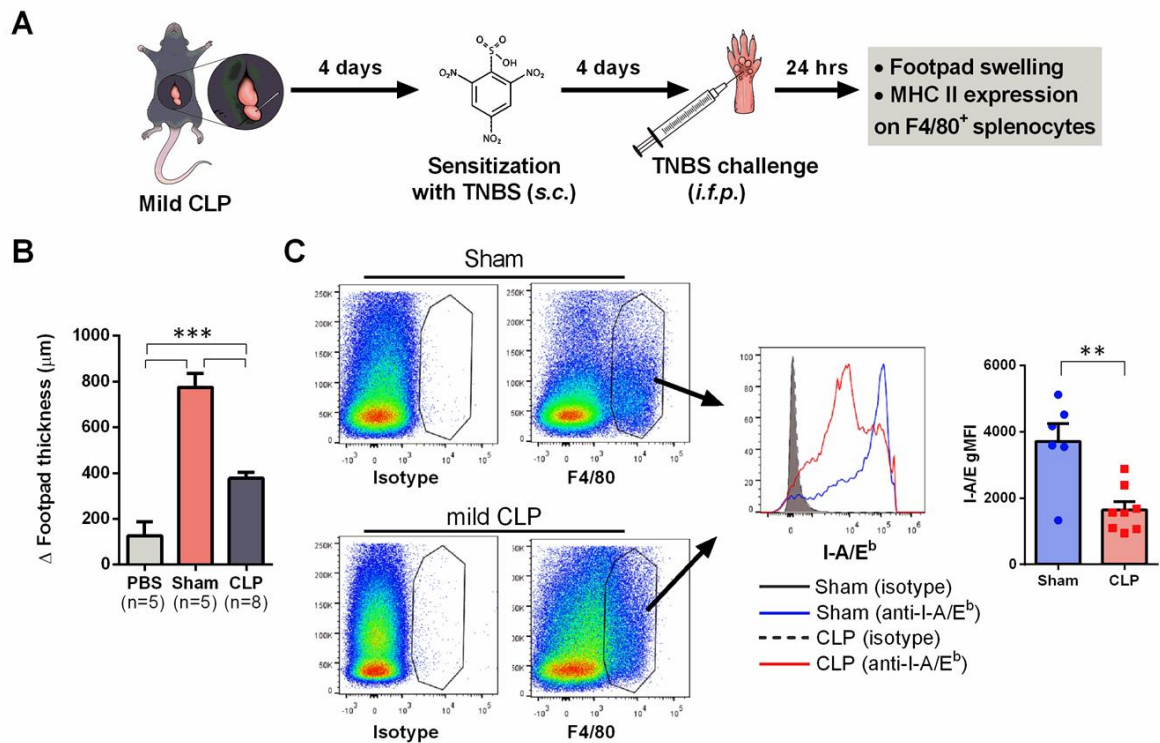


Figure 3.8: Sublethal CLP survivors become immunosuppressed.

As schematically illustrated in A, B6 mice underwent sublethal CLP or sham operation. On days 4 and 8 post-operation, animals were sensitized and challenged with TNBS via *s.c.* and *i.f.p.* routes, respectively. Twenty-four hours after the TNBS challenge, the thickness of the injected footpads was measured using a caliper, and the degree of their swelling was recorded relative to pre-recall measurement values (B). In addition, CLP survivors and sham mice were sacrificed for their spleen in which F4/80^+ macrophages were evaluated for I-A/E^b expression levels (C). Representative plots and summary data

are shown. Error bars represent SEM. Statistical comparisons were made using one-way ANOVA with a Tukey's post-hoc analysis (B) and unpaired Student's *t*-tests (C). ** and *** denote significant differences with $p < 0.01$ and $p < 0.001$, respectively.

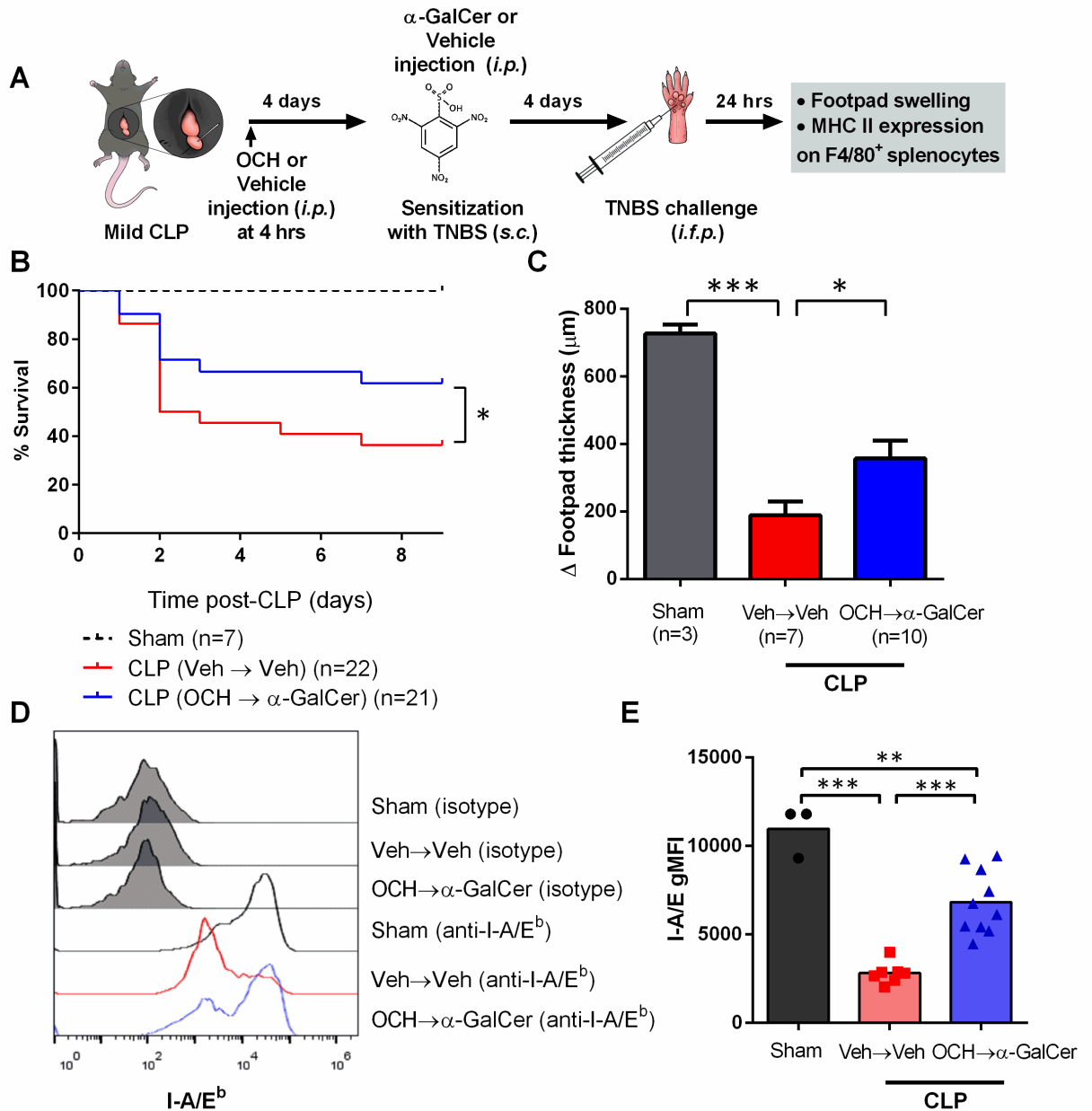


Figure 3.9: Administering α -GalCer to OCH-primed CLP survivors restores DTH reaction to TNBS and MHC II levels on macrophages.

B6 mice underwent sublethal CLP surgery before they were treated *i.p.* with indicated glycolipids (or vehicles), sensitized *s.c.* with TNBS and then challenged *i.f.p.* with TNBS following the timeline depicted in A. Kaplan- Meier survival curves were generated with

experimental endpoints defined as a weight loss of $\geq 20\%$ and/or unresponsiveness to physical provocation (B). Twenty-four hours after the TNBS recall, the thickness of the injected footpad was measured for each mouse using a caliper, from which the baseline pre-challenge thickness was deducted. Error bars represent SEM (C). On day 9 post-CLP, survivors were sacrificed, and splenic F4/80+ macrophages were assessed by flow cytometry for their expression level of I-A/Eb. Representative histograms (E) and summary geometric mean fluorescence intensity (gMFI) data (F) are shown. One-way ANOVA with Tukey's post-hoc tests was employed to determine statistically significant differences with $p < 0.05$, $p < 0.01$ and $p < 0.001$, which are denoted by *, ** and ***, respectively. Sham controls underwent a surgical procedure in which their cecum was neither ligated nor perforated. They also did not receive any treatments with glycolipids (or vehicles).

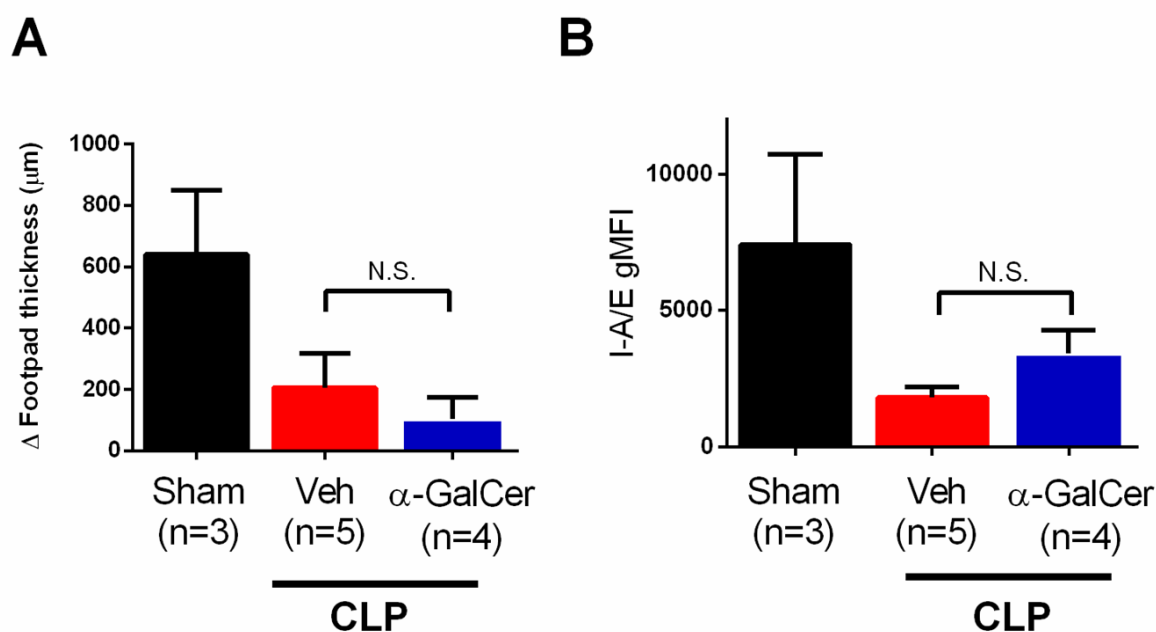


Figure 3.10: α -GalCer administration without prior OCH priming fails to alleviate CLP-induced immunosuppression.

Four days after CLP or sham surgery, survivors were injected *i.p.* with 4 μ g of α -GalCer or with vehicle. Animals were sensitized and challenged with TNBS on days 4 and 8 post-operation via *s.c.* and *i.f.p.* routes, respectively. Twenty-four hours later, footpad swelling was recorded (A), and the expression level of I-A/E^b on F4/80⁺ splenic macrophages was assessed by flow cytometry (B). Error bars represent SEM.

3.3.6 PBS-25, but not C20:2, is efficacious as the initial hit in the biphasic immunotherapy of CLP-induced sepsis

A number of glycolipids with activities similar to OCH have been previously reported. We set out to ascertain whether the beneficial bioactivity of OCH as the initial hit was unique to this molecule or could be mimicked by other T_H2-skewing iNKT cell agonists. One such

agonist is C20:2 (33), which we previously used as a solitary treatment for FIP-induced acute sepsis (21). As depicted in Fig. S3.11A and consistent with our previous findings (21), administering a single dose of C20:2 to B6 mice resulted in an early IL-4 burst and a subsequent IFN- γ response. However, priming with C20:2 resulted in a dwarfed recall response to α -GalCer (Fig. 3.11B), indicating that iNKT cells had been anergized. Unlike OCH, C20:2 has a relatively long acyl chain. Therefore, we tested two other glycolipids, namely PBS-25 and PBS-128 (34, 35), for their capacity to replace OCH since they are more similar to OCH in terms of their lipid chain length.

In our head-to-head comparisons, a single i.p. injection of PBS-25 yielded a higher IL-4:IFN- γ ratio than did PBS-128 (Fig. 3.12A). Moreover, priming naïve B6 mice with either PBS-25 or PBS-128 did not induce iNKT cell anergy since IL-4 and IFN- γ responses to second stimulation with α -GalCer were intact in both conditions (Fig. 3.12B). However, compared to PBS-128, an initial PBS-25 treatment potentiated less IL-4 and more IFN- γ production in response to α -GalCer (Fig. 3.12B). Therefore, we pursued the usage of PBS-25 in our sepsis model. We found treatment with PBS-25 \rightarrow α -GalCer to significantly reduce the mortality of CLP (Fig. 3.12C), which was reminiscent of the OCH \rightarrow α -GalCer treatment (Fig. 3.4C and Fig. 3.9B). These results suggest that the structure of iNKT cell agonists contributes, at least partially, to their anergy-inducing property, or lack thereof.

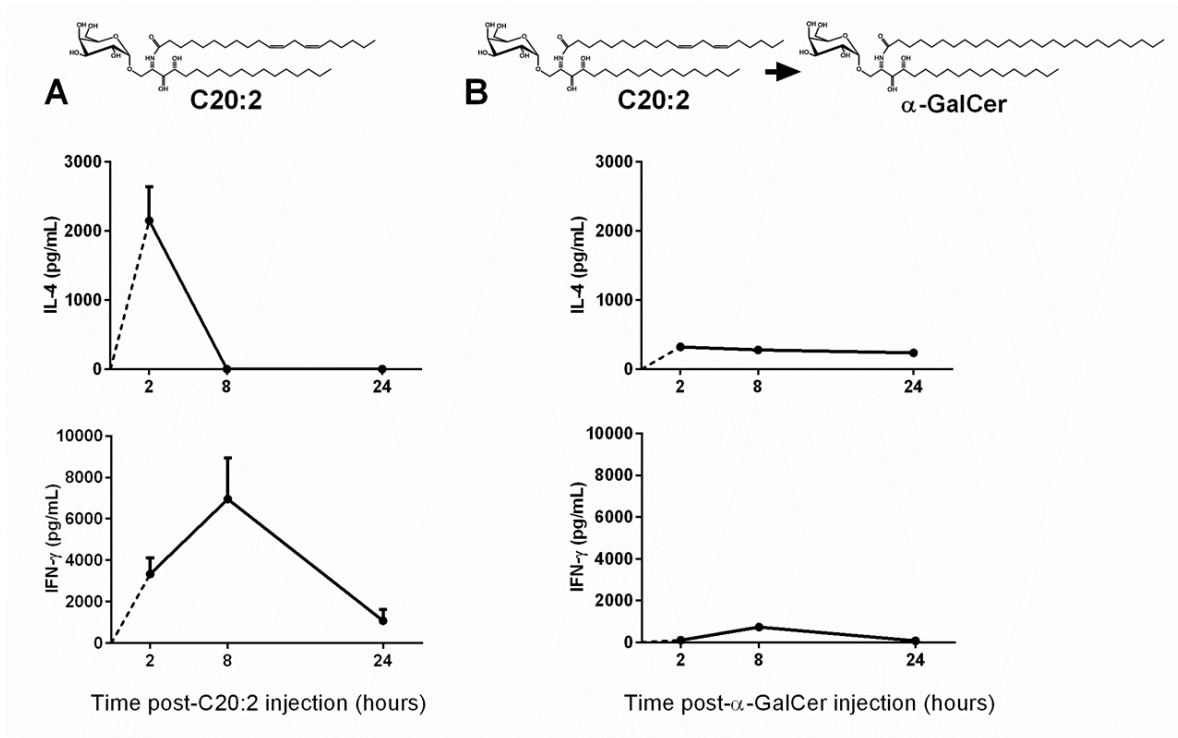


Figure 3.11: Pre-exposure to C20:2 compromises *i*NKT cell responses to α -GalCer.

Mice ($n=3$) were injected i.p. with 4 μ g of C20:2. Two, 8 and 24 hours later, they were bled for serum IL-4 and IFN- γ analyses by ELISA (A). A separate cohort ($n=3$) was primed i.p. with 4 μ g of C20:2 four days before animals received a second i.p. injection with α -GalCer. Serum IL-4 and IFN- γ levels were measured 2, 8 and 24 hours after the second injection (B). Error bars represent SEM

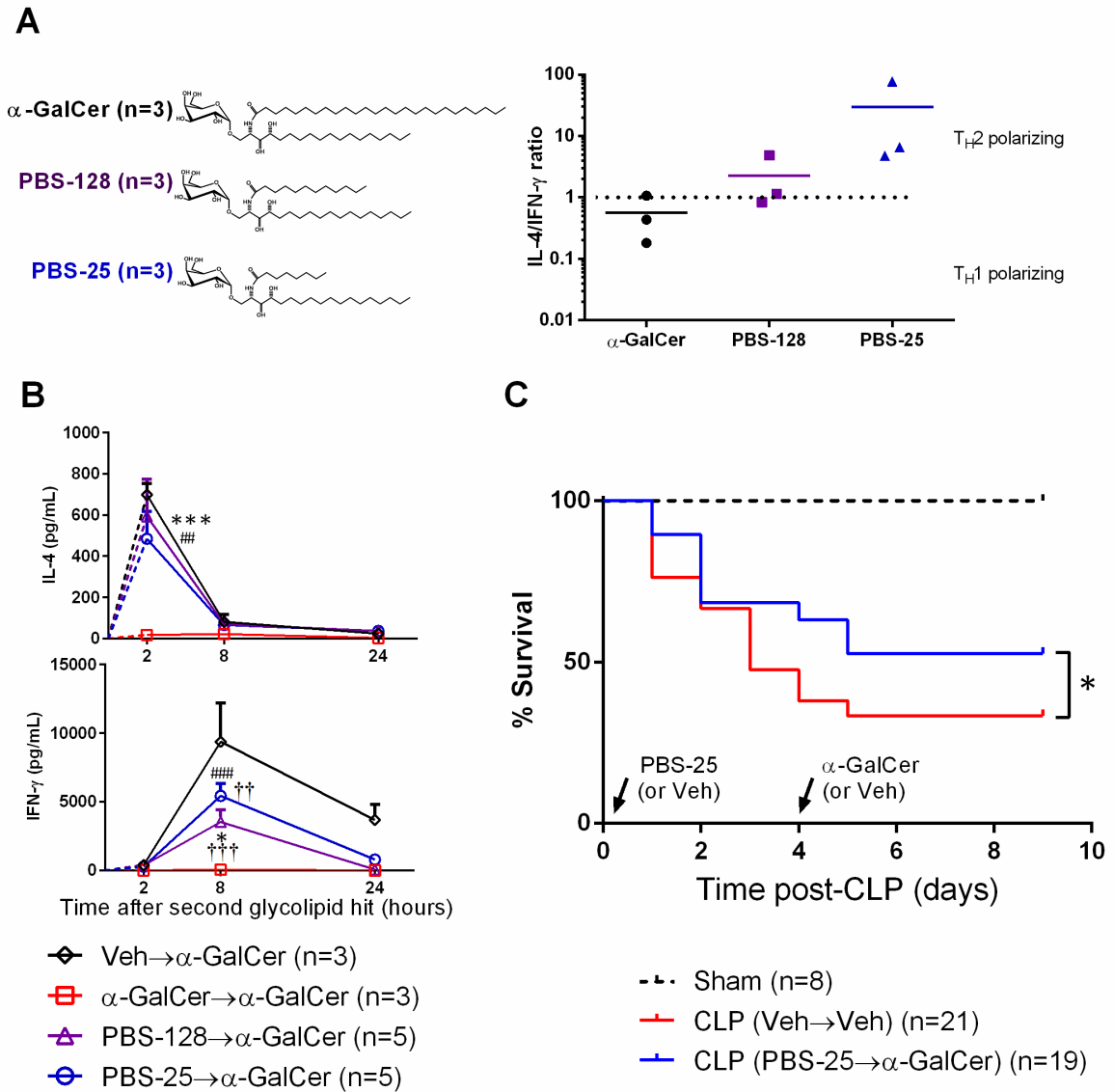


Figure 3.12: *In vivo* priming with PBS-25 does not compromise subsequent iNKT cell responses to α -GalCer, and can serve as the initial hit in the immunotherapy of biphasic sepsis.

Four μ g of α -GalCer, PBS-128 or PBS-25, glycolipid agonists of iNKT cells with varying acyl chain lengths, were injected i.p. into naïve B6 mice, which were bled 2, 8 and 24 hours later for serum cytokine analyses. Peak levels of IL-4 and IFN- γ were used to calculate the IL-4:IFN- γ ratio for each agonist (A). Separate cohorts of naïve B6 mice were given 4 μ g

of α -GalCer, PBS-128, PBS-25 or vehicle i.p. Four days later, all animals were challenged with a 4- μ g i.p. injection of α -GalCer as the second hit, and serum IL-4 and IFN- γ concentrations were quantitated by ELISA at indicated time points. Error bars represent SEM. Statistical comparisons were performed using unpaired Student's t-tests. * and *** denote differences between cohorts receiving PBS-128 \rightarrow α -GalCer and α -GalCer \rightarrow α -GalCer, with $p < 0.05$ and $p < 0.001$, respectively. †† and ††† indicate differences between PBS-25/PBS-128 \rightarrow α -GalCer- and vehicle \rightarrow α -GalCer-treated mice with $p < 0.01$ and $p < 0.001$, respectively. Significant differences between PBS-25 \rightarrow α -GalCer- and α -GalCer \rightarrow α -GalCer-treated mice with $p < 0.01$ and $p < 0.001$ are denoted by ## and ###, respectively (B). Kaplan-Meier survival curves were generated using data from B6 mice that were subjected to sublethal CLP before they were treated with PBS-25 \rightarrow α -GalCer or with vehicle \rightarrow vehicle. A log-rank test was employed to perform statistical analysis, and * indicates $p < 0.05$ (C).

3.4 Discussion

Accumulating evidence suggests that iNKT cells play important roles in acute polymicrobial sepsis. Hu *et al.* demonstrated a substantial drop in hepatic iNKT cell frequencies of C57BL/6 and BALB/c mice after severe CLP (20). This was accompanied by elevated CD25 and CD69 levels on the surface of residually detectable iNKT cells indicating their activation. Moreover, iNKT cell deficiency in $J\alpha 18^{-/-}$ mice was protective against CLP. Heffernan and co-workers subsequently reported that following CLP, iNKT cells migrate out of the liver to accumulate in the peritoneal cavity, the site of polymicrobial infection, where they influence the phagocytic activity of macrophages (36). In addition,

these investigators demonstrated a lower bacterial burden in the peritoneal cavity of septic $J\alpha 18^{-/-}$ mice compared with their wild-type (WT) counterparts. In a prospective clinical study, we found the peripheral blood iNKT:T cell ratio to be higher in patients with sepsis than in non-septic trauma patients (21). We also reported that the severity of FIP-induced sepsis was low in $J\alpha 18^{-/-}$ mice but worsened when these animals were reconstituted with WT iNKT cells. Finally, we found a single injection of OCH within 20 minutes of the fecal challenge to reduce the MSS scores and to prolong the survival of septic mice.

The above studies have painted a generally pathogenic picture of iNKT cells during acute sepsis but have fallen short of addressing the role(s) and/or the therapeutic potentials of iNKT cells in sepsis-induced immunosuppression, a problem that is common in the clinic and potentially fatal. The consequent opportunistic infections that arise can be stubbornly difficult to resolve even with broad-spectrum antibiotics and infection source control (1). A retrospective review of macroscopic postmortem findings indicated that about 77% of surgical ICU patients who had died from sepsis had continuous septic foci (37), suggesting a failure to clear infection with the inciting pathogen, nosocomial microbes, or both. Immunosuppression in patients with protracted sepsis is evidenced by weak DTH skin reactions (31) and frequent reactivation of one or more latent viruses, such as cytomegalovirus, Epstein-Barr virus, herpes simplex virus and human herpesvirus-6 (38).

To date, iNKT cells have not been studied in biphasic sepsis models. In the current investigation, we optimized and used a mild, sublethal form of CLP with hyperinflammatory and immunosuppressive phases that mimic real-life scenarios (6, 23). There has been a general consensus that sequential targeting of iNKT cells induces anergy, which can be obviously counterintuitive in therapeutic settings. Here, we have provided

strong evidence to the contrary when glycolipid combinations are carefully chosen. We designed and employed a step-wise treatment regimen in which OCH (or PBS-25) and α -GalCer were administered to septic mice sequentially and in a phase-tailored fashion. This novel approach reduced the mortality of CLP and remedied the problem of iNKT cell anergy. As a result, OCH-exposed iNKT cells retained responsiveness to α -GalCer, which was administered in the protracted phase of sepsis to mitigate immunosuppression.

Since OCH was synthesized and found to polarize iNKT cells towards a T_H2 -type phenotype (25), it has been used to ward off T_H1 -mediated immunopathology in a wide range of conditions. For instance, we employed OCH to reduce morbidity and/or pathology encountered in cardiac allotransplantation (39) and in the HLA-DR4-transgenic mouse models of rheumatoid arthritis (40) and toxic shock syndrome (41). We also demonstrated the therapeutic benefit of OCH in the FIP model of acute sepsis (21).

In the vast majority of our experiments, we used OCH as the ‘initial hit’ enabling iNKT cells to counter sepsis-induced hyperinflammation without undergoing anergy. Importantly, the impact of OCH in this capacity could be readily phenocopied through treatment with PBS-25, but not with C20:2. Although a potent T_H2 -polarizing compound, C20:2 is a diene with a relatively long and hydrophobic acyl chain and as such structurally dissimilar to OCH (33). By contrast, PBS-25 is more similar to OCH in terms of solubility in aqueous environments, lipid chain length and affinity for CD1d (34, 35). Therefore, we propose that the structure of iNKT cell agonists, especially the length of their lipid chains, is a determinant of their ability or inability to escape anergy. This should in turn inform whether an agonist can be used in multi-dose treatment modalities for a variety of conditions, including sepsis.

In our system, the second hit with α -GalCer rescued innate and adaptive responses represented by NK cell-mediated cytotoxicity and DTH, respectively. These responses largely depend on IFN- γ , a prominent pro-inflammatory cytokine that rose sharply but transiently following the second hit. It was therefore not surprising that the IFN- γ -inducible chemokines CXCL9 and CXCL10 were also elevated. These chemokines bind and signal through CXCR3 to regulate NK cell and T cell trafficking to the site of infection during sepsis (42). We also detected increased levels of the potent pleiotropic cytokines IL-2 and TNF- α along with CCL2, IL-5 and eotaxin. CCL2 is a monocyte chemoattractant with important roles in bacterial clearance in septic animals (43). IL-5 and eotaxin, another IFN- γ -inducible chemokine (44), are key to eosinophil growth and recruitment. Of note, higher IL-5 levels and eosinophil counts may be associated with survival in clinical sepsis (45, 46) although the reportedly protective property of IL-5 may not be always linked to eosinophils (47). The interplay between IL-5, eotaxin and eosinophils in the contexts of protracted sepsis and sepsis-induced immunosuppression will need to be further clarified.

Serum levels of several T_H2-type and anti-inflammatory cytokines, namely IL-4, IL-10, IL-13 and TGF- β , remained unaltered after sequential treatments with OCH and α -GalCer. Therefore, this protocol changed the overall cytokine landscape of sepsis in favor of a pro-inflammatory profile, which should serve the host well during an immunosuppressive phase.

IFN- γ is a major culprit of early sepsis immunopathology (6). However, its controlled release during protracted sepsis likely benefits the host by restoring immunocompetence. In our model, systemic administration of α -GalCer in the absence of OCH pretreatment was neither deleterious (Fig. 3.5) nor capable of reversing immunosuppression (Fig. 3.10).

Intriguingly, however, priming with OCH before α -GalCer treatment culminated in upregulated MHC II on macrophages, augmented NK cell-mediated cytotoxicity and restored DTH reactions, all of which depend, at least partially, on the activity of IFN- γ . We speculate that the OCH \rightarrow α -GalCer treatment achieves this feat by inducing a transient IFN- γ spike, which should be adequate to reverse immunosuppression. By the same token, sustained IFN- γ , which is avoided in our regimen, would unleash excessive inflammatory responses leading to tissue injury and/or immunological exhaustion. This needs to be taken into serious consideration when designing other iNKT cell-based therapies as the second hit. α -C-GalCer, a C-glycoside analog of α -GalCer with strong IFN- γ and IL-12 production capacities, has been described (48, 49). In addition, α -GalCer-pulsed dendritic cells (DCs) may be tested as the second hit in lieu of the free-floating glycolipid (50), especially if more than one treatment will be necessary. This approach would not only circumvent iNKT cell anergy but may also optimize DC functions in orchestrating adaptive responses (11, 51) to counter sepsis-induced immunosuppression (52). Regardless of the nature of the second hit, a desirable agent or modality will need to be one that will be inflammatory enough to boost antimicrobial immunity during protracted sepsis but not too inflammatory causing tissue damage and organ failure.

The next important question is whether α -GalCer can be combined with other treatments to maximize the host's ability to fight secondary infections during protracted sepsis. In a set of preliminary experiments, we found the addition of a programmed cell death-1 (PD-1)-blocking monoclonal antibody (mAb) to our second hit to further boost the IFN- γ response but not IL-4 production (Fig. 3.13). The rationale for these experiments was two-fold. First, interfering with PD-1 signaling is known to prevent α -GalCer-induced anergy

(53). This may be particularly important when the second hit may need to be delivered repeatedly. Second, a link has been established between PD-1 and the immunological shortcomings of septic patients, including their T cell exhaustion. Boomer *et al.* found high frequencies of PD-1⁺ CD4⁺ T cells and PD-ligand 1 (PD-L1)⁺ APCs among the splenocytes of septic patients harvested rapidly after their death (54). Moreover, within the postmortem lungs of septic patients, the expression of PD-1 by CD4⁺ cells and that of PD-L1 by plasmacytoid pre-dendritic cells were enhanced in comparison with control tissues from lung cancer resections. Brahmandam *et al.* reported that PD-1 blockade after CLP rescues the DTH response of septic mice (55). In a separate study, blocking PD-L1 either before or after CLP reduced the mortality of sepsis (56). Also importantly, this approach lowered the bacterial burden in the peripheral blood and within the peritoneal cavity of septic mice. We are currently investigating how PD-1-based immune checkpoint inhibitors perform as part of our second hit (i.e., OCH → α -GalCer plus anti-PD-1/PD-L1) in alleviating sepsis-induced immunosuppression.

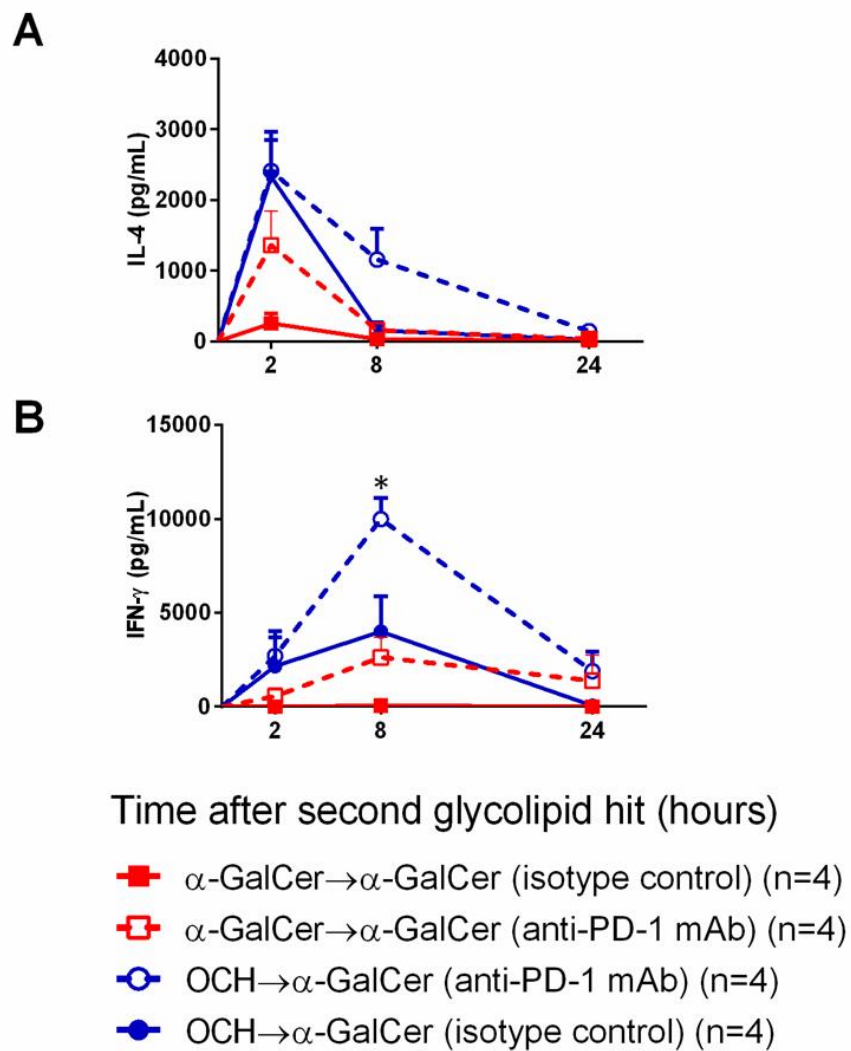


Figure 3.13: Blocking PD-1 at the time of the second glycolipid hit enhances α -GalCer-induced IFN- γ production.

B6 mice were primed with 4 μ g of α -GalCer or OCH. Four days later, indicated cohorts were injected with 100 μ g of an anti-PD-1 mAb (clone RMP1-14) or an isotype control 20 minutes before they received a 4- μ g i.p. injection of α -GalCer. Mice were bled 2, 8 and 24 hours after the second glycolipid hit, and serum IL-4 (A) and IFN- γ (B) levels were quantified by ELISA. Error bars represent SEM. * denotes $p < 0.05$ using an unpaired

Student's *t*-test comparing anti-PD-1 treated and isotype treated animals that received OCH → α -GalCer.

Another attractive pathway that can be targeted is coupled to the IL-7 receptor system, which promotes cellular viability and growth. Exposing septic patients' T cells to recombinant human IL-7 (rhIL-7) *ex vivo* enhances their proliferation, IFN- γ production, STAT5 phosphorylation, and Bcl-2 upregulation in response to T cell receptor triggering (57). Recently, in a prospective, randomized, placebo-controlled trial, the safety of rhIL-7 (CYT107) administration to septic patients and its ability to reverse CD4+ and CD8+ lymphopenia were verified (58). We noted a significant decrease in serum IL-7 levels of septic mice that had been sequentially treated with OCH and α -GalCer (Fig. 3.6). Therefore, adding IL-7 to our second hit may present a potential therapeutic opportunity by preventing immune cell apoptosis and improving T cell functions to bolster innate and adaptive antipathogen immunity.

In summary, in this investigation, we have taken advantage of the tremendous versatility of iNKT cells to design a novel, phase-tailored protocol for the treatment of sepsis-induced hyperinflammation and immunosuppression. iNKT cells constitute attractive therapeutic targets in sepsis for several reasons. First, the prognosis of sepsis is partially determined by the speed with which its treatment gets underway. iNKT cells are among the few T cell types that launch swift responses to antigenic stimulation and may, as such, make a difference quickly (59). Second, the availability of multiple T_H2- and T_H1-glycolipid agonists of iNKT cells should allow for testing additional new and carefully timed protocols similar to what we have described herein. Third, iNKT cell stimulation results in secondary activation of downstream effector cell types. As such, targeting iNKT cells will

have wide-ranging impacts on ensuing host responses. We assessed the cytolytic effector function of transactivated NK cells in this work. However, there are numerous other effectors that can be influenced, ideally to the septic host's benefit. Fourth, glycolipid agonists of iNKT cells work beyond the species barrier (60). In fact, some of the same glycolipids employed in mouse studies have shown promise in clinical trials for malignancies and viral diseases (18). Therefore, we anticipate the findings of this study to be translatable. Fifth, iNKT cells are restricted by CD1d, which is monomorphic (61). Therefore, glycolipid ligands of iNKT cells, such as those used in this work, should be useful in genetically diverse human populations. This is a tempting possibility that warrants further investigation.

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Chapter 4

4. Glycolipid stimulation of iNKT cells expands a unique tissue-resident population of pre-mNK cells endowed with oncolytic and anti-metastatic properties

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4.1 Introduction

Invariant natural killer T (iNKT) cells comprise a specialized subset of innate-like T lymphocytes with remarkable immunomodulatory properties. They express a semi-invariant TCR with a distinctive V α 14-J α 18 configuration that uniquely recognizes naturally occurring and synthetic glycolipid Ags presented within the close cleft of CD1d (1, 2). Unlike their conventional counterparts, iNKT cells occur in a partially activated state and harbor pre-formed mRNA transcripts coding for several inflammatory cytokines, which they can release amply and speedily (3).

iNKT cells have been heralded for their roles in anticancer immune surveillance (4, 5). They can express perforin, granzymes (GZMs), Fas ligand, TNF- α and TRAIL (6-8), and directly destroy glycolipid-pulsed mouse target cells *in vivo* (9) and human CD1d+ tumor cells *in vitro* (8, 10). Arguably, however, the most pronounced feature of iNKT cells is their ability to transactivate APCs (11, 12) and multiple effector cell types, including NK cells (13, 14), conventional CD8+ T cells (15) and $\gamma\delta$ T cells (16).

CD1d-restricted agonists of iNKT cells are typified by α -galactosylceramide (α -GalCer). This α -anomeric glycolipid was initially extracted from a marine sponge, *Agelas mauritanicus*, in a screen for anticancer compounds (2, 17) but also exists, in minute quantities, in mammalian cells (18). Human iNKT cells can recognize mouse CD1d and vice versa (19). Furthermore, both mouse and human iNKT cells are responsive to α -GalCer (4). Therefore, findings of tumor models in which α -GalCer is therapeutically tested are potentially translatable to the clinic. In addition, the monomorphic nature of

CD1d dictates that α -GalCer-based or -adjuvanted treatments should target iNKT cells in diverse human populations beyond the MHC restriction barrier.

In mice, α -GalCer has been employed against thymoma (20), melanoma (21) and pancreatic adenocarcinoma (22) among other cancers. The beneficial properties of α -GalCer, α -GalCer-pulsed dendritic cells (DCs) and α -GalCer-expanded iNKT cells have also been explored and exploited in clinical trials for multiple human neoplasms (4, 5). Despite encouraging results arising from such efforts, the full therapeutic potential of glycolipid-activated iNKT cells remains to be fully realized, and the range of downstream effectors they transactivate may need to be revisited or even revised.

While investigating *in vivo* responses to α -GalCer, we serendipitously found a dramatically expanded tissue-resident population with prominent anticancer functions, which turned out to be precursors to mature NK (pre-mNK) cells. Pre-mNK cells are phenotypically defined as B220⁺CD11c⁺MHC-II⁺NK1.1⁺ cells, and compose an intermediate in the differentiation pathway of conventional NK cells. They were initially called IFN-producing killer dendritic cells (IKDCs), due ostensibly to their versatility in migrating to lymph nodes to present Ags, in synthesizing copious amounts of IL-12, type I IFN and IFN- γ , and in exerting tumoricidal activities (23, 24). Subsequently, IKDCs were reported to belong to the NK lineage as evidenced by their strict developmental dependence on IL-15 (25-27) and their propensity to acquire a mature NK (mNK) cell phenotype upon adoptive transfer (28). In fact, pre-mNK cells were demonstrated to be superior to mNK cells in terms of IFN- γ and TNF- α secretion (25).

We herein report that priming with α -GalCer expands, activates and alters select tissue-resident pre-mNK cells through an IL-12/IL-18-dependent mechanism and as such adds a powerful new weapon to the immune system's anticancer arsenal. Our findings define a novel communication axis involving two innate-like effector cell types, namely iNKT and pre-mNK cells, with clear implications for cancer immunotherapy. We propose that the roles fulfilled by pre-mNK cells in the context of α -GalCer-based treatments may have been overlooked due to their phenotypic resemblance to plasmacytoid DCs (pDCs) and mNK cells.

4.2 Materials and Methods

4.2.1 Mice

WT B6 mice were purchased from Charles River Canada Inc. (St. Constant, Quebec) or bred in our institutional barrier facility. β 2M^{-/-} and GFP⁺ mice, on a B6 background, were provided by Drs. Anthony Jevnikar and Steven Kerfoot (Western University), respectively. Age- and sex-matched adult mice were used in all experiments. Our animal use protocols (AUPs 2010-241 and 2018-093) were reviewed and approved by the Western University Animal Use Subcommittee.

4.2.2 Cell lines

YAC-1 mouse lymphoma cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, GlutaMAX-I, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 120 U/mL penicillin, 100 μ g/mL streptomycin, and 10 mM HEPES. The chicken OVA-expressing T cell lymphoma line EG7-OVA was supplied by Drs. Jack

Bennink and Jonathan Yewdell (NIAID, NIH) and maintained in RPM1-1640 medium containing 10% FBS and 400 $\mu\text{g}/\text{mL}$ G418. B16-F10 mouse melanoma cells were grown in FBS-supplemented MEM α medium. The B3Z hybridoma with specificity for SIINFEKL:H-2Kb was a gift from Dr. Sameh Basta (Queen's University, Kingston, Ontario). B3Z cells were cultured in IMDM medium and 10% FBS in the presence of G418.

4.2.3 *In vivo* treatments

Mice were injected i.p. with 4 μg α -GalCer (Funakoshi Co. Ltd) or with a vehicle containing 0.5% Tween 20, 56 mg/mL sucrose and 7.5 mg/mL histidine. Where indicated and except in serum transfer experiments, a CD1d-blocking mAb (clone 20H2) or a rat IgG1 control (HRPN) was injected i.p. at 500 $\mu\text{g}/\text{dose}$ 2 hours before and 24 and 48 hours after α -GalCer treatment. In a pilot experiment, an anti- γc mAb (3E12) or a rat IgG2b control (LTF-2) was administered using a similar regimen except the experiment was ended 24 hours post-glycolipid treatment. To deplete B220⁺ or CD19⁺ cells, mice were given 200- μg i.p. injections of an anti-B220 mAb (RA3.3A1/6.1) or an anti-CD19 mAb (1D3) on days 1 and 3 post- α -GalCer priming. All the above mAbs and isotype controls were purchased from Bio X Cell (West Lebanon, NH).

FTY720 (Sigma) was reconstituted in water, diluted in PBS, and injected at 1 mg/kg or 3 mg/kg i.p. (29) 2 hours prior to and 24 hours following α -GalCer treatment.

Recombinant mouse IFN- γ , IL-12 and IL-18 (PeproTech) were diluted in sterile PBS and administered once via tail vein at final concentrations of 10, 4 and 200 ng, respectively.

4.2.4 Serum cytokine quantifications

Mouse peripheral blood was collected via the saphenous vein, and serum was isolated through centrifugation for 15 minutes at 17×1000 g. Samples were stored at -80°C until use. ELISA kits from ThermoFisher Scientific was employed to quantitate the cytokine content of each sample.

4.2.5 Cytofluorimetric analyses

α -GalCer- and vehicle-treated mice were sacrificed by cervical dislocation. Spleens and livers were homogenized in PBS using a glass plunger. Hepatic parenchymal cells were removed through centrifugation, without brake, at $700 \times g$ for 12 minutes in a 33.75% Percoll PLUS solution (GE Healthcare). Lungs were cut into small pieces using sterile scissors. The resulting preparation was then treated for 1 hour with 0.5 mg/mL collagenase IV (Sigma) in RPMI-1640 containing 10% FBS while being rotated inside a 37°C incubator. Splenic, hepatic and pulmonary cell preparations were briefly exposed to ACK lysis buffer to eliminate erythrocytes, washed, and filtered through 70- μm pores of a cell strainer to remove debris. To block Fc γ receptors, splenic cells, HMNCs and non-parenchymal lung mononuclear cells were incubated for 5 minutes at 4°C with 20 μL of the 2.4G2 hybridoma supernatant containing a CD16/CD32 mAb. Cells were then stained with fluorophore-conjugated mAbs to surface B220 (clone RA3-6B2), CD11b (M1/70), CD11c (N418), CD27 (LG.7F9), CD49b (DX5), CD69 (H1.2F3), CD107a (LAMP-1)(1D4B), CD122 (TM-b1), CD218a (P3TUNYA), FasL (MFL3), I-Ab (AF6-120.1), NK1.1 (PK136), TRAIL (N2B2), and/or intracellular GZM A (GzA-3G8.5), GZM B

(NGZB), IFN- γ (XMG1.2), Ki67 (SolA15), perforin (eBioOMAK-D) and TNF- α (MP6-XT22). All the above mAbs and corresponding isotype controls were from ThermoFisher.

Cell surface staining was conducted at 4°C for 30 minutes. Intracellular staining of freshly isolated cells was performed using an eBioscience Fixation & Permeabilization Buffer Set. To detect cytolytic molecules, splenic cells or HMNCs were first co-cultured with YAC-1 tumor cells in the presence of 10 μ g/mL brefeldin A (BFA)(Sigma) for 4 hours at 37°C and 6% CO₂. Cells were then washed, stained for pre-mNK cell surface markers, fixed, permeabilized, stained for indicated intracellular molecules, washed again and analyzed. For CD107a staining, anti-CD107a mAb was present in co-cultures that also contained 2 μ M monensin (BioLegend) in addition to BFA.

Surface staining for S1PR1 was conducted in two steps. First, cells were incubated with an unconjugated anti-S1PR1 mAb (clone #713412 from R&D Systems) for 30 minutes at 4°C. This was followed by addition of allophycocyanin-labeled goat anti-rat IgG F(ab')₂ fragments (R&D Systems) for 30 minutes at 4°C in dark. Control samples were incubated first with a control rat IgG2a (clone 2A3) and subsequently with the secondary reagent.

Stained cells were interrogated using a FACSCanto II cytometer, and data were analyzed using FlowJo software (Tree Star).

4.2.6 Customized gene expression examination

Two days after α -GalCer or vehicle administration, hepatic B220⁺CD11c⁺NK1.1⁺ cells were purified using a BD FACSAria III cytometer after live gating based on forward and side scatter profiles and doublet exclusion. The purity of sorted pre-mNK cells was always

greater than 95%. A Purelink RNA Mini Kit (ThermoFisher) was utilized to extract RNA, which was then converted to cDNA using the Invitrogen SuperScript VILO MasterMix. Quantitative PCR was carried out in Custom TaqMan Array 96-Well Fast Plates (ThermoFisher) using a StepOnePlus Real-Time PCR System. The $\Delta\Delta CT$ method was employed to assess changes in gene expression.

4.2.7 **Detection of peptide Ag presentation**

Sorted hepatic pre-mNK cells were pulsed with 1 μM SIINFEKL for 45 minutes at 37°C. They were then washed and co-incubated at a 1:1 ratio with B3Z cells for 24 hours at 37°C and 6% CO₂. After washing in PBS, cells were treated with a lysis buffer containing 0.125% Nonidet P-40, 9 mM MgCl₂ and 0.1 mM 2-mercaptoethanol in the presence of 5 mM ortho-nitrophenyl- β -D-galactopyranoside (ONPG) as the substrate. Four hours later, the OD of supernatants was determined at 415 nm.

To detect Ag cross-presentation *in vivo*, B6 mice were injected i.p. with α -GalCer or vehicle 24 hours before they received 1×10^6 EG7-OVA tumor cells i.v. Four days later, the frequency of SIINFEKL-presenting pre-mNK cells among HMNCs was determined by flow cytometry using a PE-conjugated mAb that recognizes SIINFEKL:H-2Kb complexes (clone 25-D1.16) (30).

4.2.8 **Adoptive cell and serum transfer**

Five days after α -GalCer injection into GFP⁺ mice, animals were euthanized, and hepatic B220⁺CD11c⁺NK1.1⁺ and B220⁻CD11c⁻NK1.1⁺ cells were FACS-purified. Sorted populations, which were always >95% pure, were injected i.v. into WT B6 recipients,

which were subsequently sacrificed for their liver on days 5 and 15 post-transfer. Non-parenchymal HMNCs were isolated and analyzed for their pre-mNK cell content.

For adoptive serum transfer experiments, α -GalCer-injected donor WT B6 mice were terminally bled at 2-, 6-, 12- and 24-hour time points. Two hundred μ L of pooled sera collected at each individual time point were transferred via tail vein into a recipient WT B6 mouse. In a separate setting, 60 μ L of serum from the 6-hour time point were combined with 200 μ L from the 12- or the 24-hour time point, as indicated, and injected into a B6 mouse. In both scenarios, recipient animals were given a solitary 500- μ g i.p. dose of an anti-CD1d mAb (20H2) 2 hours before they received serum.

In several experiments, IFN- γ , IL-12 and/or IL-18 were magnetically removed from serum samples. Briefly, sera were incubated for 10 minutes at room temperature with 10 μ g/mL of biotinylated mAbs to IFN- γ (clone DB1 from ThermoFisher), IL-12 (C17.8, ThermoFisher) and/or IL-18 (93-10C, Medical and Biological Laboratories). Streptavidin-conjugated RapidSpheres (STEMCELL Technologies) were then added to each cocktail followed by magnetic separation of the particles as per manufacturer's instructions. A small aliquot of each cytokine-replete and -depleted sample was stored at -80°C for cytokine measurements to confirm the procedure's efficacy, and the remainder was adoptively transferred.

4.2.9 **51-Chromium (⁵¹Cr) release assays**

YAC-1 cells were labeled with 100 μ Ci of Na₂⁵¹CrO₄ for 90 minutes at 37°C, washed with PBS, and used as target cells. Bulk HMNCs or purified hepatic pre-mNK cells from α -GalCer- and vehicle-treated mice were employed at indicated effector:target ratios against

⁵¹Cr-labeled YAC-1 cells in 96-well microplates. After 4-hour incubation at 37°C and 6% CO₂, plates were spun before a 100-μL supernatant aliquot was collected from each well and read by a γ-counter. In several experiments, a combination of EGTA (3 mM) and MgCl₂ (2 mM) was added to co-cultures to block granule exocytosis (31). Alternatively, 100 nM concanamycin A (CMA) was used to pre-treat effector cells for 1 hour, and was also present during 4-hour co-cultures (32). Experimental release (ER) values were determined in supernatant samples from wells containing both effector and target cells. Spontaneous release (SR) and total release (TR) were obtained from wells in which target cells were suspended in medium only or in 1% Triton X-100, respectively. Specific killing of YAC-1 cells was calculated using the following formula: % specific lysis = [(ER–SR) ÷ (TR–SR)] × 100.

4.2.10 *In vivo* cytotoxicity

Erythrocyte-depleted syngeneic target splenocytes were prepared from WT and β2M^{-/-} B6 mice, and labeled with 0.2 μM and 2 μM CFSE, respectively. Target cells were washed, mixed in equal numbers, and injected at 1×10⁷ cells in 200 μL PBS into the tail vein of recipient B6 mice that had been treated 5 days earlier with α-GalCer or vehicle. In several experiments, B220⁺ or CD19⁺ cells were depleted. Recipient mice were euthanized 3 hours later, and CFSE-labeled target cells were detected among HMNCs by flow cytometry. A constant number of CFSE^{low} events, typically 2×10³, were acquired for each sample, and percent specific killing of target cell populations was calculated using the following equation: % specific cytotoxicity = {1 - [(CFSE^{high} event number in recipient mouse ÷ CFSE^{low} event number in recipient mouse) ÷ (CFSE^{high} event number among

mixed target cells before injection \div CFSE^{low} event number among mixed target cells before injection)]} \times 100.

4.2.11 Lung metastasis model

B6 mice received α -GalCer or vehicle 24 hours prior to an i.v. injection of 5×10^5 B16-F10 melanoma cells. In a limited number of experiments, α -GalCer-treated animals also received an anti-B220 mAb or an anti-CD19 mAb as described above. Fourteen days after melanoma cell injections, α -GalCer- and/or vehicle-treated mice were euthanized, and pulmonary metastatic nodules were enumerated.

4.2.12 Statistical analyses

Statistical comparisons were made using Graphpad Prism 5 software. We used unpaired Student t-tests and ANOVA as appropriate. Statistically significant differences were reported as $p < 0.05$, $p < 0.01$ and $p < 0.001$, which were denoted by *, ** and ***, respectively.

4.3 Results

4.3.1 *In vivo* priming with α -GalCer gives rise to a suddenly enlarged tissue-resident population with phenotypic pre-mNK cell characteristics

While investigating hepatocellular responses to α -GalCer, we found a prominent cell population with forward and side scatter profiles consistent with those of lymphocytes (Fig. 4.1A). At first glance, this was not surprising since α -GalCer induces an early and robust proliferative burst in the iNKT cell compartment (33). Intriguingly, however, a substantial proportion of the enlarged population expressed CD11c, a classic mouse DC marker that is not typically expressed by iNKT cells. This prompted us to further their characterization. We found them to also express B220 and NK1.1 (Fig. 4.1A), which are usually considered B cell/pDC and NK cell markers, respectively. The only cell type that concomitantly expresses CD11c, B220 and NK1.1 is the pre-mNK cell (23, 24) whose tissue presence has been reported in the bone marrow, spleen and lymph nodes (23, 24, 26, 34, 35), but never in the liver before.

Wright-Giemsa staining of sorted B220⁺CD11c⁺NK1.1⁺ cells revealed mononuclear cells with a basophilic cytoplasm (Fig. 4.2). Upon further cytofluorimetric analyses, these were T/B lineage-negative (CD3⁻/CD19⁻) cells that also stained positively with monoclonal antibodies (mAbs) against CD49b, CD122 (IL-2R β) and MHC class II molecules (Fig. 4.1B), which established their identity as pre-mNK cells at least by immunophenotypic standards (36).

We confirmed that the observed pre-mNK cell accumulation was secondary to iNKT cell activation since it could be significantly reversed by a CD1d-blocking mAb (clone 20H2) (Fig. 4.1C). This mAb was efficient in preventing iNKT cell responses since IL-4 and IFN- γ were virtually undetectable in the serum 2 and 6 hours after α -GalCer injection, respectively (Fig. 4.1C).

Our kinetic experiments demonstrated gradual accumulation of pre-mNK cells after glycolipid priming, which became evident after 24 hours and reached a plateau between three and five days (Fig. 4.3A). It was most pronounced in the liver, but could also be observed within the spleen and the lungs (Fig. 4.3B).

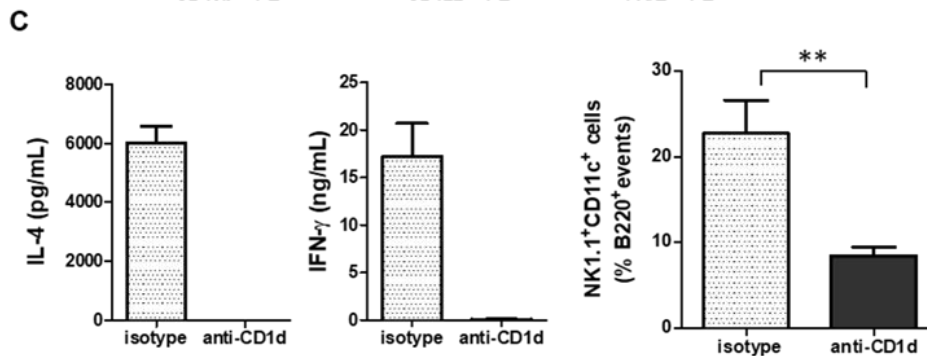
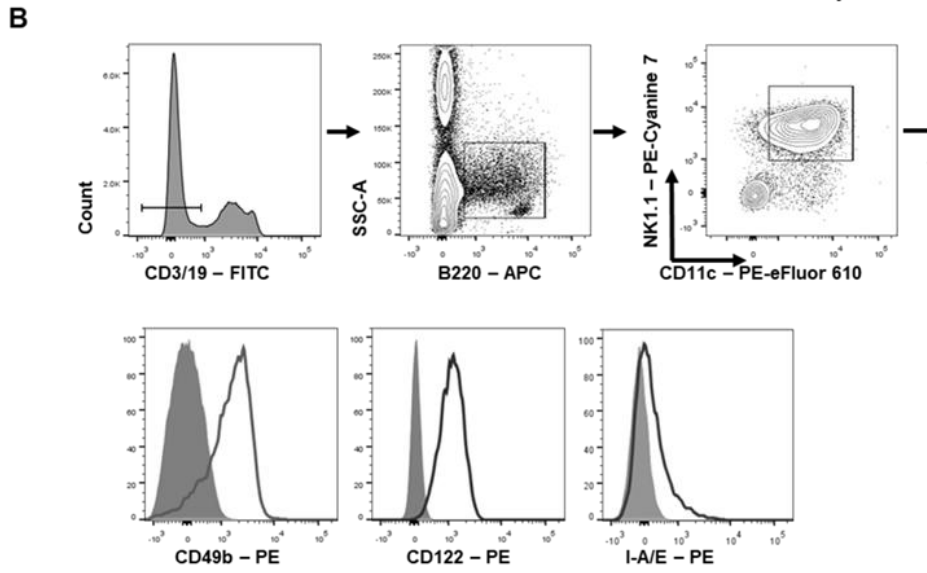
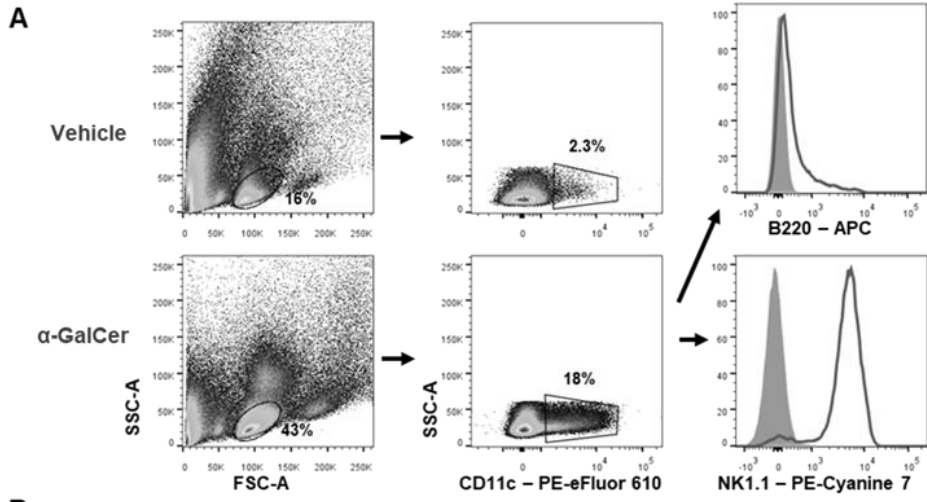


Figure 4.1: *In vivo* stimulation of iNKT cells with α -GalCer enlarges a hepatocellular population phenotypically resembling pre-mNK cells.

(A) B6 mice (n=3) were injected i.p. with either vehicle or α -GalCer. Five days later, HMNCs were isolated and analyzed cytofluorimetrically for their forward and side scatter characteristics and for their reactivity with mAbs against CD11c, B220 and NK1.1. Representative plots are illustrated. (B) CD3-CD19- HMNCs from α -GalCer-treated mice (n=3) were further immunophenotyped using mAbs to B220, CD11c, CD49b, CD122 and I-A/E. (D) B6 mice (n=4/group) were pre-treated with a CD1d-blocking mAb or isotype control 2 hours before and once every 24 hours after α -GalCer administration as applicable. Animals were bled, and serum IL-4 and IFN- γ concentrations were quantified at 2 and 6 hours post- α -GalCer injection, respectively. On day 3, B220⁺CD11c⁺NK1.1⁺ cell frequencies among bulk HMNCs were determined by flow cytometry. Error bars represent mean \pm SEM values, and ** denotes a statistically significant difference with p<0.01 using unpaired Student t-test. Filled histograms in A and C represent staining with appropriate isotype controls.

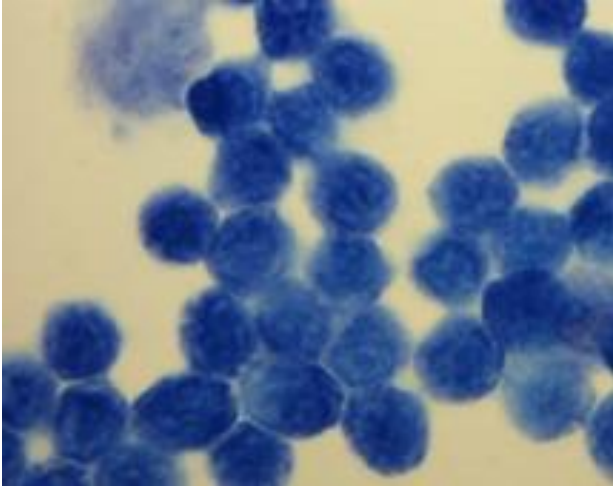


Figure 4.2: B220⁺CD11c⁺NK1.1⁺ cells are mononuclear with a basophilic cytoplasm.

B220⁺CD11c⁺NK1.1⁺ cells from α -GalCer-treated mice were sorted, pooled (n=2 in each of two independent experiments) and stained with Wright-Giemsa

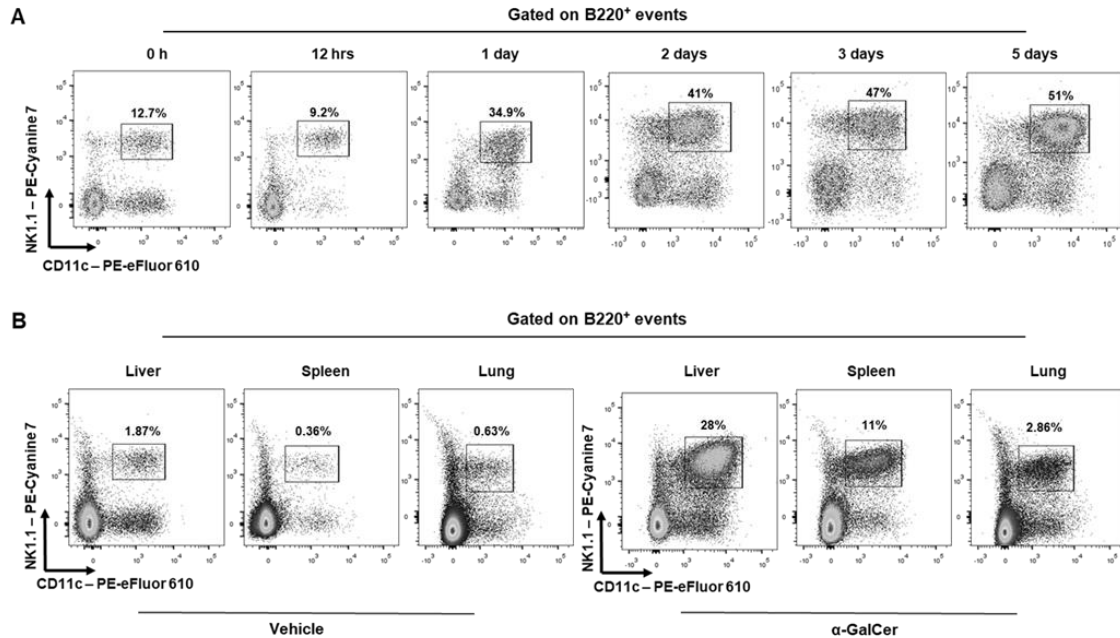


Figure 4.3 α -GalCer-induced pre-mNK cell accumulation is rapid and relatively widespread.

(A) The kinetics of hepatic pre-mNK cell accumulation was studied in α -GalCer-primed mice ($n=3$). (B) Five days after vehicle or α -GalCer administration ($n=4$ /group), the frequencies of B220⁺CD11c⁺NK1.1⁺ pre-mNK cells were determined among bulk splenocytes and non-parenchymal hepatic or pulmonary mononuclear cells. Representative plots from two independent experiments are shown.

4.3.2 **Transactivated pre-mNK cells lose their B220 expression and mature upon adoptive transfer**

The functional maturity of cells belonging to the NK lineage has been linked to their differential expression of CD27 and CD11b (37). We found CD27^{high}CD11b^{low} cells, which are among the most immature subsets of NK lineage cells (36, 37), to comprise a sizeable proportion of our population of interest (Fig. 4.4). It was therefore pertinent to determine whether and how quickly α -GalCer-transactivated hepatic pre-mNK cells may mature. This is experimentally achieved via adoptive cell transfer into another animal and monitoring for a loss of B220 (28). To this end, α -GalCer was administered to GFP-transgenic (GFP⁺) C57BL/6 (B6) mice followed, 5 days later, by cytofluorimetric isolation of hepatic B220⁺CD11c⁺NK1.1⁺ (pre-mNK) and B220⁻NK1.1⁺ (control ‘conventional’ NK) cells, which were then transferred into separate cohorts of wildtype (WT) B6 mice and tracked. As hypothesized, pre-mNK cells lost their B220 dramatically on day 5 and almost completely on day 15 post-transfer (Fig. 4.5). We also noticed a gradual disappearance of CD11c⁺ cells, albeit to a much lesser extent. The loss of cell surface receptor expression was not a global effect because NK1.1 and CD122 levels were maintained in both pre-mNK and conventional NK cells (Fig. 4.5).

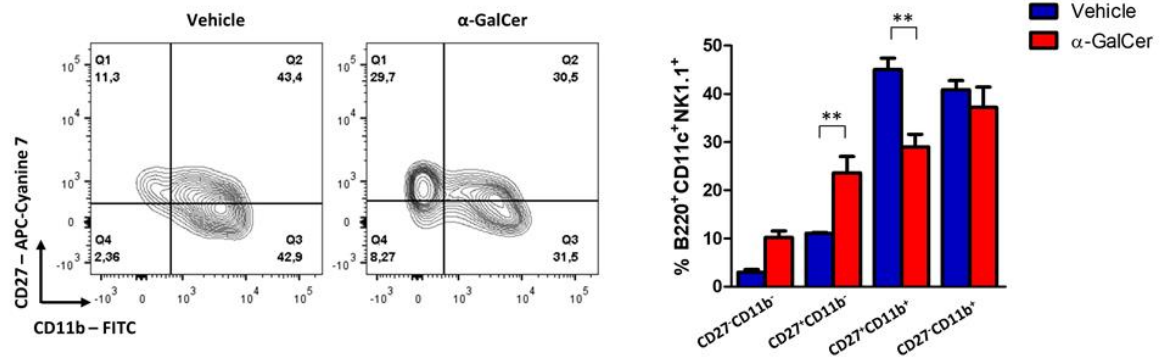


Figure 4.4: Treatment with α -GalCer increases the frequency of immature CD27⁺CD11b⁻ pre-mNK cells among HMNCs

Five days after treatment with α -GalCer or vehicle, HMNCs were isolated and the expression of CD27 and CD11b on pre-mNK cells was assessed by flow cytometry. Representative plots (left panel) and cumulative data from 3 mice per group are illustrated. Error bars represent SEM. Statistical comparisons were made using two-way ANOVA with Bonferroni post-hoc analysis. ** denotes $p < 0.01$.

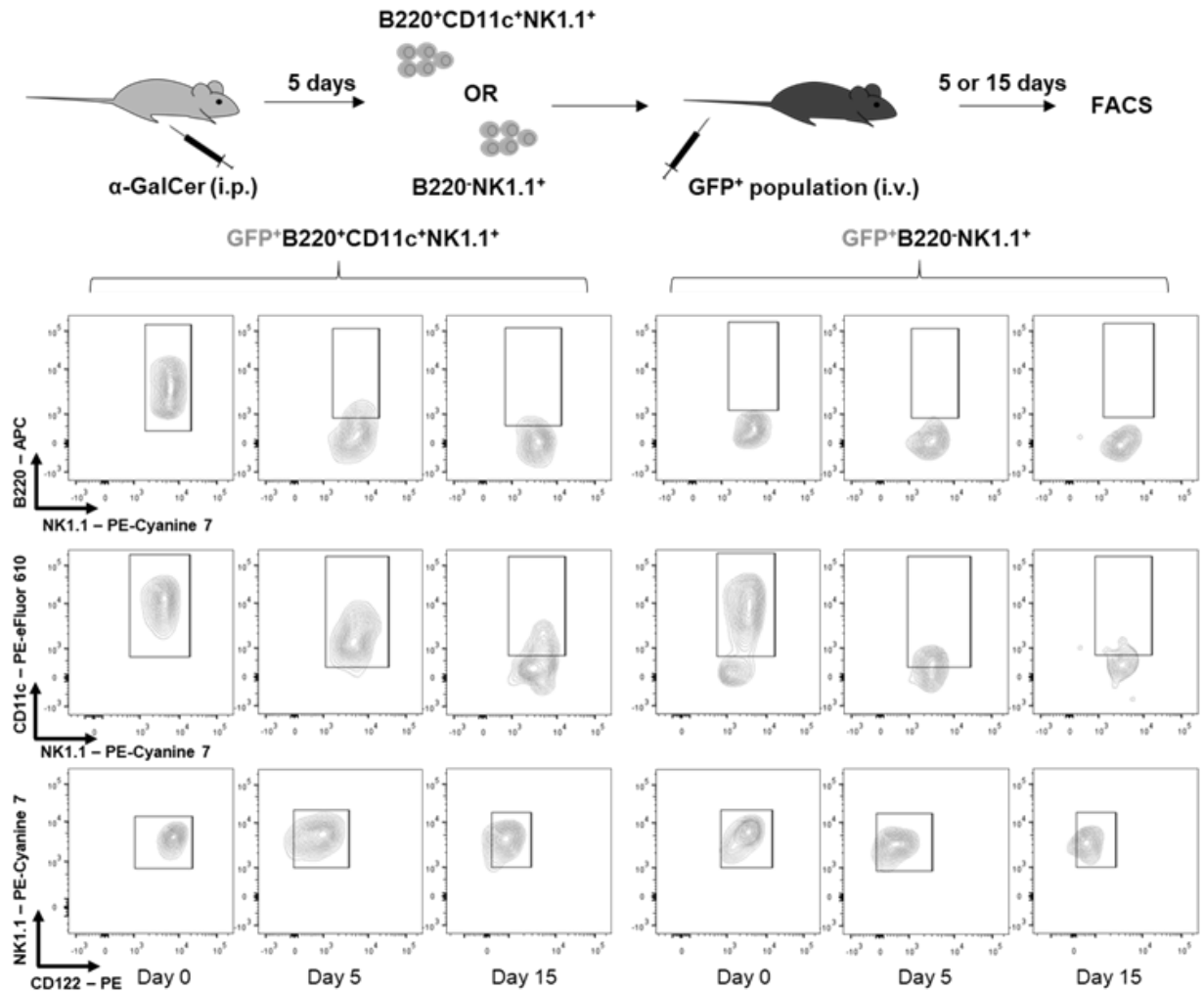


Figure 4.5: α -GalCer-transactivated pre-mNK cells lose B220 expression upon adoptive transfer.

Five days after α -GalCer administration to GFP⁺ mice, hepatic B220⁺CD11c⁺NK1.1⁺ and B220⁻NK1.1⁺ cell populations were FACS-sorted and transferred into separate cohorts of WT B6 recipients (n=2 per group). Mice were sacrificed on day 5 or day 15 post-adoptive transfer for their liver in which GFP⁺ cells were traced and examined for their expression levels of B220, CD11c, NK1.1 and CD122. Representative FACS plots are illustrated.

4.3.3 α -GalCer triggers hepatic pre-mNK cell proliferation *in situ*

Next, we set out to determine whether intrahepatic pre-mNK cells had expanded locally or emigrated from other sites. We performed a comprehensive gene expression analysis of pre-mNK cells sorted from α -GalCer- (n=3) and vehicle-treated mice (n=12). The proliferation marker Ki67 was the most upregulated gene, with a 16-fold increase in mRNA transcript levels in pre-mNK cells from α -GalCer-treated mice relative to control (Fig. 4.6A). Proliferating cell nuclear antigen (Pcna) was also increased 4-fold (Fig. 4.6A). In contrast, there was a 4-fold drop in the expression of sphingosine-1-phosphate receptor 1 (S1pr1) (Fig. 4.6A), which encodes a G protein-coupled receptor that mediates lymphocyte egress from lymphoid organs and their trafficking into the periphery (38). These results suggested that pre-mNK cells had undergone rigorous proliferation following α -GalCer administration as opposed to infiltrating the liver.

We validated the observed changes in Ki67 and S1PR1 expression at the protein level (Fig. 4.6B-C). In addition, we examined the surface expression of CD69 on hepatic pre-mNK cells since CD69 can directly suppress S1PR1 and, in doing so, serves as a tissue retention molecule (39, 40). We found an inverse correlation between S1PR1⁺ and CD69⁺ pre-mNK cell frequencies in α -GalCer-treated animals. Accordingly, a relatively sharp decline in the former population was reciprocally accompanied by an equally sharp rise in the latter on day 1 post-glycolipid injection (Fig. 4.6C).

To more definitively rule out the possibility that α -GalCer mobilizes pre-mNK cells to amass in select tissues, we employed two separate doses of FTY720 (aka. fingolimod), an immunomodulatory compound that causes S1PR1 downregulation and inhibits

lymphocytes' emigration from lymphoid organs (38). FTY720 administration 2 hours before and 24 hours after α -GalCer injection significantly lowered T cell numbers, but not pre-mNK cell numbers in the liver and in the spleen (Fig. 4.6D).

Collectively, the above findings indicate that tissue-resident pre-mNK cells downregulate their expression of S1PR1, upregulate CD69, and proliferate locally in response to systemic α -GalCer treatment.

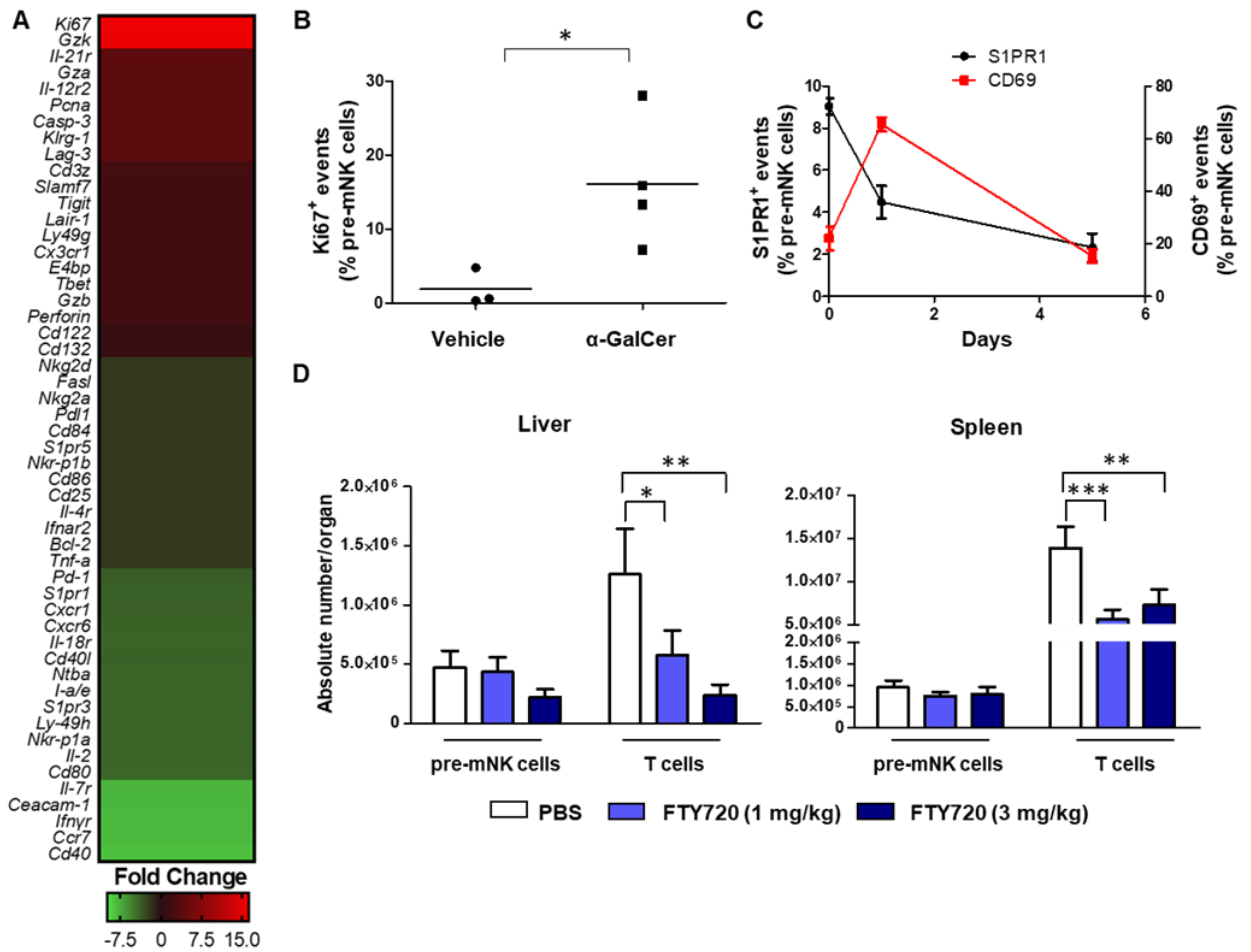


Figure 4.6: Tissue accumulation of pre-mNK cells following α -GalCer treatment is due to their *in situ* expansion.

(A) B6 mice were injected i.p. with vehicle (n=12) or α -GalCer (n=3). Two days later, pre-mNK cells were FACS-sorted and pooled before RNA was extracted for quantitative RT-PCR. A heat map was generated to demonstrate fold changes in transcript levels of indicated genes in pre-mNK cells isolated from α -GalCer-primed mice relative to those sorted from vehicle-treated controls. (B) Two days after treatment with vehicle- or α -GalCer, HMNCs were stained for surface B220, CD11c and NK1.1 as well as intracellular Ki67, and the percentages of Ki67⁺ cells among pre-mNK cells were determined by flow cytometry. Each symbol represents an individual mouse. (C) Surface expression of CD69 and S1PR1 by hepatic pre-mNK cells was assessed at indicated time points after α -GalCer administration (n=4/time point). Error bars represent SEM. (D) Mice were injected with indicated doses of FTY720 or with PBS 2 hours before and 24 hours after they received α -GalCer. Five days later, HMNCs and splenocytes were harvested and subjected to staining with anti-CD3, -B220, -CD11c and -NK1.1 mAbs. B220⁺CD11c⁺NK1.1⁺ pre-mNK cells and CD3⁺NK1.1⁻ T cells were then enumerated by flow cytometry. Error bars represent SEM for 6 mice per group from two independent experiments. Statistical comparisons were made using unpaired Student's t-test (B) or a two-way ANOVA with Bonferroni post-hoc test (D). *, ** and *** denote differences with p<0.05, p<0.01 and p<0.001, respectively.

4.3.4 **IL-12 and IL-18 mediate pre-mNK cells' tissue expansion following glycolipid administration**

We sought to uncover the mechanism underlying pre-mNK cell expansion in our system. α -GalCer stimulation of iNKT cells induces the production of a myriad of cytokines that modify the biological behavior of multiple downstream effector cell types. Our transcript analyses pointed to altered expression of several cytokine receptors in transactivated pre-mNK cells (Fig. 4A), suggesting that inflammatory cytokines may be involved. IL-15 is known to promote pre-mNK cell expansion (41). However, α -GalCer treatment did not change the expression levels of two IL-15R subunits, namely Cd132 [common γ (γ c) chain, which is also shared by receptors for IL-2, -4, -7, -9 and -21] and Cd122 (which constitutes a component of both IL-2R and IL-15R) (Fig. 4.4A). We did not include IL-15 α in our array simply because pre-mNK cells are reportedly devoid of this molecule (41). IL-15 α is found instead on the surface of Ag-presenting cells that trans-present IL-15 to other immunocytes (42).

We then shifted our focus onto other cytokines that activate NK cells and other innate-like lymphocytes. Of note, Il-21r and Il-12r2 transcripts were each elevated over 4-fold (Fig. 4.6A). *In vivo* blockade of CD132 before and after α -GalCer administration failed to prevent pre-mNK cell expansion, thus ruling out a role for γ c chain cytokines, including IL-15 and IL-21 (data not shown). We then zeroed in on IL-12 and cytokines with which it synergizes. We injected mice with recombinant IL-12, IL-18 and IFN- γ in several combinations in lieu of α -GalCer. The only combination that induced hepatic pre-mNK cell accumulation was IL-12 plus IL-18 (Fig. 4.7A). We also observed a moderate

reduction in IL-18r mRNA levels two days after α -GalCer treatment (Fig. 4.6A), a finding we validated by flow cytometry at a slightly later time point. Three days after the injection of α -GalCer or vehicle, $36.5 \pm 2.4\%$ and $62.3 \pm 1.4\%$ of hepatic pre-mNK cells expressed CD218a (IL-18Ra), respectively (n=3/group). In addition, we found a marginal decrease in the gMFI of CD218a in α -GalCer-primed animals ($1,180 \pm 4$) compared with controls ($1,317 \pm 19$). These changes likely reflect activation-induced IL-18R downregulation at later time points following α -GalCer treatment.

Previous studies have shown that serum IL-12 reaches its peak level at 6 hours post- α -GalCer injection (43, 44). In addition, IL-18 typically follows IL-12 and plateaus between 12 and 24 hours after *in vivo* priming with select inflammatory stimuli (45). In order to first demonstrate that circulating cytokines mediate the accumulation of pre-mNK cells, we obtained serum samples at several time points after α -GalCer treatment, which were then pooled and transferred into naïve B6 mice (Fig. 4.7B). This was followed by pre-mNK cell enumeration in the liver. Transferring pooled sera prepared at 2-, 6, or 12-hour time point did not result in pre-mNK cell expansion, and neither did combined sera collected at 6- and 24-hour time points (Fig. 4.7B). Strikingly, however, a combination of samples from 6- and 12-hour time points worked synergistically to recapitulate the effect of α -GalCer (Fig. 4.7B). Of note, in these experiments, we injected a CD1d-blocking mAb into the recipients prior to serum transfer. This was to avoid false positive results due to the presence of free-floating α -GalCer in transferred sera.

The above time points are consistent with the peaks of serum IL-12 and IL-18 among other soluble mediators. To confirm the roles of IL-12 and IL-18, we used a previously established protocol to remove cytokines from serum samples. Unlike cytokine-replete

sera, IL-12/IL-18-depleted samples failed to expand hepatic pre-mNK cells (Fig. 4.7C). Therefore, α -GalCer-triggered pre-mNK cell expansion is dependent on the synergistic functions of IL-12 and IL-18.

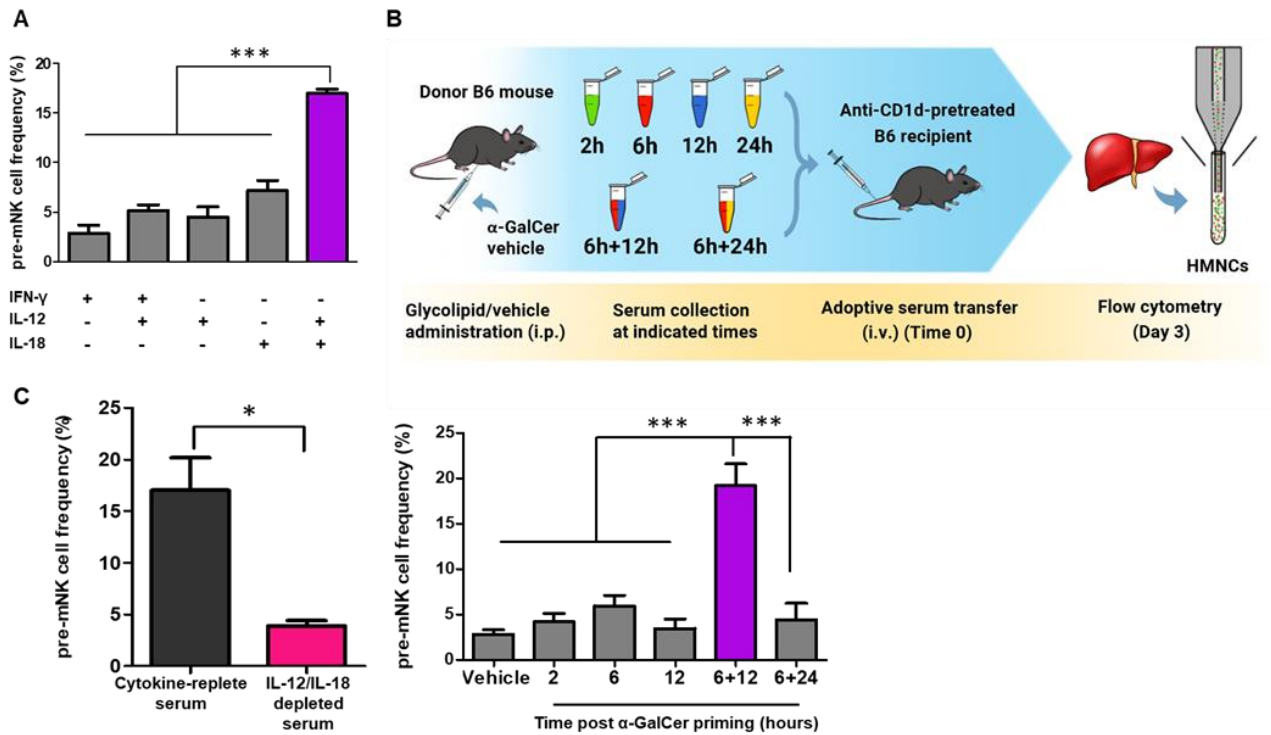


Figure 4.7: Hepatic pre-mNK cell expansion in response to α -GalCer is dependent on IL-12 and IL-18.

(A) Naïve B6 mice (n=3-4 per group) were injected with indicated recombinant cytokines followed, 3 days later, by determination of pre-mNK cell frequencies in the liver by flow cytometry. (B) B6 mice were primed with α -GalCer or injected with vehicle before they were bled at indicated time points. Sera were isolated, pooled as indicated, and adoptively transferred into B6 mice (n=3/group) that had received a CD1d-blocking mAb two hours

earlier. Three days after serum transfer, hepatic pre-mNK cell frequencies were determined by flow cytometry. (C) Sera collected at 6 and 12 hours post- α -GalCer injection were pooled. An aliquot was depleted of IL-12 and IL-18. Cytokine-replete and -depleted serum samples were then transferred into separate cohorts of anti-CD1d-pretreated mice (n=3) and pre-mNK cell percentages were determined. Error bars represent SEM. Statistical analyses were performed using a one-way ANOVA with Tukey's post-hoc analysis (A) or unpaired Student's t-test (C). * and *** denote differences with $p < 0.05$ and $p < 0.001$, respectively.

4.3.5 α -GalCer treatment causes pre-mNK cells to lose their cross-presentation capacity

Splenic and bone marrow-derived pre-mNK cells are capable of cross-presenting peptide Ags to T cells, thus engaging the adaptive arm of antitumor immunity (35, 46, 47). We explored whether α -GalCer-transactivated hepatic pre-mNK cells can similarly serve as Ag-presenting cells. When pulsed with SIINFEKL, a synthetic peptide corresponding to the immunodominant epitope of chicken ovalbumin (OVA257-264), purified hepatic pre-mNK cells were able to activate B3Z cells (Fig. 4.8A), a CD8⁺ hybridoma line that recognizes SIINFEKL in the context of H-2Kb. Therefore, hepatic pre-mNK cells had retained their MHC-I expression and peptide presentation capacity. It was, however, more important to determine whether pre-mNK cells' *in vivo* interactions with malignant cells could result in tumor Ag-derived peptide cross-presentation. To this end, we inoculated vehicle- and α -GalCer-pretreated B6 mice with EG7-OVA lymphoma cells via tail vein. Four days later, non-parenchymal hepatic mononuclear cells (HMNCs) were isolated, and

the percentage of SIINFEKL-presenting pre-mNK cells was determined using a mAb against SIINFEKL:H-2Kb complexes (clone 25-D1.16)(30). Interestingly, a “25-D1.16⁺” fraction was clearly detectable among pre-mNK cells sorted from vehicle-treated mice, and at a lowered frequency among those isolated from glycolipid-treated animals (Fig. 4.8B). Therefore, α -GalCer administration impairs Ag cross-presentation by pre-mNK cells, a finding that is also consistent with decreased I-a/e, Cd40 and Cd80 transcript levels (Fig. 4.6A).

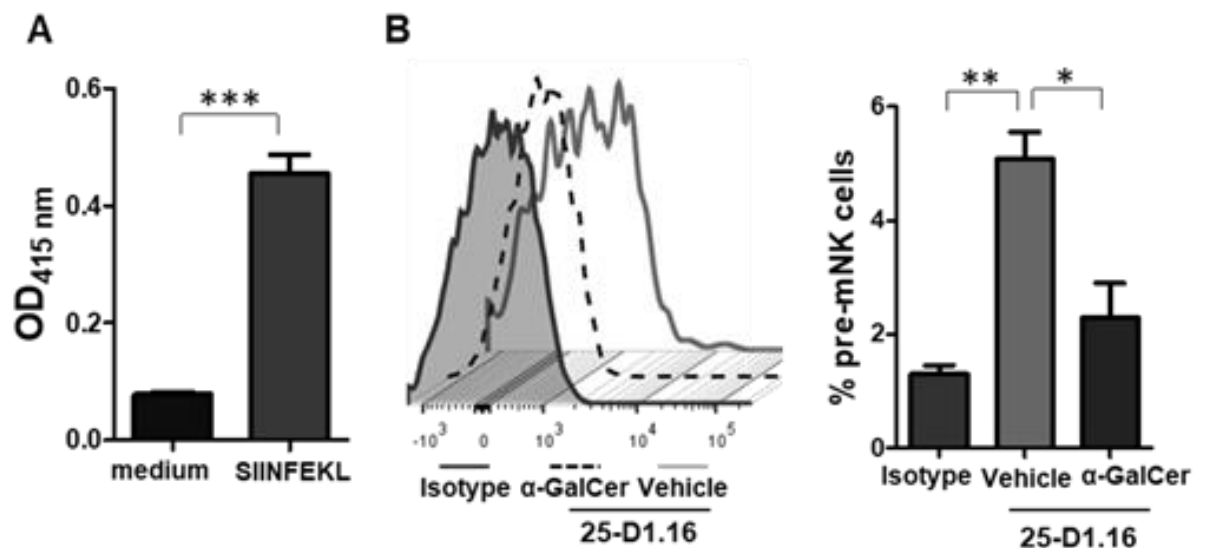


Figure 4.8: α -GalCer-transactivated hepatic pre-mNK cells are unable to cross-present tumor Ags.

(A) B6 mice (n=4 from two independent experiments) were euthanized 5 days after they were injected with α -GalCer. Hepatic pre-mNK cells were FACS-sorted and pulsed with SIINFEKL before they were co-cultured with B3Z cells. After 24 hours, cells were lysed in the presence of ONPG, and the OD415nm of supernatants was determined 4 hours later.

(B) EG7-OVA tumor cells were injected i.v. into B6 mice that had received α -GalCer- or

vehicle 24 hours earlier (n=3 per group). Four days after the metastatic tumor challenge, SIINFEKL:H-2Kb complexes were detected via staining with 25-D1.16. Histograms representing two independent experiments yielding similar results are depicted. Error bars represent SEM. An unpaired Student's t-test (A) and one-way ANOVA with Tukey's post-hoc analysis (B) were used for statistical comparisons. *p<0.05, **p<0.01, ***p<0.001.

4.3.6 **α -GalCer-transactivated pre-mNK cells kill tumor cells via granule exocytosis and exhibit anti-metastatic activity**

Pre-mNK cells reportedly express TRAIL and kill tumor targets (23). It was therefore critical to test whether the expanded hepatic pre-mNK cells retained their oncolytic arsenal and functions. We found unfractionated HMNCs from α -GalCer-treated mice to readily and dose-dependently destroy YAC-1 lymphoma cells (Fig. 4.9), a classic mouse NK cell target. To directly assess pre-mNK cell-mediated cytotoxicity, we also employed purified hepatic pre-mNK cells from glycolipid-primed animals against YAC-1 cells. In certain experiments, CMA or a combination of EGTA and MgCl₂ was added to co-cultures. CMA increases the pH of lytic granules to accelerate the degradation of perforin (48). EGTA/MgCl₂ chelates extracellular Ca⁺⁺, which is required at several steps during the perforin/GZM pathway (49, 50). Purified pre-mNK cells from α -GalCer-treated mice could efficiently lyse YAC-1 cells (Fig. 4.10A). However, this response was completely abolished in the presence of either EGTA/MgCl₂ or CMA in co-cultures (Fig. 4.10A), clearly implicating the granule exocytosis pathway in our system. This notion was supported by increased expression of CD107a, a degranulation marker, among hepatic pre-mNK cells after they had established an immunological synapse with YAC-1 cells (Fig.

4.10B). To identify the cytotoxic effector molecules pre-mNK cells utilized to kill tumor cells, we determined the frequencies of FasL⁺, TRAIL⁺, TNF- α ⁺, GZM A⁺ and GZM B⁺ pre-mNK cells from α -GalCer-treated mice after they had engaged YAC-1 cells. Surprisingly, GZM A, but not TRAIL (or any other mediators for that matter), was upregulated (Fig. 4.10C). Also interestingly, α -GalCer treatment alone had resulted in elevated levels of GZM A on a per cell basis as judged by the geometric mean fluorescence intensity (gMFI) of its staining (Fig. 4.10C).

To measure pre-mNK cell-mediated cytotoxicity *in vivo*, we modified and used a previously described protocol (51). We co-injected CFSE^{low} WT B6 splenocytes (control target cells) and CFSE^{high} β 2 microglobulin (β 2M)^{-/-} splenocytes (NK-sensitive target cells) into α -GalCer-treated mice. Labeled targets were tracked using their differential CFSE fluorescence. The peak corresponding to β 2M^{-/-} target cells was always smaller, indicating that they had been eliminated (Fig. 4.10D). To determine the partial contribution of pre-mNK and conventional NK cells, we used an anti-B220 mAb that depletes the former but not the latter. This approach resulted in significantly reduced cytotoxicity (Fig. 4.10D), indicating that pre-mNK cells were partially responsible for elimination of β 2M^{-/-} target cells. To rule out a role for B220⁺ B cells in this model, we used an anti-CD19 mAb in parallel. This mAb should remove B cells but not pre-mNK cells. As anticipated, anti-CD19 administration failed to diminish the lysis of β 2M^{-/-} cells (Fig. 4.10D).

Finally, we tested the anti-metastatic capacity of transactivated pre-mNK cells using the B16-F10 melanoma model. This model was chosen because it is responsive to α -GalCer treatment (52), which we first verified (Fig. 4.11 and Fig. 4.10E). In addition, pre-mNK cells reportedly fulfill a protective role against metastatic B16-F10 melanoma (23). We

found depletion of B220⁺ cells, but not CD19⁺ cells, to partially repress α -GalCer's anti-metastatic activity and almost double the number of pulmonary metastatic nodules (Fig. 4.10E). Taken together, the above results demonstrate that α -GalCer selectively boosts the expression of GZM A by hepatic pre-mNK cells and imparts a primarily oncolytic and anti-metastatic phenotype to these unique cytotoxic lymphocytes.

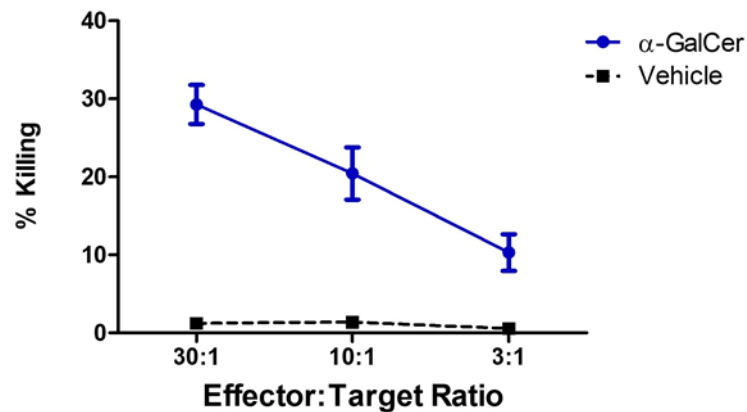


Figure 4.9: Bulk HMNCs from α -GalCer-treated mice are able to lyse YAC-1 cells.

HMNCs were isolated from vehicle- or α -GalCer-treated mice and used against ⁵¹Cr-labeled-YAC-1 target cells at indicated ratios. Percent specific cytotoxicity was calculated after 4 hours using a formula that is described in Methods. Error bars represent SEM (n=3).

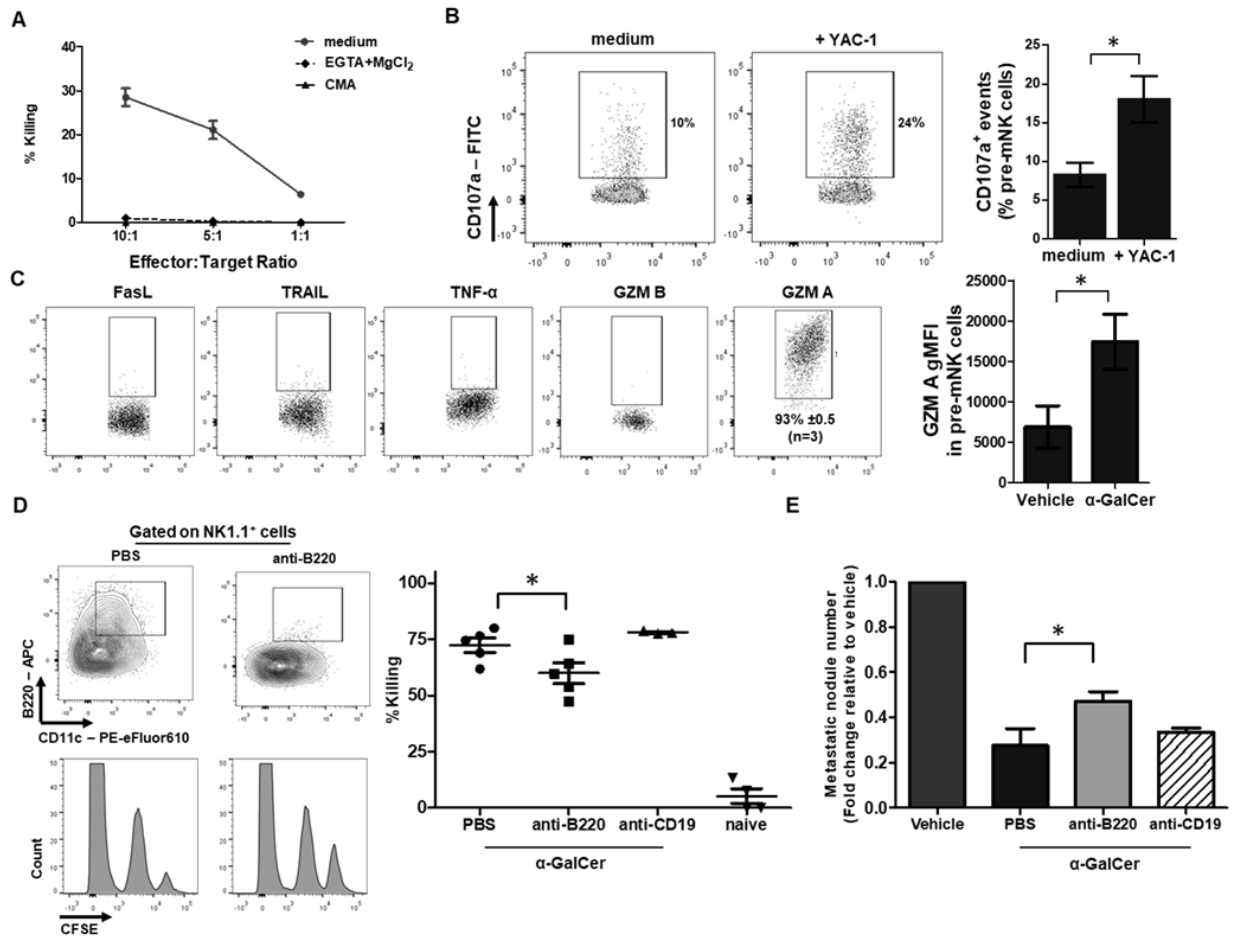


Figure 4.10: Transactivated pre-mNK cells are oncolytic and partially protective against metastatic B16 melanoma.

(A) Hepatic pre-mNK cells were FACS-sorted from α -GalCer-primed mice ($n=4$) and used as effector cells against ^{51}Cr -labeled YAC-1 target cells in 4-hour co-cultures containing or lacking a combination of EGTA and MgCl_2 or CMA. The ^{51}Cr activity of culture supernatants was quantitated using a γ -counter. (B) Bulk HMNCs were isolated from α -GalCer-treated mice and co-incubated with YAC-1 cells in the presence of BFA and monensin. Four hours later, the surface expression of CD107a on pre-mNK cells was determined by flow cytometry. Representative plots and cumulative data from two independent experiments ($n=5$ per group) are shown. (C) HMNCs from α -GalCer-primed

mice (n=3) were co-incubated with YAC-1 cells in the presence of BFA before surface or intracellular staining for indicated effector molecules. The expression of each molecule by pre-mNK cells was analyzed by flow cytometry. In addition, the gMFI of GZM A staining in pre-mNK cells is shown shortly after HMNC isolation (n=3/group). (D) CFSE^{low} WT splenocytes (control target cells) and CFSE^{high} $\beta 2M^{-/-}$ splenocytes (MHC-I-deficient target cells) were mixed in equal numbers and injected i.v. into naïve mice or mice that had been primed with α -GalCer and also injected with an anti-B220 mAb, an anti-CD19 mAb or PBS as described in Methods. Three hours later, target cells were identified in the liver using their differential CFSE fluorescence, and percent *in vivo* cytotoxicity against each target population was calculated using a formula that is also provided in the Methods. Representative contour plots demonstrate the efficacy of anti-B220 in depleting pre-mNK cells. For *in vivo* killing assays, representative histograms and cumulative data from two independent experiments are shown. (E) Five hundred thousand B16-F10 melanoma cells were injected i.v. into vehicle-treated mice or α -GalCer-primed animals that had previously received anti-B220, anti-CD19, or PBS. Two weeks later, metastatic nodules in the lungs were enumerated. Results are depicted as fold change in nodule numbers relative to vehicle-treated mice in three independent experiments. All error bars represent SEM. Statistical analyses were performed using Student's t-tests (B and C) or one-way ANOVA with Neuman-Keuls post-hoc test (D and E). * denotes $p < 0.05$.

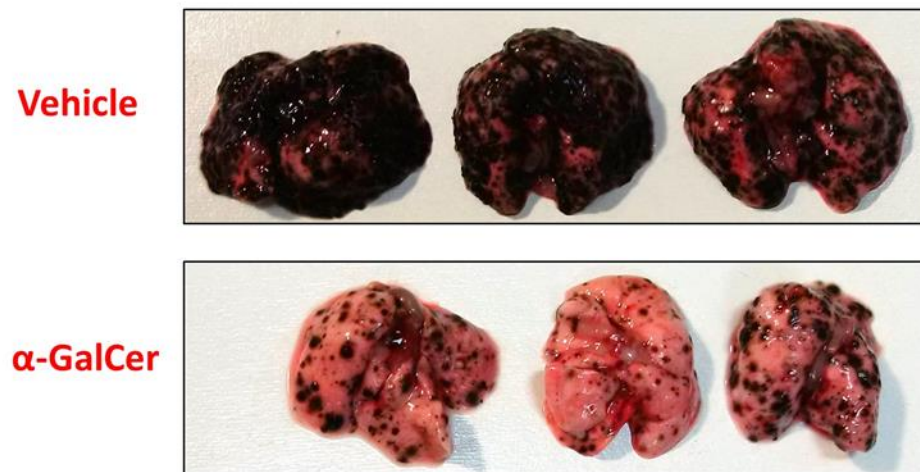


Figure 4.11: α -GalCer administration lowers the pulmonary metastatic burden of B16-F10 melanoma.

Five hundred thousand B16-F10 melanoma cells were injected *i.v.* into B6 mice that had been pretreated 24 hours earlier with either vehicle or α -GalCer. Two weeks after the injection of tumor cells, lungs were harvested and imaged. Representative images shown from two independent experiments are shown.

4.4 Discussion

Although pre-mNK cells were discovered more than a decade ago, only a precious little is known about immunoregulatory mechanisms that control or modify their abundance and activities. In this work, we have identified and characterized a novel population of pre-mNK cells that amass in select tissues, especially in the liver, following α -GalCer administration and that are endowed with potent cytolytic and anti-metastatic properties.

Previous investigations have found pre-mNK cells in the spleen, lymph nodes and bone marrow, at low frequencies (23, 24, 26, 34, 35). We now describe a liver- and lung-resident population that can be dramatically enlarged through α -GalCer priming, likely due to their local expansion as opposed to their recruitment from other sites. This is in agreement with the notion that pre-mNK cells are rapidly recycling cells by nature (28). We found increased levels of Ki67 and CD69, decreased S1PR1 expression, and the failure of FTY720 to prevent the observed accumulation. We are cognizant of previous reports that S1PR1 and S1PR5 can both mediate NK cell trafficking (53, 54). However, we focused our efforts on FTY720-sensitive S1PR1 because our gene array analyses showed only a modest change in *S1pr5* transcript levels in pre-mNK cells (Fig. 4.6A). Interestingly, Walzer *et al.* found that NK cells from *S1PR5*^{-/-} mice were unable to home to several tissue compartments with the notable exception of the liver (53), the prominent site of pre-mNK cell accumulation following α -GalCer treatment in our model. Although tissue recruitment was not responsible for the observed numerical rise in hepatic pre-mNK cells, it will be important to explore the migratory properties of these cells in the future.

Pre-mNK cell differentiation is typically dependent on IL-15 receptor signaling (41), and their proliferation can be triggered by a combination of IL-15 and IL-18 (27). However, α -GalCer treatment did not alter IL-15 receptor levels in hepatic pre-mNK cells, and our mechanistic experiments revealed a previously unappreciated role for IL-12 and IL-18 in pre-mNK cell expansion. We also found a higher percentage of IL-18R⁺ pre-mNK cells in vehicle-treated mice when compared with their α -GalCer-transactivated counterparts. IL-18R downregulation in α -GalCer-treated mice may have followed an early burst of IL-12, which may have initially elevated the expression level of IL-18R. IL-12-induced IL-18R upregulation has been previously reported in other cell types (55). IL-12 and IL-18 are known for their ability to activate innate and innate-like lymphocytes, and our findings widen their range of functions by introducing pre-mNK cells as one of their cellular targets.

The cross-talk between iNKT and pre-mNK cells represents a new intercellular communication cascade following treatment with α -GalCer. The importance of this cascade is several-fold. First, pre-mNK cells can now be viewed as a downstream effector cell type mediating some of the beneficial effects of α -GalCer. Given pre-mNK cells' phenotypic and functional similarities to several other cell types (e.g., DCs and mNK cells), they may have been given a mistaken identity in the past. Many investigations have taken advantage of an anti-NK1.1 mAb to delineate the roles of α -GalCer-transactivated NK cells in destroying tumor cells *in vitro* (56, 57) and in lowering metastatic tumor burden *in vivo* (58). While this approach is widely accepted and still valid, it may ignore pre-mNK cells' potential participation in such responses. In this study, pre-mNK cells were indeed partially responsible for oncolytic and anti-metastatic activities of α -GalCer. By the same token, Fujii *et al.* described a splenic CD3⁻CD11c⁺ population capable of producing IFN- γ two

hours after an i.v. injection of α -GalCer (12). This was before IKDCs/pre-mNK cells found their way into the literature. Therefore, the possibility that such IFN- γ -producing “DCs” may have contained a pre-mNK cell component is not far-fetched.

Pre-mNK cells can serve as Ag-presenting cells in adaptive immunity (24, 35, 46, 47). They typically express MHC-I and MHC-II, which should enable them to activate CD8⁺ and CD4⁺ T cells. We were able to detect SIINFEKL:H-2Kb complexes on the surface of hepatic pre-mNK cells after i.v. inoculation of an OVA-expressing lymphoma cell line. This provides direct evidence in support of a role for pre-mNK cells in tumor Ag cross-presentation *in vivo*. Interestingly however, while α -GalCer-transactivated pre-mNK cells maintained their expression of MHC-I, they lost their ability to cross-present SIINFEKL. This may be due, at least partially, to the expansion of the CD27⁺CD11b⁻ fraction of hepatic pre-mNK cells by α -GalCer (Fig. 4.4) since Terme *et al.* reported that pre-mNK cells' cross-presentation capacity resides within their CD11b⁺ subset (46).

α -GalCer-transactivated hepatic pre-mNK cells exhibited cytotoxicity against NK targets, namely YAC-1 thymoma and β 2M^{-/-} splenocytes. They relied on granule exocytosis for their oncolytic function and expressed high levels of GZM A, which was evident even before conjugate formation with YAC-1 cells. The tumoricidal activity of pre-mNK cells was initially reported to be TRAIL-dependent (23). However, α -GalCer-transactivated pre-mNK cells lack TRAIL and likely employ GZM A to kill tumor targets. Of note, we also noticed a ~16-fold increase in GZM K expression by these cells at the mRNA level (Fig. 4.6A). However, a reliable anti-mouse GZM K mAb is not available at this point to enable validation of this finding at the protein level.

The above results, together with the intact anti-metastatic activity of α -GalCer-transactivated pre-mNK cells, indicate that exposure to this clinically relevant glycolipid skews pre-mNK cell responses away from an Ag-presenting phenotype in favor of NK-like antitumor behaviors. This may be a unique property of α -GalCer and potentially other glycolipid agonists of iNKT cells. In fact, stimulation with CpG oligodeoxynucleotides skews pre-mNK cells in the opposite direction and results in their loss of NK-like functions, upregulation of MHC-II and costimulatory molecules, and acquisition of DC-like Ag-presenting activity (24).

Taieb et al. found IKDCs/pre-mNK cells to produce more IFN- γ in comparison with mNK cells and to exert prolific cytotoxicity against B16-F10 melanoma cells (23). These cells proliferated in response to a combination of imatinib mesylate and IL-2 and infiltrated pulmonary metastases of B16-F10. *Taieb et al.* also isolated B220⁺NK1.1⁺ pre-mNK cells from mice that had been treated with imatinib plus IL-2 and adoptively transferred them into melanoma-bearing Rag2^{-/-}Il2rg^{-/-} mice. They found a lower tumor burden in animals that had received pre-mNK cells, but not in recipients of mNK cells. In our work, we used a WT, non-transgenic mouse model and cell-depleting mAbs to establish a similarly protective role for α -GalCer-transactivated pre-mNK cells. In contrast, *Wilson et al.* suggested that pre-mNK cell depletion augments the protective effects of adoptively transferred melanoma-specific CD4⁺ T cells in melanoma-bearing RAG^{-/-} mice (59). It is noteworthy that in order to deplete pre-mNK cells, the authors used a mAb (clone HB220) that targets the CD45RB isoform of B220. In our hands, this mAb was inefficient in appreciably removing pre-mNK cells (data not shown). In addition, the experimental systems used in the two studies are fundamentally different. We examined the efficacy of

α -GalCer and α -GalCer-transactivated pre-mNK cells in a pulmonary metastasis model in WT mice. By comparison, Wilson *et al.* addressed the role of pre-mNK cells in regulation of CD4⁺ T cell-mediated immunity against primary melanoma established in immunodeficient mice. Such stark differences likely account for the different conclusions reached.

Although pre-mNK cells may express higher levels of certain inflammatory and effector molecules in comparison with mNK cells on a per cell basis (23, 25), their therapeutic value is shadowed by their relative paucity. However, this limitation may be remedied by treatments that expand pre-mNK cells without hampering their anticancer potentials. This may be particularly important for tumors that arise from the liver or metastasize to this organ, for instance through the portal vein circulation, since hepatic pre-mNK cell expansion can be profound. We posit that iNKT cell-based glycolipid immunotherapies should be regarded as examples of such treatments.

CD56^{bright} and HLA-DR⁺ subsets of human NK cells have been proposed to be the mouse pre-mNK cell counterparts (36). In contrast to ‘classical’ CD56^{dim} NK cells, which are often considered the main executors of NK cell-mediated anti-tumor responses, the CD56^{bright} population has been viewed as an immunomodulatory subset. However, this paradigm was shifted by a recent report that upon priming with IL-15, CD56^{bright} NK cells exhibit markedly enhanced degranulation, cytotoxicity and cytokine production (60). In a phase I clinical trial, imatinib mesylate plus IL-2 expanded the HLA-DR⁺ NK cell population and improved the progression-free survival and overall survival of patients with refractory solid tumors (61). On the other hand, α -GalCer-based therapies have shown

promise in several clinical trials for cancer (4). It is tempting to speculate an additive or synergistic effect through combining imatinib, IL-2 and α -GalCer.

Finally, it will be interesting to determine whether the antitumor activities of pre-mNK cells can be further amplified through treatment with T_H1 -polarizing α -GalCer analogs such as α -C-GalCer (62) or α -GalCer/ α -C-GalCer pulsed DCs that may be superior to free-floating glycolipids (63). Much work lies ahead in characterizing circulating and tissue-resident pre-mNK cell subsets in mice and humans and in deciphering the regulatory mechanisms and therapeutic modalities that dictate or alter their functional attributes.

4.5 References

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Chapter 5

5. Discussion and Conclusion

5.1 Discussion

iNKT cells are a potent immunomodulatory population that serves as a bridge between innate and adaptive arms of immunity. The impact of iNKT cell activation on the overall immune response is truly pleiotropic and thus have tremendous potential as an immunotherapeutic. For instance, iNKT cells: i) are rapidly activated; ii) produce massive amounts of immunoregulatory cytokines and chemokines, iii) can transactivate many downstream effector cells; iv) have a range of endogenous and exogenous glycolipid agonists which provide more nuanced targeting approaches; and v) glycolipid agonists presented on the monomorphic CD1d molecule has direct implications for the clinic and importantly, circumvents many stumbling blocks associated with genetically diverse human populations. In this thesis, I outlined two disparate diseases in cancer and sepsis, where a dysregulated immune response contributes to a pivotal aspect of the pathogenesis. Moreover, whether immunomodulatory manipulation of iNKT cells via its glycolipid agonists to mitigate or reverse the immunopathology present in sepsis and cancer was unclear. To this end, I sought to illuminate the potential of glycolipid-mediated activation of iNKT cells as a therapeutic candidate in two distinct models of polymicrobial sepsis and cancer.

5.1.1 **INKT cells can be manipulated using a two-pronged glycolipid immunotherapy to improve sepsis morbidity, mortality, and immunosuppression.**

In chapter 3, I employed the CLP model, considered the gold standard for polymicrobial sepsis (1), and administered a two-pronged glycolipid immunotherapy (OCH \rightarrow α -GalCer) to sequentially curb the proinflammatory and immunosuppressive phases of sepsis. As a result, treatment conferred a significant reduction in disease severity during the first 48 hours post CLP, which translated to a marked increase in overall survival (Fig 3.4B-C). Furthermore, survivors that received OCH \rightarrow α -GalCer exhibited a reversal of immunosuppression compared to controls, as measured by DTH test and MHC II expression of splenic macrophages (Fig. 3.9C-E). Multiplex cytokine readouts on day 4 post CLP showed that OCH \rightarrow α -GalCer treated mice exhibited an altered cytokine milieu with proinflammatory cytokines, (IL-2, IFN- γ , and TNF- α) and chemokines (CCL2, CXCL9, and CXCL10) all significantly elevated compared to untreated septic mice (Fig 3.6). Finally, I show that that OCH \rightarrow α -GalCer treatment also augments the transactivation of downstream NK cells compared to control (Fig 3.7). Collectively, these findings build a case that a glycolipid-based immunotherapy approach effectively targets iNKT cells to have pleiotropic effects that confer a survival advantage, alters the cytokine environment, and restores immunocompetence in the host. These findings, at first glance, may seem at odds with previous studies purporting a pathogenic role of iNKT cells in sepsis (2-4). Importantly, these studies examined the contribution of iNKT cells at baseline and thus has no bearing on the type of response iNKT cells may have when therapeutically manipulated. Indeed, iNKT cells are capable of producing enormous amounts of proinflammatory

cytokines rapidly, and therefore, it is most likely the case that during sepsis, iNKT cells are easily susceptible to contribute to the early cytokine storm which has deleterious effects on the host. Despite this, here we report that therapeutic intervention with glycolipid agonists can skew the iNKT cell response to confer a protective effect. This is most aptly observed in the clear differences in the cytokine environment between treated and untreated septic mice (Fig 3.6).

Mice primed with either OCH or select T_H2-polarizing glycolipids (PBS-25 and PBS-128) failed to induce iNKT cell anergy. Although several mechanisms that contribute to iNKT cell anergy have been described, these may be acting independently from each other. Alterations in acyl chain length on the sphingosine moiety has been linked with iNKT cell anergy and is a factor which effects the loading and stability of glycolipid compounds on CD1d, which in part, contributes to the polarization of differential T_H1 or T_H2 responses (5). However, acyl chain variances may not be essential in inducing iNKT cell anergy since mice primed with α -GalCer-loaded dendritic cells conferred hyperresponsiveness to secondary activation (6), which suggests the co-stimulatory/-inhibitory signals provided by the APC is a determining factor. Alternatively, the mouse homolog of HLA-E, Qa-1, which is highly upregulated on iNKT cells after free-floating α -GalCer but not OCH injections, has been identified as a key regulator of iNKT cell anergy (7). A systematic approach is necessary in delineating which of these pathways are involved that allows the circumvention of iNKT cell anergy by OCH, PBS-25, and PBS-128 and whether these are contributing to their protective effect in polymicrobial sepsis.

Although the specific mechanism by which OCH \rightarrow α -GalCer treatment improves survival and immunosuppression has yet to be fully elucidated, one can speculate that the effect of

the glycolipid immunotherapy is multi-factorial. Piliponsky et al. recently reported that adequate TNF- α levels during sepsis was essential for myeloid cell effector function, bacterial clearance, and survival (8). We also observed significantly elevated levels of TNF- α in the serum of treated mice (Fig. 3.6). It is tempting to speculate that increased TNF- α levels may play a part in our model as well. To add another layer of complexity, Roquilly et al. recently linked sepsis-induced immunosuppression to “paralyzed DCs” which exhibited drastically reduced antigen presenting capacity and cytokine production (9). It is noteworthy that iNKT cells possess the remarkable ability to activate and mature APCs (10-12); therefore, it is possible that the upregulation of MHC II expression on splenic macrophages and the restoration of DTH responses in the immunotherapy treated mice were largely due to the intercellular cross-talk between iNKT cells and APCs (Fig 3.9C-E).

Importantly, I also delineated that our two-pronged glycolipid immunotherapy did not work indiscriminately with any T_H2 polarizing agonist. I showed that substituting OCH with C20:2 or PBS-25 glycolipids, either resulted in anergy (Fig 3.11A-B) or responsiveness, respectively (Fig 3.12B). Notably, a significant survival advantage could be recapitulated when septic mice were treated with PBS-25 \rightarrow α -GalCer (Fig. 3.12C).

In this chapter, a number of statistical comparisons were performed using repeated Student's t test when comparing multiple groups (Fig 3.1; Fig 3.2; Fig. 3.4; Fig. 3.12; Fig 3.13). Although these analyses were adjusted for the number of comparisons made which increases the threshold of statistical significance, there are still limitations to this method over a conventional ANOVA analysis for multiple groups. Notably, no comparison lost its

statistical significance when re-analyzed using either a one-way or two-way ANOVA, as appropriate.

There is a cacophony of complex pathways that are involved in sepsis pathology that culminates to a severely dysregulated immune response leading to organ failure. It is unlikely that any one pathway takes precedence over all others, and thus finding a cure has proven to be incredibly complicated and difficult. More recent preclinical studies are now uncovering previously unsuspecting cell types such as basophils (8), and mast cells (13) to contribute to sepsis. In this chapter, I provide evidence that another unconventional cell type, iNKT cells, can be targeted using phase-tailored glycolipid agonists to skew the immune response towards homeostasis during sepsis. Future investigations are required to further elucidate the specific mechanism by which this immunotherapy confers protection against sepsis.

5.1.2 α -GalCer-activated iNKT cells mediate the expansion and activation of pre-mNK cells and skews them towards an anti-cancer phenotype.

In chapter 4, I serendipitously discovered a distinct B220⁺CD11c⁺NK1.1⁺ population that dramatically accumulated in the liver after a α -GalCer i.p injection (Fig 4.1A). To rule out the possibility that α -GalCer had an off-target effect to cause this accumulation, I employed a blocking mAb, anti-CD1d, effectively blocking activation of iNKT cells by α -GalCer. As expected, I confirmed that this distinct population in the liver was mediated by iNKT cells (Fig 4.1B). Previously classified as IKDCs and later renamed to pre-mNK cells, these cells were heralded to be prolific tumoricidal activity, produce proinflammatory cytokines, like

IFN- γ and TNF- α , and surprisingly were shown to present antigens to cognate CD4 and CD8 T cells (14-16). Following further immunophenotyping, we confirmed that pre-mNK cells also accumulated in the spleen and lungs following α -GalCer administration, albeit most prominently in the liver (Fig. 4.3B).

Considering the rapid nature of pre-mNK cell accumulation in the liver after α -GalCer injection (Fig. 4.3A), I was curious to see whether pre-mNK cells were being expanded in their respective tissues or whether these cells were being recruited from the periphery. Indeed, hepatic pre-mNK cells upregulated proliferation marker, Ki67, after α -GalCer injection, at both the transcript (Fig 4.6A) and protein level (Fig 4.6B). Furthermore, hepatic pre-mNK cells downregulated transcript levels of immune cell trafficking receptor, S1PR1 (Fig 4.6A), which interestingly enough, had an inverse relationship with tissue retention molecule, CD69 (Fig 4.6C). These findings suggested that pre-mNK cells were being trapped in the liver upon α -GalCer administration and were proliferating *in situ*. Using a chemical inhibitor which blocks cell recruitment, FTY720, I confirmed *in vivo*, that α -GalCer-mediated activation of iNKT cells induced the expansion of a resident population of pre-mNK cells in the liver (Fig 4.6D).

The relative paucity of pre-mNK cells *in vivo* make them difficult to isolate and target for cellular therapeutics. Currently, there are no known methods that specifically expand pre-mNK cell numbers *in vivo* (17). Previous studies have shown combinational therapy with chemotherapeutic drug, imatinib mesylate and IL-2, non-specifically expanded pre-mNK cells (14), whereas *in vitro* experiments have found pre-mNK cells proliferate in response to IL-15 stimulus (18). Thus, I wanted to elucidate the signaling pathways by which iNKT cells induced pre-mNK expansion. I hypothesized pre-mNK cell expansion was mediated

through a soluble mediator, therefore an adoptive serum transfer assay was performed. Interestingly, we showed that only the combination of serum collected at 6 and 12 hours post α -GalCer injection induced pre-mNK cell expansion in a naïve host (Fig 4.7B). I also showed that I could recapitulate this phenotype with exogenous recombinant IL-12 and IL-18 injection (Fig. 4.7A). To link these two findings, I depleted IL-12 and IL-18 in the serum sample and found that pre-mNK cell expansion was significantly attenuated (Fig. 4.7C). Together, these findings uncover another aspect of iNKT cell mediated pre-mNK cell expansion through IL-12 and IL-18 signaling.

One distinguishing feature of pre-mNK cells is their ability for antigen presentation (19, 20). However, I observed that α -GalCer-expanded pre-mNK cells lost the ability to cross present tumor peptides *ex vivo* (Fig 4.8B). Importantly, Terme et al. reported that the cross-presentation function of pre-mNK cells were limited to the CD11b⁺ subset (21). In our own model, pre-mNK cells in vehicle treated mice predominantly expressed CD11b, however, α -GalCer-mediated expansion resulted in a significant decrease in the CD11b⁺ subset (Fig. 4.4). Therefore, the loss of antigen presentation may be at least partially due to the decrease in CD11b expressing subsets.

Finally, I confirmed that expanded pre-mNK cells still retained their prolific tumoricidal activity. Following cell sorting by flow cytometry, I plated purified pre-mNK cells, with cancer target cells which showed their efficient killing of these targets were mediated by degranulation (Fig 4.10A). Intracellular cytokine staining revealed that granzyme A was the likely effector molecule mediating the killing (Fig. 4.10C). This was important as pre-mNK cells were thought to mediate killing by TRAIL and this was the first report which showed an alternative pathway (14). Using two different assays, we showed that α -GalCer-

expanded pre-mNK cells contributed significantly to NK cell mediated killing *in vivo* (Fig. 4.10E-F).

Here we show for the first time that iNKT cells can be targeted via α -GalCer to induce the expansion and activation of pre-mNK cells, allowing them to participate in metastatic surveillance. This is especially important as it reveals a novel target of α -GalCer as an immunotherapeutic. Certainly, numerous studies have already shown the protective effects of α -GalCer administration in cancer bearing mice (12, 22-25), however the extent to which expanded pre-mNK cells were contributing to these phenotypes is unknown and warrants further investigation.

5.2 Limitations

Limitations remain in our interpretations of the data that may require further validation and are interesting lines of investigations to pursue. In chapter 3, we found that OCH \rightarrow α -GalCer treatment was beneficial in enhancing survival and rescuing immunosuppression in septic mice. One limitation to this interpretation is whether OCH injection as the initial hit is necessary in alleviating sepsis pathology. Although I have shown that α -GalCer treatment alone on day 4 after CLP neither changed survival (Fig 3.5) nor rescued immunosuppression (Fig 3.10), this does not address whether an alternate combination of a two-pronged glycolipid immunotherapy would confer a therapeutic benefit. This is a valid objection and is currently being investigated, albeit not in this dissertation. Another limitation lies within the CLP model. While it is true that CLP is considered the gold standard for mouse models of sepsis, there are limitations in capturing the complexities of clinical sepsis. Notably, mice subjected to CLP are left to recover on their own without any

source control interventions by antibiotics or peritoneal washes. Mice that are immediately treated with antibiotics following CLP have been shown to make full recovery with minimal septic pathology (26), therefore the pathology observed in CLP subjected mice does not fully mimic what is observed in the clinic where virtually all septic patients are treated with some form of anti-microbial agent. Nevertheless, the CLP model is essential in determining the physiological changes in responses to a severe infectious insult.

In chapter 4, I discovered that pre-mNK cells were transactivated and involved in the anti-cancer response. One key finding was that when using the established B16 metastatic melanoma model, mice depleted of pre-mNK cells *in vivo* exhibited an increase in overall tumor burden (Fig 4.10). Although this model is routinely used to determine the immune response against metastatic forms of cancer, there are a few limitations with this interpretation. First, intravenous injection of B16 melanoma cells circumvents the basic nature of aggressive metastatic cells that extravasate through the physical barrier into the blood. Second, the accumulation of nodules observed in the lungs of B16-melanoma injected mice are not selected for their metastatic ability but rather their capacity to colonize a tissue for growth. Finally, it is unclear whether the decrease in overall tumor burden is a direct result of cytotoxic activity by immune effector cells or other factors, such as nutrient deprivation, that would prevent neoplastic proliferation in secondary tissues. Thus, interpretation of the data should be made with careful considerations to these limitations.

5.3 Conclusion

The overarching focus of this thesis is to emphasize that the impressive immunomodulatory capacity of iNKT cells can be harnessed using glycolipid agonists to shape the course and nature of the ensuing immune response. Among the diverse choice of glycolipid agonists, α -GalCer has been the most extensively studied (27) and is discussed extensively in this thesis. It should be noted that potential side effects have been observed in mouse models, dependent on the route of α -GalCer injection. For instance, both intravenous and intranasal administration of α -GalCer has been shown to induce a cytokine storm (28) and airway hypersensitivity (29), respectively. Nevertheless, this thesis shows how one can efficaciously approach glycolipid immunotherapies to treat two devastating diseases in sepsis and cancer. In chapter 3, I outlined how strategic tailoring of glycolipid agonists can curb the biphasic pathology of sepsis. These findings are quite timely as treatments aiming to restore the severe immune dysregulation caused by sepsis is being intensively investigated (30, 31). Future studies may consider combinational therapies including glycolipid agonists with other immune boosting agents such as recombinant IL-7 or anti-PD-1 therapies. In chapter 4, I discovered a novel mechanism by which glycolipid agonists can be used to mediate the involvement of pre-mNK cells in the anti-cancer response. There are still many unanswered questions that need to be addressed going forward. First, what is the fate of the expanded pre-mNK populations following α -GalCer administration? It would be interesting to see whether these populations contract back to baseline levels or whether their augmented numbers are sustained. Second, are pre-mNK cells regulated by MHC molecules the same way mNK cells are? Finally, a definitive human equivalent of pre-mNK cells still needs to be identified and whether expansion can be induced, via α -

GalCer, has yet to be determined. This thesis uncovers new and exciting methods in which glycolipid agonists of iNKT cells can be used to galvanize the host's immune response in combating severe immune-related diseases.

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Appendices

Copyright Approval



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Eugene M. Oltz, Ph.D.

May 27, 2020

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Curriculum Vitae

JOSHUA CHOI

Western University; Department of Microbiology and Immunology; London, Ontario,
Canada.

EDUCATION

- 2015- **PhD in Microbiology & Immunology**, Western University
Thesis: Therapeutic application of invariant natural killer T cells in sepsis
Supervisor: Dr. S.M.Mansour Haeryfar
- 2012-2014 **MSc in Biology**, Lakehead University
Thesis: The Role of *Haemophilus influenzae* Lipooligosaccharide in
the Activation of Innate and Adaptive Immunity.
Supervisor: Dr. Marina Ulanova
- 2005-2010 **HBSc**, University of Toronto

HONOURS and AWARDS

- 2019 PSAC 610 Award for Research Excellence, \$500
- 2019-2020 Recipient of “Careers in Immunology” Fellowship granted by *The American Association of Immunologists*, \$35000
- 2019 Awarded invitation by Western University to compete in Canadian Student Health Research Forum (CSHRF) (Nomination given to top 5% of PhD students in Canada)
- Recipient of CIHR Gold Award for poster presentation at CSHRF (Awarded to top 4% of 276 PhD students), \$500
- 2016-2019 Microbiology and Immunology Travel Award, Western University, \$1000/year
- 2018 The Queen Elizabeth II Graduate Scholarship in Science and Technology, \$15000
- 2018 The American Association of Immunologists Trainee Abstract Award, \$500
- 2018 Canadian Society for Immunology Travel Award, \$500
- 2017 IIRF 2017 Oral Talk Award winner
- 2017 The Queen Elizabeth II Graduate Scholarship in Science and Technology, \$15000
- 2016 Neter Award Finalist, Buffalo Microbial Pathogenesis Conference

2015	Dr. FW Luney Graduate Entrance Scholarship, Western University, \$3000
2015	Ontario Graduate Scholarship, \$15000
2013	Hilda Simons Graduate Scholarship in Science, Lakehead University \$1000
2013	CUPE local 3905 Graduate Award, \$1000
2012-2014	Faculty Research Scholarship, Lakehead University, \$4000

PUBLICATIONS

Published

- 1) **Choi J**, Rudak PT, Lesage S, Haeryfar SMM. 2019. Glycolipid stimulation of iNKT cells expands a unique population of pre-mNK cells endowed with oncolytic and anti-metastatic properties. *The Journal of Immunology*. 203(7): 1808-19
- 2) Rudak PT, Gangireddy R, **Choi J**, Burhan AM, Summers KL, Jackson DN, Inoue W, Haeryfar SMM. 2019. Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes. *Brain Behavior and Immunity* Aug;80:793-804
- 3) **Choi J**, Meilleur CE, Haeryfar SM. 2019. Tailoring *In Vivo* Cytotoxicity Assays to Study Immunodominance in Tumor-specific CD8+ T Cell Responses. *J. Vis. Exp.* (147), e59531, doi:10.3791/59531
- 4) Nix EB , **Choi J**, Anthes C, Gaultier GN, Thorgrimson J , Cox AD, Tsang RSW, McCready WG, Boreham D, Ulanova M. 2018. Characterization of natural bactericidal antibody against Haemophilus influenzae type a in Canadian First Nations: A Canadian Immunization Research Network (CIRN) Clinical Trials Network (CTN) study. *PLoS One* 13(8):e0201282
- 5) Rudak PT, **Choi J**, Haeryfar SMM. 2018. MAIT cell-mediated cytotoxicity: roles in host defense and therapeutic potentials in infectious diseases and cancer. *Journal of Leukocyte Biology* 104 (3):473-486
- 6) Alhazmi A, **Choi J**, Ulanova M. 2017. Syk inhibitor R406 down-regulates inflammation in an *in vitro* model of *Pseudomonas aeruginosa* infection. *Can J Physiol Pharmacol*, 10:1-9.
- 7) Memarnejadian A, Gouklani H, Mohammadi S, Moosazadeh M, **Choi J**. 2018. Prevalence of HIV-1 pre-treatment drug resistance in a southern province of Iran, 2016-2017. *Arch Virol*, 163(1):57-63

- 8) Shaler CR, **Choi J**, Rudak PT, Memarnejadian A, Szabo PA, Tun-Abraham ME, Rossjohn J, Corbett AJ, McCluskey J, McCormick JK, Lantz O, Hernandez-Alejandro R, Haeryfar SMM. 2017. Mucosa-associated invariant T cells launch rapid, robust and distinct hyperinflammatory responses to bacterial superantigens and quickly acquire an anergic phenotype that impedes their cognate antimicrobial function: Defining a novel mechanism of superantigen-induced immunopathology and immunosuppression. *PLoS Biology* 15(6):e200193
 - a. Highlighted in a commentary ('primer') article published in the same journal [Sandberg JK, et al: Bacterial deception of MAIT cells in a cloud of superantigen and cytokines. *PLoS Biology* 2017, 15(7): e2003167]
 - b. Featured in a PLoS Biology press release entitled "From helpful to harmful: Superantigens reveal MAIT cells' dark side"
- 9) Szabo PA, Rudak PT, **Choi J**, Xu SX, Schaub R, Singh B, McCormick JK, Haeryfar SMM. 2016. Invariant NKT cells are pathogenic in the HLA-DR4-transgenic humanized mouse model of toxic shock syndrome and can be targeted to reduce morbidity. *The Journal of Infectious Disease* 215 (5): 824-829
- 10) Kivinen J, Togtema M, Mulzer G, **Choi J**, Zehbe I, Curiel L, Samuel P. 2015. Sonoporation efficacy on SiHa cells *in vitro* at raised bath temperatures – experimental validation of a prototype sonoporation device. *Journal of Therapeutic Ultrasound* 3(19):1-13
- 11) **Choi J**, Nix EB, Gaultier GN, Cox AD, McCready W, Ulanova M. 2015. Naturally occurring bactericidal antibodies specific for *Haemophilus influenzae* lipooligosaccharide are present in healthy adult individuals. *Vaccine* 33:1941-7.
- 12) **Choi J**, Cox AD, Li J, McCready W, Ulanova M. 2014. Activation of Innate Immune Responses by *Haemophilus influenzae* lipooligosaccharide. *Clinical and Vaccine Immunology* 21(5): 769-776

Under Revision

- 1) **Choi J**, Mele TS, Porcelli SA, Savage PB, Haeryfar SMM. Harnessing the versatility of iNKT cells in a novel step-wise approach to sepsis immunotherapy. Submitted to *Journal of Immunology* (Manuscript ID: 20-00220-FL)

In Submission

- 1) Rudak PT, **Choi J**, Parkins KM, Summers KL, Jackson DN, Foster PJ, Skaro AI, Leslie K, McAlister VC, Kuchroo VK, Inoue W, Lantz O, Haeryfar SMM.

Chronic stress spares invariant T cells but impairs their fitness in a cell-intrinsic glucocorticoid receptor-dependent fashion. Submitted to Cell Reports

In Preparation

- 1) (Choi J et al)

ORAL PRESENTATIONS

- 1) Invited seminar presentation to the Kuchroo lab, Harvard University. “Exploiting the immunomodulatory potentials of iNKT cells in sepsis and cancer.” Feb 20, 2020
- 2) *The Canadian Society for Immunology (CSI)*: “iNKT cell activation expands resident pre-mNK cells and skews their responses towards an anti-metastatic phenotype.” April 14th 2019
- 3) *The Canadian Society for Immunology (CSI)*: “Glycolipid stimulation of invariant NKT cells mobilizes premNK cells and potentiates their participation in immune surveillance against metastatic cancer.” June 3rd 2018, London ON CAN.
- 4) *The American Association of Immunologists (AAI)*: “Glycolipid stimulation of invariant NKT cells mobilizes precursors of mature NK cells and potentiates their participation in immune surveillance against metastatic cancer.” May 5th 2018, Texas USA
- 5) *Ebenzer Student Group Guest speaker*: “The morality of truth in the 21st century.” Mar 5, 2018. Western University
- 6) *12th Annual Infection & Immunity Research Forum*: “Glycolipid stimulation of invariant NKT cells mobilizes precursors of mature NK cells and potentiates their participation in immune surveillance against metastatic cancer.” Oct 27th, 2017. Western University, London, CAN
- 7) *28th Annual Buffalo Conference on Microbial Pathogenesis*: “Modulation of iNKT cell functions by their glycolipid agonists reveals divergent effects on cell-mediated cytotoxicity: implications for anti-pathogen immunity”. May 18, 2016 Buffalo, NY, USA.

PUBLISHED ABSTRACTS

- 1) **Choi J**, Rudak PT, Lesage S, Haeryfar SMM. 2019. INKT cell activation expands resident pre-mNK cells and skews their responses towards an anti-metastatic phenotype. Canadian student health research forum; Winnipeg, Manitoba CAN
- 2) **Choi J**, Rudak PT, Lesage S, Haeryfar SMM. 2019. INKT cell activation expands resident pre-mNK cells and skews their responses towards an anti-metastatic phenotype. Canadian Society for Immunology; Banff, Alberta CAN
- 3) Rudak PT, **Choi J**, Haeryfar SMM. 2019. Sustained psychological stress impairs antitumour immunity orchestrated by invariant NKT cells via intrinsic glucocorticoid receptor signaling. Canadian Society for Immunology; Banff, Alberta CAN
- 4) **Choi J**, Rudak PT, Lesage S, Haeryfar SMM. 2019. INKT cell activation expands resident pre-mNK cells and skews their responses towards an anti-metastatic phenotype. London Health Research Day; London ON, CAN
- 5) **Choi J**, Chan C, Lesage S, Haeryfar SMM. 2018. Glycolipid stimulation of invariant NKT cells mobilizes precursors of mature NK cells and potentiates their participation in immune surveillance against metastatic cancer. Canadian Society for Immunology; Western University, London CAN
- 6) Rudak PT, **Choi J**, Haeryfar SMM. 2018. Elevated Glucocorticoid release during psychological stress diminishes antitumor immunity promoted by invariant natural killer T cells. Canadian Society for Immunology; Western University, London CAN
- 7) **Choi J**, Chan C, Lesage S, Haeryfar SMM. 2018. Glycolipid stimulation of invariant NKT cells mobilizes precursors of mature NK cells and potentiates their participation in immune surveillance against metastatic cancer. The American Association for Immunologists; Austin, Texas, USA.
- 8) Rudak PT, **Choi J**, Haeryfar SMM. 2018. Glucocorticoid receptor signaling during prolonged psychological stress compromises the ability of invariant NKT cells to participate in antitumor immune surveillance. The American Association for Immunologists; Austin, Texas, USA.
- 9) Ulanova M, Nix EB , **Choi J**, Anthes C, Gaultier GN, Thorgrimson J , Cox AD, Tsang RSW, McCready WG, Boreham D. 2018. Natural Immunity to Haemophilus influenzae Type A in First Nations living in communities with different burden of invasive disease. AMMI Canada; Vancouver, CAN
- 10) **Choi J**, Mele T, Cepinskas G, Haeryfar SMM. 2017. Therapeutic application of invariant natural killer T cell glycolipid agonists for sepsis. Canadian Society for Immunology; Banff, Alberta, CAN
- 11) Rudak PT, **Choi J**, Haeryfar SMM. 2017. Potent suppression of invariant NKT cell responses in a model of chronic psychological stress. Canadian Society for Immunology; Banff, Alberta, CAN

- 12) **Choi J**, Mele T, Cepinskas G, Haeryfar SMM. 2017. Therapeutic application of invariant natural killer T cell glycolipid agonists for sepsis. London Health Research Day; London, Ontario, CAN.
- 13) **Choi J**, Mele T, Cepinskas G, Haeryfar SMM. 2016. Therapeutic application of invariant natural killer T cell glycolipid agonists for sepsis. Infection and Immunity Research Forum; London, Ontario, CAN.
- 14) **Choi J**, Memarnejadian A, Haeryfar SMM. 2016. Modulation of iNKT cell functions by their glycolipid agonists reveals divergent effects on cell-mediated cytotoxicity: implications for anti-pathogen immunity. 28th Annual Buffalo Conference on Microbial Pathogenesis. Buffalo, NY, USA.
- 15) Shaler CR, Rudak P, **Choi J**, Memarnejadian A, Abraham MET, McCormick JK, Lantz O, Alejandro RH, Haeryfar SMM. 2016. Human Mucosa-associated invariant T cells are hyper-responsive to bacterial superantigens, rapidly becoming anergic and unresponsive to secondary bacterial challenge. Canadian Society for Immunology; Ottawa, Ontario, CAN.
- 16) **Choi J**, Memarnejadian A, Haeryfar SMM. 2016. Successive administration of invariant natural killer T cell glycolipid agonists exerts divergent effects on cell-mediated cytotoxicity and cytokine production. Canadian Society for Immunology; Ottawa, Ontario, CAN.
- 17) Memarnejadian A, **Choi J**, Shaler RC, Foster PJ, Haeryfar SMM 2016. Invariant natural killer T cells play a pathogenic or a protective role in a minimal residual disease model of prostate cancer depending on their activation state. Canadian Society for Immunology; Ottawa, Ontario, CAN.
- 18) **Choi J**, Haeryfar SMM. 2016. A T helper 2-polarizing glycolipid agonist of invariant natural killer T cells does not induce anergy to a subsequent encounter with α -galactosylceramide. London Health Research Day; London, Ontario. CAN.
- 19) **Choi J**, Haeryfar SMM. 2015. A T helper 2-polarizing glycolipid agonist of invariant natural killer T cells does not induce anergy to a subsequent encounter with α -galactosylceramide. Infection and Immunity Research Forum, London Ontario.
- 20) **Choi J**, Cox AD, McCready W, Ulanova M. 2014. The role of Lipooligosaccharide of *Haemophilus influenzae* in the activation of human adaptive immune response. Northern Health/NBRHC Research Conference, Sioux Lookout.
- 21) **Choi J**, Cox AD, McCready W, Ulanova M. 2013. Activation of Innate Immune Response by *Haemophilus influenzae* Lipooligosaccharide. Northern Health/NBRHC Research Conference, North Bay.

RESEARCH EXPERIENCE

2015-present **Ph.D. Candidate**, Schulich Medicine and Dentistry: Department of Microbiology and Immunology

- Investigating T cell-mediated cytotoxicity in response to polymicrobial infection
- 2014-2015 **Research Assistant**, Northern Ontario School of Medicine
- Looked at the bactericidal activity of anti-LOS antibodies in sera samples.
 - Investigated the prevalence of *Haemophilus influenzae* type a infections in Northern Ontario, Aboriginal communities.
 - Proficient in bacterial culture, and serum bactericidal assays.
- 2012-2014 **M.Sc. student**, Department of Biology, Lakehead University
- Completed Master thesis in the field of infectious disease caused by *Haemophilus influenzae* with respect to potential vaccine and adjuvant development.
 - Developed expertise in cell culture, flow cytometry, real-time PCR, and ELISA.
- 2013 **Research technician**, Thunder Bay Regional Research Institute
- Performed GFP staining followed by fluorescent microscopy of HIFU-treated cells.

TEACHING EXPERIENCE

- 2020 **Lecturer**, Western University
MICROIMM 3300B
- 2016- 2020 **Teaching Assistant**, Western University
Immunology Laboratory MICROIMM 3620G
- 2018 **Teaching Assistant**, Western University
Clinical Immunology MICROIMM 4300A
- 2016 **Graduate Student Teaching Award Nomination**, Western University
- 2012-2014 **Teaching Assistant**, Lakehead University
Introduction to Microbiology BIOL2713F
Introduction to Human Physiology BIOL2030W

SUPERVISORY EXPERIENCE

- 2019-2020 Honours Student Jennifer Jiao
- 2017-2018 Honours Student Cindy Chan
- 2013-2014 Honours Student Gabrielle Gaultier
- 2012-2013 Honours Student Devin Dasey

MEMBERSHIPS

- The American Association of Immunologists (Since 2018)
The Canadian Society for Immunology (Since 2016)

ADMINISTRATIVE EXPERIENCE

- 2018 **Volunteer at the Canadian Society for Immunologists (CSI) conference**

- 2013-2014 **Northern Ontario School of Medicine Journal Club Coordinator**
- Organization and management of weekly scholarly presentations
- 2013 **Graduate Student Association representative, Lakehead University**
- Maintained weekly office hours to answer graduate students' questions and concerns

RELEVANT EXPERIENCES

- 2016 **University Heights Middle School Science Fair Judge**
- 2012-2014 **Primary Immunodeficiency Canada: Video Conference Series Attendee**
- Presentation of notable PID case studies nationwide with differential diagnoses