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The impacts of psychological stress on innate-like invariant T cell survival, phenotype, and function

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Abstract

The nervous system serves numerous critical roles in the regulation of immune responses. Consequently, psychological stress can result in immunosuppressive states that are conducive to the development of infection and cancer. Yet, whether stress impacts the functions of innate-like T lymphocytes including invariant natural killer T (*i*NKT) and mucosa-associated invariant T (MAIT) cells, which participate in early host defense against pathogens and tumors, remains poorly understood. In this thesis, I leveraged multiple established methods with which to induce psychological stress in mice. I demonstrate that T_H1- and T_H2-type immune responses initiated by *i*NKT cells are abrogated during stress, effects which are lost upon habituation to homotypic stressors. Instead, *i*NKT cells in stressed mice trigger an abnormal systemic inflammatory response characterized by striking levels of interleukin (IL)-10, IL-23, and IL-27. These dysregulated responses are driven by *i*NKT cell-intrinsic glucocorticoid receptor (GR) signaling. Accordingly, *i*NKT cells upregulate the co-inhibitory molecule T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) in a GR-dependent manner and blockade of TIGIT partially restores their functional capacity in stressed mice. Ultimately, in a GR-dependent fashion, *i*NKT cells from stressed mice fail to prevent pulmonary metastases of B16 melanoma. MAIT cells also upregulate TIGIT and are incapable of mounting optimal T_H1- and T_H2-type responses during stress. Lastly, these inhibitory effects are not simply due to cell death since human and mouse *i*NKT and MAIT cells are unusually refractory to glucocorticoid-induced apoptosis. Collectively, my findings reveal a mechanism of stress-induced immunosuppression with implications for *i*NKT or MAIT cell-based immunotherapies.

Keywords

Psychological stress, sympathetic nervous system, hypothalamic-pituitary-adrenal axis, glucocorticoids, invariant natural killer T cells, mucosa-associated invariant T cells, apoptosis, TIGIT, cytokines, immunosuppression

Summary for Lay Audience

The nervous system is critical for regulating the immune system. During psychological stress, normal interactions between the nervous system and the immune system can become disrupted, leading to a reduced ability of the immune system to respond to microbial infections and tumors. However, we still have a poor understanding of why there exists a relationship between stress, weakened immune responses, and greater susceptibility to infectious diseases and cancer. For example, how stress affects particular subsets of immune cells known as invariant natural killer T (*i*NKT) cells and mucosa-associated invariant T (MAIT) cells, which react rapidly to microbes and tumors and alert other immune cell types of impending danger, is essentially unknown. In this thesis, I investigated how stress impacts the function of *i*NKT and MAIT cells using multiple methods with which to induce psychological stress in mice. I demonstrate that pro-inflammatory cytokine production by activated *i*NKT cells is diminished in stressful environments, but only when mice are incapable of predicting the stressor being applied. Instead, *i*NKT cells from stressed mice trigger an abnormal immune response typified by anti-inflammatory cytokine production. Next, I found that these atypical responses are a consequence of glucocorticoids, a major stress hormone, acting directly on *i*NKT cells. Moreover, these glucocorticoids cause *i*NKT cells to increase their expression of the inhibitory molecule T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), which limits their responsiveness, and blocking this pathway partially restores their functional capacity during stress. Ultimately, *i*NKT cells in stressed mice fail to protect against cancer including lung metastases derived from melanoma. Consistent with the above results, MAIT cells in stressed mice also upregulate TIGIT and have a diminished ability to produce pro-inflammatory cytokines. Since mediators of stress are capable of killing immune cells, it was important to discern whether these effects on invariant T cell function were due to their death. To the contrary, I discovered that *i*NKT and MAIT cells are unusually resistant to stress-induced cell death. Collectively, these findings reveal previously unidentified modes of stress-induced immunosuppression and implicate the stress response as a hurdle for harnessing *i*NKT and MAIT cells to prevent disease.

Dedication

I dedicate this dissertation to my mother, Maude, whose endless encouragement fostered my scientific curiosity.

Co-Authorship Statement

The research presented throughout this thesis was primarily conducted by the author, Patrick Rudak, with guidance from Dr. Mansour Haeryfar. The following information describes the specific contributions of co-authors listed in published works:

Chapter 1: Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *The Journal of Leukocyte Biology*. 2018 Sep;104(3):473-486. DOI: 10.1002/JLB.4RI0118-023R

Rudak PT collected the relevant literature, generated experimental data (not included in this thesis), and wrote the manuscript's first draft. Choi J generated some of the experimental data (not included in this thesis). Haeryfar SMM conceived the theme of the review, participated in experimental design (not included in this thesis), and edited the manuscript.

Chapter 3: 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 physically spares but functionally impairs innate-like invariant T cells. *Cell Reports*. 2021; 35(2): 108979. DOI: 10.1016/j.celrep.2021.108979

Rudak PT designed and performed experiments, analyzed and interpreted data, and wrote the initial manuscript. Choi J and Parkins KM assisted with performing experiments and analyzing data. Summers KL, Jackson DN, and Foster PJ interpreted data. Skaro AI, Leslie K, and McAlister VC provided clinical samples and interpreted data. Kuchroo VK provided experimental reagents and designed experiments. Inoue W designed experiments and interpreted data. Lantz O provided experimental reagents and tools, designed experiments, interpreted data and edited the manuscript. Haeryfar SMM conceived the idea, obtained funding, administered the project, designed experiments, analyzed and interpreted data, and edited the final manuscript.

Chapter 4: Rudak PT, Gangireddy R, Choi J, Burhan AM, Summers KL, Jackson DN, Inoue W, Haeryfar SMM. Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes. *Brain, Behavior, and Immunity*. 2019 Aug;80:793-804. DOI: 10.1016/j.bbi.2019.05.027

Rudak PT designed and performed experiments, analyzed and interpreted data, and wrote the initial manuscript. Gangireddy R and Choi J assisted with performing experiments and analyzing data. Burhan AM, Summers KL, and Jackson DN interpreted data. Inoue W designed experiments and interpreted data. Haeryfar SMM conceived the idea, obtained funding, administered the project, designed experiments, analyzed and interpreted data, and edited the final manuscript.

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See co-authorship statement for Chapter 3.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
Ag	antigen
αCGC	α-C-galactosylceramide
αGC	α-galactosylceramide
ACK	ammonium-chlorine-potassium [buffer]
ANS	autonomic nervous system
AP-1	activator protein 1
APC	antigen-presenting cell
5-ARU	5-amino-6-D-ribitylaminouracil
AVP	arginine vasopressin
β2M	β-2-microglobulin
B6	C57BL/6 [mice]
BMDC	bone marrow-derived dendritic cell
BTLA	B and T lymphocyte attenuator
cAMP	cyclic adenosine 5'-monophosphate
CCR	C-C chemokine receptor
CD	cluster of differentiation
CNS	central nervous system
CRH	corticotropin-releasing hormone

CS	corticosterone
Ct	cycle threshold
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CVS	chronic variable stress
CXCR	C-X-C motif chemokine receptor
DC	dendritic cell
DEX	dexamethasone
DN	[$CD4^{-}CD8^{-}$] double negative
DP	[$CD4^{+}CD8^{+}$] double positive
EGR	early growth response protein
ER	experimental release
FBS	fetal bovine serum
Foxp3	forkhead box P3
6-FP	6-formylpterin
GATA-3	GATA binding protein 3
GILZ	glucocorticoid-induced leucine zipper
GITR	glucocorticoid-induced TNF receptor-related protein
gMFI	geometric mean fluorescence intensity
GR	glucocorticoid receptor
GRE	glucocorticoid response element

GZM	granzymes
HC	hydrocortisone
HCV	hepatitis C virus
HMNC	[non-parenchymal] hepatic mononuclear cell
HPA	hypothalamic-pituitary-adrenal [axis]
IAV	influenza A virus
ICOS	inducible T cell costimulator
IFN	interferon
IL	interleukin
*i*NKT	invariant natural killer T [cells]
i.p.	intraperitoneal
*i*T	invariant T [cells]
*i*TCR	invariant T cell receptor
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITT	immunoglobulin tail tyrosine[-like motif]
i.v.	intravenous
KLRG1	killer cell lectin-like receptor subfamily G member 1
LAG-3	lymphocyte activation gene 3
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
LAMP-1	lysosome-associated membrane protein 1

LPS	lipopolysaccharide
mAb	monoclonal antibody
MAIT	mucosa-associated invariant T [cells]
MDM	monocyte-derived macrophages
MDSC	myeloid-derived suppressor cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cell
MR1	MHC-related protein 1
NE	norepinephrine
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer [cells]
NKT	natural killer T [cells]
NPY	neuropeptide Y
OHDA	6-hydroxydopamine
O/N	overnight
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
PAMPs	pathogen-associated molecular patterns
PB	peripheral blood

PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death-1
PKC	protein kinase C
PLC	phospholipase C
PLZF	promyelocytic leukemia zinc finger
PMA	phorbol 12-myristate 13-acetate
PNS	peripheral nervous system
pre-mNK	precursors to mature NK [cells]
PSNS	parasympathetic nervous system
qPCR	quantitative polymerase chain reaction
RORγt	RAR-related orphan receptor gamma t
RRS	repeated restraint stress
SEM	standard error of the mean
SLAM	signaling lymphocytic activated molecules
SNS	sympathetic nervous system
SR	spontaneous release
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells

T_{conv}	conventional T [cells]
TCR	T cell receptor
TH	tyrosine hydroxylase
T_H	T helper-type
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains
TIM-3	T cell immunoglobulin and mucin-3
TME	tumor microenvironment
TNF	tumor necrosis factor
TR	total release
T_{reg}	regulatory T [cells]
VISTA	V-domain immunoglobulin suppressor of T cell activation
Veh	vehicle
*v*NKT	variant NKT [cells]
WT	wild-type

Preface

As I am sure has been the case for many students in the past, my graduate thesis project was not initially meant to follow the trajectory that it did. Upon joining the Haeryfar lab as a Master's student, I embarked on an exploratory project aiming to determine the regulatory consequences of sympathetic nervous system (SNS)-derived neurotransmitters on invariant natural killer T (*i*NKT) cell function. This project yielded exciting results rather quickly. Within a few months, I demonstrated in multiple experimental systems that norepinephrine is reproducibly suppressive for *i*NKT cell responses, providing an initial phenotype to expand upon for a potentially publishable study. Eventually, I aimed to determine whether similar effects could be observed in a bona fide rodent model of psychological stress and was elated to find just that. However, to my disappointment at the time, my early mechanistic work indicated that the inhibitory effects of stress on *i*NKT cell function were not mediated by the SNS. Therefore, I could not reconcile the data from my SNS-related work with that from the *in vivo* stress model. After a brief period of denial, I then refocused my attention towards the hypothalamic-pituitary-adrenal axis and found that the suppression of *i*NKT cell responses during stress was indeed imposed by the actions of glucocorticoids. Ultimately, this observation formed the crux of my primary PhD project. In the end, this project became much more fascinating to me than I could have ever expected. Thus, my graduate training has reinforced two valuable lessons – approach all experiments without any preconceived notions and allow the data to guide you without hesitation even if a considerable amount of effort has gone into supporting another hypothesis. I will certainly carry these lessons with me as a scientist moving forward.

As I partially alluded to above, the studies presented within this thesis have a unifying theme of how psychological stress regulates the biology of two distinct subsets of innate-like invariant T cells, namely *i*NKT cells and mucosa-associated invariant T (MAIT) cells. The primary focus of the first study presented was to comprehensively describe the impacts of stress on invariant T cell survival and function, many of which effects were eventually ascribed to the actions of glucocorticoids. In the second study, I analyzed the consequences of stress on the surface phenotype of invariant T cells, focusing specifically on their expression of several known co-inhibitory molecules. In this line of inquiry, I discovered that glucocorticoid release during stress surprisingly prompted invariant T cells to upregulate

TIGIT, a targetable receptor that has previously been demonstrated to limit protective immune responses. Taken together, the data held within this thesis provide a framework for identifying markers associated with stress-induced immunosuppression in other physiological and cellular contexts and in human cohorts. Overall, this work may be of broad interest to those in the field of neuroimmunology and for those aiming to harness the properties of invariant T cells for therapy including tumor immunologists and vaccine biologists.

1 Chapter 1: Introduction

Parts of chapter 1.3 have been adapted from a published review article: Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *The Journal of Leukocyte Biology*. 2018 Sep;104(3):473-486. DOI: 10.1002/JLB.4RI0118-023R. Please refer to Appendix A to view the appropriate copyright permission obtained to reuse this work.

1.1 Neuroimmunomodulation

For over a century, immunology largely evolved without any perspectives or input from researchers in other respective branches of physiology (1, 2). For this reason, it is not surprising that the immune system had historically been regarded as a generally autonomous system governed independently of the nervous system (1). In recent decades, technological advances and multidisciplinary approaches have challenged this view of immunity, revealing that the nervous system provides multiple regulatory signals to the immune system and vice versa (1, 2). Now, it is firmly established that the nervous system and the immune system cooperate through bidirectional communication to achieve the shared goal of preserving organismal homeostasis (3). This relatively novel paradigm has been instrumental in spawning widespread interest in neuroimmune interactions, manifesting into the burgeoning and interdisciplinary field of study termed neuroimmunology (4).

Given the wide breadth of research covered under the umbrella of neuroimmunology, investigators in the field typically devote much of their focus towards either "immune-to-brain" signaling or "brain-to-immune" signaling (4). Likewise, while both directions of neuroimmune communication will be discussed in this subchapter, how immune responses dictate the function of the nervous system will be described relatively briefly. Instead, because of its far greater relevance to the central theme of this dissertation, the regulatory effects of the nervous system on the immune system will comprise the majority of the scope of this subchapter. In particular, how the nervous system influences the magnitude and characteristics of immune responses during physiological stress will be described in detail.

1.1.1 The neuroimmune interface: general anatomy and mechanisms

Anatomically, the nervous system can be divided into the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS), consisting of nerve fibers that connect the CNS to peripheral tissues (5). The PNS can further be subdivided into the somatic nervous system, which is responsible for relaying

commands of voluntary movement to skeletal muscles, and the autonomic nervous system (ANS), which controls involuntary bodily processes such as heart rate, thermoregulation, and digestion (5). The ANS is composed of the sympathetic nervous system (SNS), which is classically described to govern the “fight-or-flight” response, and the parasympathetic nervous system (PSNS), which is also known as the “rest-and-digest” system (6). The most basic unit of autonomic nerves is comprised of two connected neurons: one preganglionic neuron and one postganglionic neuron. The preganglionic neuron emanates from the brainstem or spinal cord and carries signals towards a central and peripherally located autonomic ganglion – a cluster of cell bodies formed from postganglionic neurons. Afterwards, the postganglionic neuron relays messages towards target cells via its axon terminals which infiltrate peripheral tissues (6). All preganglionic neurons are cholinergic; they transmit signals to postganglionic neurons via the neurotransmitter acetylcholine (5). However, postganglionic neurons in the PSNS and SNS differ by the chemical mediator they transmit. While postganglionic parasympathetic neurons are also cholinergic, postganglionic sympathetic neurons are noradrenergic and primarily utilize norepinephrine (NE) to relay signals (2, 5). Importantly, the collective functions of the ANS represent one of two cardinal pathways by which the nervous system exerts control over immune responses (see below and Chapter 1.1.2) (4).

The neuroendocrine system constitutes the other chief physiological axis leveraged by the CNS to regulate immunity (7). Forming a central component of the limbic system, the hypothalamus acts as the main control and command centre for coordinating internal responses to environmental cues (8). Its direct connection to the hypophyseal portal system allows the hypothalamus to dictate hormonal output by the pituitary gland and, by extension, distant endocrine organs such as the adrenal glands (4). These hormones are then released into the general circulation, and feedback loops signaling back to the hypothalamus allow it to tightly control their levels as appropriate (8). Ultimately, these hormones migrate throughout the bloodstream and function to regulate the activity of target cells in distal tissues, including cells of the immune system (see Chapter 1.1.3) (7).

On the other end of the neuroimmune functional interface, immune cells can regulate neural activity through their production and release of cytokines (1). Indeed, cytokines are now recognized to influence virtually all behavior-related activities governed by the CNS, including sleep, hunger, motivation, anxiety, and others (9). This relationship is perhaps most clearly exemplified by “sickness behavior” induced during infection (10). Consistent with these notions, certain cytokine therapies such as interferon (IFN)-α treatment for chronic viral infections are frequently associated with adverse psychiatric effects resembling sickness behavior, including symptoms of depression (11). However, much remains to be learned about the role of cytokines in CNS function, and unexpected effects of cytokines in this regard continue to be identified. For example, meningeal T cell-derived IFN-γ production in the CNS was recently demonstrated to be critical for the proper development of social behaviors (12).

The principal mission of the immune system is to defend against infection, providing a straightforward and fundamental scenario in which immune-to-brain communication can be witnessed (3). During pathogen exposure, innate immune cells rapidly secrete cytokines such as interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α, triggering key inflammatory events that are integral for optimizing antimicrobial immunity (9). These processes include, but are certainly not limited to, the febrile response (e.g., to promote macrophage and neutrophil activity), vascular permeabilization (e.g., to allow effector cells to infiltrate infected tissues), activation and mobilization of additional immune cells, and the initiation of adaptive immune responses (13, 14). Perhaps unsurprisingly, IL-1α, IL-1β, IL-6, and TNF-α also appear to be the primary cytokines involved in communication with the CNS during infection (9). The mechanisms by which these cytokine signals reach the CNS are plentiful and include: i) local cytokines in the periphery are recognized by afferent sensory nerves, most notably those held within the vagus nerve; ii) at sufficient quantities, cytokines reaching the circulation can be actively transported through specific cytokine receptors at the blood-brain-barrier; iii) cytokines in the bloodstream diffuse into the brain through vascularized, leaky regions of the brain, most notably the circumventricular organs and choroid plexus; and iv) brain tissue-resident immune cells (e.g., microglia) respond to cytokines and/or pathogen-associated molecular patterns (PAMPs) in the circulation by producing pro-

inflammatory cytokines of their own (1, 3, 9). As an important note, neurons are well-equipped with cytokine receptors, allowing them to respond accordingly to the presence of the corresponding cytokine (15).

Albeit crucial for mounting adequate host defenses against invading pathogens, inflammatory responses can lead to undesirable tissue damage if left unchecked (16). As such, inflammatory cytokines act as internal stressors (8), alerting the CNS of danger and engaging its immunomodulatory capabilities to help guide the appropriate response to an immediate threat (16). In turn, the CNS employs neuroendocrine circuits and/or the ANS to amplify certain immunological processes involved in pathogen clearance while also assisting with the resolution of inflammation, thereby avoiding overwhelming tissue damage and restoring homeostasis (16). As an example, acetylcholine release by efferent terminals of the vagus nerve, which is classically described as being the main nerve of the PSNS, is used by the CNS to send broad anti-inflammatory signals into the periphery (*aka*., the cholinergic anti-inflammatory pathway) (3).

Adding another layer of complexity to the neuroimmune axis, immune cells can also be primary sources of immunomodulatory neurotransmitters, including NE (17), serotonin (18), dopamine (19), and acetylcholine (20), and production of neurotransmitters can be induced by neural processes (21). Upon secretion, these neurotransmitters exert their effects in an autocrine or paracrine manner to enhance, repress, or shape immune responses (22). As a prime example, it was discovered that memory T cells in the spleen secrete acetylcholine after vagus nerve stimulation, which in turn attenuates TNF-α production by macrophages during lipopolysaccharide (LPS)-induced endotoxemia (23). More recently, it was found that IL-21-induced acetylcholine production by T cells during lymphocytic choriomeningitis virus infection is crucial for vasodilation and the subsequent recruitment of antiviral T cells to sites of infection (24). Due to these and other similarities between neurons and immune cells, it has been proposed that, together, the nervous and immune systems comprise interconnected “super-systems” responsible for orchestrating all processes serving to maintain host integrity (2, 25, 26).

The connection between the nervous system and the immune system has important implications for health and disease (3, 9). Disruption or dysregulation of appropriate neuroimmune interactions may lead to disorders related to immunological function (27). On one hand, inadequate immune responses resulting from a lack of stimulatory signals or an excess of inhibitory signals may increase susceptibility to infectious diseases or cancer (4). On the other hand, excessive immune responses due to a surplus in stimulatory signals or a lack of inhibitory signals may lead to autoimmunity or inflammatory conditions (28).

A sustained stress response presents a prime situation in which proper neuroimmune interactions appear to break down (29). During the stress response, hyperactivation of the SNS and the hypothalamic-pituitary-adrenal (HPA) axis, which govern this response, can facilitate several detrimental immunoregulatory effects over the long term (30). Below, the physiological pathways related to the stress response will be described in greater detail. A general overview of the known effects of stress on immune responses will also be provided.

1.1.2 Sympathetic nervous system

Part of the earliest evidence for the role of the nervous system in the regulation of the immune system was the observation that postganglionic neurons of the ANS innervate lymphoid tissues where immune cells develop and reside (31). These would later be found to be predominantly sympathetic neurons due to their positive staining for tyrosine hydroxylase, the rate-limiting enzyme in the NE synthesis pathway (32). Now, the SNS is well-recognized to densely innervate all primary (thymus, bone marrow) and secondary (lymph nodes, mucosa-associated lymphoid tissues, spleen) lymphoid tissues, providing a direct physical connection between the nervous system and the immune system during stress responses (Figure 1.1) (33). Within lymphoid tissues, sympathetic nerve endings come into close proximity with immune cells, and some may even form synaptic-like contacts with target immune cells (32). This is particularly true for regions that tend to be heavily infiltrated by macrophages, dendritic cells (DCs), plasma cells, and T cells (2, 34). As an example, sympathetic nerves in the spleen terminate close to T cell-rich

regions such as the periarteriolar lymphoid sheaths, marginal zone, and marginal sinus (2, 34).

As previously mentioned, NE is the primary neurotransmitter relayed by postganglionic sympathetic nerve fibers upon stimulation (5, 6). NE release in lymphoid tissues is considered to occur in a non-synaptic fashion since it diffuses for a relatively large distance and length of time before reaching target cells (2). Upon release, NE exerts its effects on target cells by engaging α- and β-adrenergic receptors, a class of G protein-coupled receptors (35). Depending on the adrenergic receptor subtype it interacts with, NE modulates cellular transcription by increasing or decreasing intracellular concentrations of cyclic adenosine 5'-monophosphate (cAMP) or by activating phospholipase C (PLC) (35). In turn, cAMP levels adjust the activation state of protein kinase A, which controls the phosphorylation and activation of the cAMP-responsive-element binding protein transcription factor (36). PLC raises inositol triphosphate and diacylglycerol levels, which affect transcription by mobilizing intracellular Ca^{++} stores and activating Ca^{++}/calmodulin-dependent kinases and protein kinase C (2). Importantly, many types of immune cells have been shown to express α- and/or β-adrenergic receptors, allowing NE to directly modulate their functional characteristics (37). Indeed, adrenergic receptor signaling has been demonstrated to modify the activity of several transcription factors that are critical for immune cell function including activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (2, 38, 39).

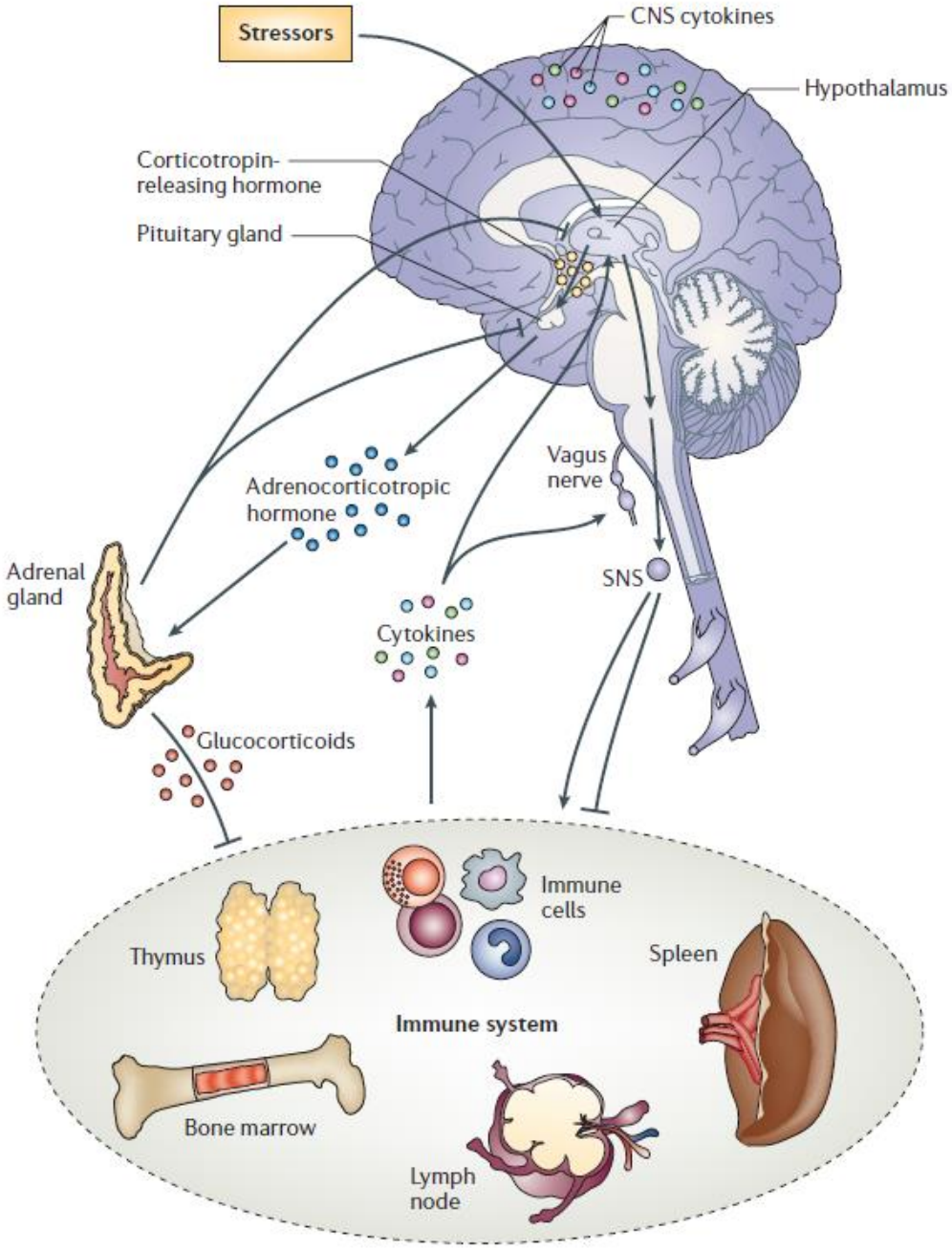

Figure 1.1: Major physiological pathways involved in modulating immune responses during stress.

The internal effects of the stress response are brought about primarily by the hyperactivation of the SNS and the HPA axis. The SNS regulates immune responses through dense innervation of primary and secondary lymphoid organs, where it releases immunomodulatory neurotransmitters. The SNS also controls the release of

catecholamine hormones such as epinephrine from the adrenal glands (not shown). The HPA axis facilitates the release of glucocorticoid hormones into the circulation, which typically have broad anti-inflammatory effects. In turn, cytokines released from immune cells act as internal stressors that signal back to the CNS as described in Chapter 1.1.1. This figure was modified from (16).

Of the adrenergic receptors to which NE can bind, the β_2-adrenergic receptor appears to be particularly influential for immune responses. Several studies have uncovered a role for β_2-adrenergic receptor signaling in regulating the functions of innate immune cells. For instance, at physiologically relevant concentrations, NE has been demonstrated to suppress human peripheral blood (PB) natural killer (NK) cell-mediated cytotoxicity against myelogenous leukemia target cells via the β_2-adrenergic receptor *in vitro* (40). Recently, it was shown that, upon engagement by exogenous or endogenous agonists, the β_2-adrenergic receptor impairs host innate immune responses to mouse cytomegalovirus, including IFN-γ production by hepatic NK cells, resulting in greater viral load and poorer survival (41). Through stimulation of the β_2-adrenergic receptor, NE also reduces NF-κB and AP-1 activity in DCs and inhibits their production of IL-12, which in turn promotes the differentiation of cluster of differentiation (CD)4^+ T cells into T helper-type (T_H)17 cells rather than T_H1 cells (39). β_2-adrenergic receptor signaling also directly affects the functions of adaptive immune cells. Indeed, NE was demonstrated to suppress the ability of $CD4^+$ T cells to produce IFN-γ, the prototypical T_H1-type cytokine, yet does not affect their ability to produce IL-4, the prototypical T_H2-type cytokine (36). This was found to be due to the β_2-adrenergic receptor being expressed by T_H1 cells but not T_H2 cells (36). Another study showed that human $CD8^+$ memory T cells stimulated in the presence of NE upregulate the pro-inflammatory cytokines IL-1α, IL-6, and TNF-α but decrease their production of IFN-γ and IL-2 through β_2-adrenergic receptor signaling (42). Grebe et al. found that SNS-derived NE release suppresses antiviral $CD4^+$ and $CD8^+$ T cell responses to influenza A virus (IAV) *in vivo* via the β_2-adrenergic receptor (43). Inhibitory effects of β_2-adrenergic receptor signaling on T cell function have also been identified in the context of vesicular stomatitis virus infection (44), antitumor immunity (45), and CNS autoimmunity (46). Lastly, NE-induced β_2-adrenergic receptor activity was recently shown to promote the immunosuppressive functions of myeloid-derived suppressor cells (MDSCs), providing another avenue by which NE may diminish T cell responses (47). Taken together, the above studies indicate that NE serves pleiotropic roles in the regulation of immunity and that these actions are largely mediated by its agonistic effects on the β_2-adrenergic receptor.

Neuropeptide Y (NPY) is a 36-amino acid peptide neurotransmitter (48). At high levels of sympathetic stimulation, NPY is co-released with NE from the large intraneuronal storage vesicles of postganglionic neurons (49). Upon release, NPY engages 5 different receptor subtypes on target cells: NPY1R, NPY2R, NPY4R, NPY5R, and NPY6R (48). Similar to NE, NPY has been shown to suppress human and mouse NK cell-mediated cytotoxic activity (50, 51) as well as IFN-γ production by $CD4^+$ T_H1 cells (52). However, unlike NE, NPY appears to promote the production of the T_H1-type cytokine IL-12 by DCs and macrophages, which serve as professional antigen (Ag)-presenting cells (APCs) (53). Interestingly, this bimodal role for NPY in adaptive immunity, particularly via its engagement of NPY1R, may be involved in the differential implications of NPY function in immune-related diseases (54). While Hassani et al. reported that NPY exacerbates the symptoms of dextran sulfate sodium-induced colitis, a T_H1-driven inflammatory condition (55), Bedoui et al. reported that NPY reduces the disease severity of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis that the authors suggested to also be driven by T_H1-type responses (56). Of note, the conclusions of the latter study may be controversial as it has since been argued that experimental autoimmune encephalomyelitis is actually driven by T_H17-type responses and that T_H1-type responses are instead protective in this context (57).

Another important physiological axis that governs immunological functions during the stress response is the sympathetic-adrenal-medullary system (30). The adrenal glands uniquely receive innervation by preganglionic (rather than postganglionic) sympathetic nerve fibers (58). Upon sympathetic stimulation, preganglionic neurons of the SNS induce the production of epinephrine (and NE to a far lesser extent) by chromaffin cells within the adrenal medulla (58). These catecholamines are then released into the bloodstream and act as hormones to impact immune responses systemically (30). Like NE, epinephrine engages α- and β-adrenergic receptors expressed on cells at target organs (2). With respect to its effects on immune responses, epinephrine is generally similar to NE, but with certain exceptions (29). For example, in a β_2-adrenergic receptor-dependent manner, exposing DCs to epinephrine impairs their ability to produce IL-12 and causes them to promote T_H17-type cytokine production from $CD4^+$ T cells, as is the case for NE (39, 59). However, unlike NE, treating DCs with epinephrine does not diminish their

ability to promote T_H1-type cytokine production by $CD4^+$ T cells upon co-culture (39, 59).

1.1.3 Hypothalamic-pituitary-adrenal axis

The HPA axis represents the principal neuroendocrine system responsible for regulating the development, survival, and functional characteristics of immune cells, particularly during the stress response (16, 29, 30). Mechanistically, the paraventricular nucleus of the hypothalamus plays a commanding role in determining the appropriate amount of activity exhibited by the HPA axis (60, 61). It receives extensive sensory and emotional information from the internal and external environment and integrates this information to direct the ultimate level of hormonal output into the general circulation. Parvocellular neurosecretory cells of the paraventricular nucleus initiate the activity of the HPA axis by secreting corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal vessels connected to the anterior pituitary gland (8, 62). Subsequently, the anterior pituitary gland reacts by releasing adrenocorticotropic hormone (ACTH) into the peripheral bloodstream. Upon reaching the adrenal glands, ACTH stimulates the synthesis and release of glucocorticoids, a class of steroid hormones, from the zona fasciculata of the adrenal cortex. In rodents, the main glucocorticoid utilized is corticosterone; in humans, it is cortisol (63). Glucocorticoids in circulation then participate in a negative feedback loop, signaling back towards the limbic system to limit further production of CRH, AVP, and ACTH (8).

Being lipophilic, glucocorticoids passively diffuse across target cell membranes and engage the glucocorticoid receptor (GR) encoded by *Nr3c1*, which when unbound remains largely in the cytoplasm and exists in a complex with chaperone proteins and immunophilins (64). Upon ligand binding, the GR dissociates from these proteins and translocates to the nucleus where it recognizes glucocorticoid response elements (GREs) on genomic DNA (65). After forming dimers or tetramers at GREs, the GR then serves as a transcription factor to enhance or repress the expression of target genes, although non-genomic effects of the activated GR (e.g., protein-protein interactions) are also well-appreciated (66). Importantly, virtually all types of cells express the GR constitutively (64), allowing glucocorticoids to exert systemic control over immune cells (among

several other cell types) at multiple levels (Figure 1.1) (16). Of note, while glucocorticoids can also bind to the mineralocorticoid receptor encoded by *Nr3c2*, major immune cell subsets including T cells essentially lack the expression of this molecule (67).

Glucocorticoids are best known for their powerful anti-inflammatory effects (64). Glucocorticoid medications such as dexamethasone (DEX), hydrocortisone (HC)/cortisol, and prednisone are widely prescribed for chronic inflammatory conditions including, but not limited to, asthma, inflammatory bowel diseases, and rheumatoid arthritis (68). Furthermore, glucocorticoids are considered to be one of the most conspicuous mediators of stress-induced immunosuppression (30). Consistent with these well-defined functions, glucocorticoids and the GR have been demonstrated to inhibit the activity of prominent transcriptional regulators of immune responses, most notably NF-κB (69, 70) and AP-1 (71, 72). Accordingly, glucocorticoids suppress the ability of human PB monocytes to produce pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α upon stimulation with LPS (73). On numerous occasions, glucocorticoids have also been shown to inhibit the functions of NK cells, including their cytotoxic activity and levels of cytokine production (74-76). Collectively, these studies provide a clear indication that glucocorticoids, through GR signaling, are suppressive for frontline innate immune defense mechanisms. At the same time, glucocorticoids have also been found to impose strong immunosuppressive effects on adaptive immune responses. For example, recent accumulated evidence has indicated that glucocorticoids disrupt $CD8^+$ T cell-mediated antitumor immune responses (77, 78). Moreover, GR signaling has been shown to enhance the ability of MDSCs to inhibit T cell function (79).

How glucocorticoids perturb the balance of T_H1-, T_H2-, and T_H17-type and regulatory T (T_{reg}) cell responses is a pertinent aspect of their immunoregulatory effects as it may explain the underlying determinants of disease progression during stress or in other circumstances. First, glucocorticoids are well-established to potently impair T_H1-type immunity (80). The activated GR reduces the production of IL-12 from DCs and macrophages (81, 82), inhibits the activity of the T_H1-promoting transcription factors T-box expressed in T cells (T-bet) and signal transducer and activator of transcription

(STAT)4 (83, 84), and directly suppresses the generation of IFN-γ in stimulated $CD4^+$ T cells (84, 85). Conversely, glucocorticoids exhibit only marginally suppressive effects on T_H2-type immunity (80). While they modestly inhibit the production of T_H2-type cytokines such as IL-5, as well as the activity of the T_H2 polarizing transcription factor GATA binding protein 3 (GATA-3) (86), they do not affect IL-4-mediated STAT6 signaling during T_H2 cell differentiation (84). Thus, glucocorticoids appear to skew the canonical T_H1/T_H2 balance towards a T_H2-type bias. On the other hand, T_H17 responses tend to be unaffected or even promoted by GR signaling (80). For instance, glucocorticoids have been shown to increase the expression of the T_H17-promoting transcription factor RAR-related orphan receptor gamma t (RORγt) (87) and augment IL-17A production by T cells (88). Lastly, GR signaling has been found to foster the generation of T_{reg} cells as, unlike T-bet and GATA-3, glucocorticoids increase forkhead box P3 (Foxp3) expression in $CD4^+$ T cells (87, 89). Accordingly, the GR has been demonstrated to elevate T cell-derived synthesis of IL-10 on multiple occasions (87, 89, 90) and to augment the suppressive functions of T_{reg} cells (91). Taken together, glucocorticoids appear to exert pathway-dependent effects on $CD4^+$ T cell differentiation in a manner that generally suppresses T_H1-type responses, permits T_H2- and T_H17-type responses, and promotes T_{reg} responses (80).

Another mechanism by which glucocorticoids regulate the functional capacity of immune cells is by altering their surface receptor expression profiles. Endogenous glucocorticoid release during murine cytomegalovirus infection induces expression of the co-inhibitory molecule programmed cell death-1 (PD-1) on splenic NK cells, preventing their immunopathological production of IFN-γ (92). Exposure to DEX also leads to the upregulation of PD-1 by bulk peripheral blood mononuclear cells (PBMCs) (93) and by activated T cells (94). DEX treatment results in the upregulation of another co-inhibitory molecule, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), on activated T cells as well (95). Recently, glucocorticoids in the tumor microenvironment (TME) were found to induce $CD8^+$ T cells' expression of multiple co-inhibitory receptors including PD-1, T cell immunoglobulin and mucin-3 (TIM-3), and lymphocyte activation gene 3 (LAG-3), leading to dysfunctional antitumor $CD8^+$ T cell responses (78). A caveat, however, is that this study showed that glucocorticoid production was derived from tumor-associated

monocytes and macrophages rather than the HPA axis (78). GR signaling has also been demonstrated to downregulate the expression of co-stimulatory molecules such as CD28 in T cells (93), providing another route by which glucocorticoids can impair immune responses. Therefore, monitoring how the surface phenotype of immune cells changes in response to glucocorticoids may be helpful in revealing the specific molecular pathways involved in the immunomodulatory effects of the GR.

It is becoming increasingly evident that several of the anti-inflammatory actions of the activated GR are a consequence of its positive transcriptional regulation of *Tsc22d3*, which encodes glucocorticoid-induced leucine zipper (GILZ) (96, 97). Like the GR, GILZ directly inhibits the activity of NF-κB and AP-1 through protein-protein interactions (98, 99). It has also been speculated that GILZ can bind directly to DNA to repress the expression of pro-inflammatory genes (100). Another parallel with the known function of glucocorticoids is that GILZ promotes an increase in T_{reg} cell numbers (101). Recently, GILZ expression was shown to abolish the efficacy of immunostimulatory chemotherapies *in vivo* and to correlate with poorer prognosis in human cancers (102). Therefore, GILZ may represent an attractive target for potentially preventing or mitigating glucocorticoid-mediated immunosuppression (e.g., during the stress response).

1.1.4 Stress-induced immunomodulation

A stressor can be defined as any real, anticipated, or imagined threat to homeostasis or well-being (8, 62). In turn, exposure to a stressor instigates a measurable internal stress response in which immediate physiological adaptations are implemented by the SNS and HPA axis to cope with the existing or incoming threat (see Chapters 1.1.2 and 1.1.3). This intentionally broad definition is meant to reflect the potential ambiguity surrounding which circumstances can be considered genuine stressors as well as the diverse forms of stressors that may arise throughout life.

In general, stressors can be categorized as being either psychological or physical in nature (62). Psychological stressors are ubiquitous and commonly experienced within human populations (103, 104). They elicit psychogenic stress responses derived from regions of the brain associated with processing emotions (e.g., fear) such as the amygdala and

infralimbic prefrontal cortex (8). Potential examples of psychological stressors include abuse, discrimination, financial turmoil, low social status, and major life events such as the death of a loved one, divorce, relocation, and being diagnosed with a disease (105, 106). Physical stressors stimulate sensory nerves that send signals of homeostatic disturbances towards the brainstem, triggering reflexive mechanisms mediated by the ANS and alerting the paraventricular nucleus of the hypothalamus (62, 107). Examples of physical stressors include inflammation (e.g., due to infection – see Chapter 1.1.1), low external temperatures, malnutrition, sleep deprivation, strenuous exercise, drug abuse, and others (29, 107, 108).

The immunological consequences of being exposed to a stressor follow a dichotomous pattern with respect to the duration of the corresponding stress response (Figure 1.2). In general, a brief or acute stress response, typically lasting two hours or less, augments the reactive potential of immune cells (109-111). Generating inflammatory responses that are greater in magnitude compared to baseline is considered to be adaptive; it confers an evolutionary advantage by directing resources to the immune system in order to meet the demands imposed by the acute stressor (112). A classic example in which this can be explained is a predator-prey scenario. The enhanced responsiveness of immune cells to tissue damage or PAMPs would be beneficial while the host is at high risk for sustaining wounds and subsequently being exposed to opportunistic pathogen entry into the blood (112). In contrast, prolonged or chronic stress responses lasting several hours, days, or weeks are generally inhibitory for immunological functions (29, 113). The maladaptive effects of chronic stress are postulated to reflect the mismatch between our ancestral environments and those of the present day, at least in industrialized societies (114). Briefly, several factors or circumstances that may be experienced in the modern world (e.g., overwhelming workloads, pervasive social expectations, and certain forms of media) induce psychological stress responses that persist for far longer than those of the past, resulting in immunological dysfunction (114). Ultimately, prolonged or chronic stress can result in detrimental immunosuppressive states that form an underlying basis for increased susceptibility to certain diseases (30), as will be described in greater detail below.

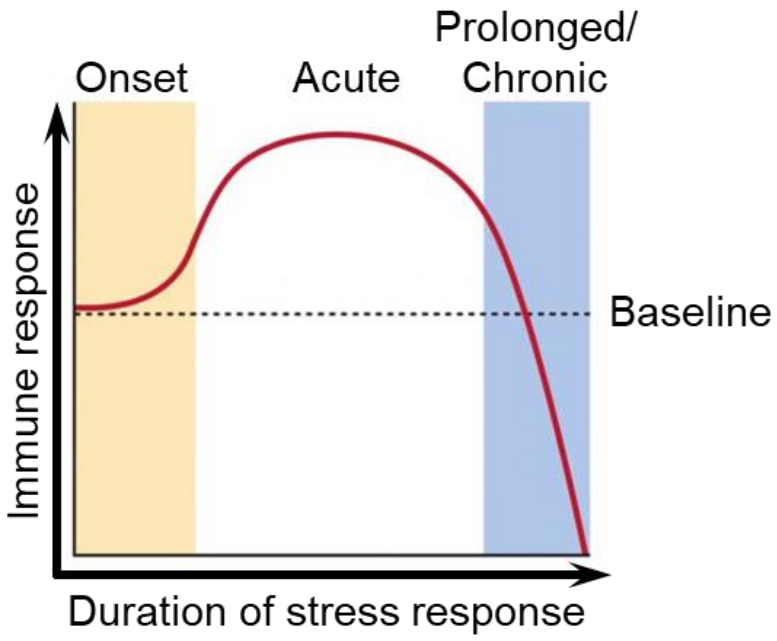

Figure 1.2: The differential impacts of acute stress and prolonged or chronic stress on the magnitude of immune responses.

A major determinant of the effect that stress responses have on immunological function is duration. Compared with steady-state conditions, acute stressors typically increase the magnitude of subsequent immune responses, an effect which is considered to be adaptive. Conversely, chronic stressors are typically suppressive for immune responses, which can increase susceptibility to the development of infectious diseases and cancer and may also prevent the efficacy of immunostimulatory drugs. This figure was modified from *Psychological Science* 4th edition, Figure 11.9 (2011).

Several associative studies of human populations have linked psychological stress to greater susceptibility to infectious diseases. Subjects self-reporting greater relative levels of psychological stress are more likely to develop symptoms of the common cold after being inoculated with various rhinoviruses, respiratory syncytial virus, or a coronavirus (115). Human immunodeficiency virus-infected patients experiencing a greater number of major life events or who receive less social support exhibit quicker progression towards acquired immunodeficiency syndrome (116). Prolonged stress has also been associated with increased frequency and duration of reoccurring herpesvirus symptoms (117). Accordingly, cohorts thought to be more prone to psychological stress, such as caregivers of dementia patients and medical students taking exams, generate poorer vaccine responses towards influenza A, hepatitis B, and rubella viruses as well as pneumococcal pneumonia (118-121). Consistent with the above human-based findings, exposing mice to prolonged physical restraint, which is a common and well-established method for inducing psychological stress in rodents (122), results in reduced control over herpes simplex virus type 1, IAV, and Theiler's murine encephalomyelitis virus infections (123-125). A mixed restraint (psychological stressor) and cold (physical stressor) model of stress also impairs host responses against *Listeria monocytogenes* infection (126). Collectively, these studies indicate that psychological stress can drastically impair host defenses against both viral and bacterial pathogens.

Epidemiological and associative studies have also suggested a relationship between psychological stress and the progression of human cancers. In a recent, ~9.5-year prospective study of 163,363 initially cancer-free participants, individuals experiencing greater levels of psychological stress were found to be more likely to succumb to death due to any cancer as well as many specific cancers including leukemia, bladder cancer, pancreatic cancer, and others (127). Similarly, a meta-analysis of prospective studies demonstrated that exposure to higher relative levels of psychological stress increases the risk of developing lung cancer and also increases the likelihood of death due to breast, hematopoietic, head and neck, and hepatobiliary cancers (128). A 20-year study of 6,284 subjects who had lost a child due to war or an accident found that the bereaved were more likely to develop haematological malignancies and melanoma compared to non-bereaved controls (129). There are now several retrospective studies that have concluded

that pharmacological blockade of receptors for mediators of stress, namely the β-adrenergic receptor for NE and epinephrine, improves prognosis for a number of different cancers (130-133). Psychological stress has also been associated with poorer tumor-infiltrating NK cell cytotoxic capacity in ovarian cancer patients (134). While the above studies do not link immunological outcomes to their findings per se, it is conceivable that dysfunctional immune responses play a role in the relationship between stress and cancer, especially since immune evasion is a recognized hallmark of cancer (135). Indeed, evidence from numerous mouse models substantiate the notion that stress-induced immunosuppression contributes significantly to tumor progression (102, 136, 137). For instance, Kamiya et al. showed that chronic restraint stress accelerates breast cancer growth and that removal of sympathetic nerves in the TME delays progression and improves T_H1-type responses within the tumor (136). A separate study demonstrated that psychological stress abrogates the efficacy of immunostimulatory anticancer drugs via GR signaling in DCs, revealing important implications for the utility of immunotherapies in stressed individuals (102).

A common conclusion in prior literature is that sustained exposure to psychological stress imposes a T_H2-type bias (138). In a study by Hu et al., splenocytes that were isolated from mice subjected to restraint stress were found to produce elevated levels of IL-4 but lower levels of IFN-γ and IL-12 upon stimulation *ex vivo* (139). Hou et al. demonstrated that chronic psychological stress increases IL-4 expression but decreases IFN-γ expression in the colon TME and in the circulation, which correlates with accelerated cancer growth (140). As described throughout Chapters 1.1.2 and 1.1.3, mediators of stress including NE, NPY, epinephrine, and glucocorticoids tend to permit or promote T_H2-type cytokine production while suppressing T_H1-type cytokine production (52, 59, 141, 142). Importantly, the T_H1 pathway is often indispensable for antitumor immunity and certain aspects of antimicrobial immunity, specifically with respect to the clearance of viral and other intracellular infections (143). On the other hand, the T_H2 pathway generally has direct antagonistic effects on protective immune responses mediated by the T_H1 pathway (143). Therefore, the stress-induced shift in the T_H1/T_H2 balance towards increased T_H2 polarization is a major proposed mechanism by which the stress response increases our susceptibility to infectious diseases and cancer (144-146).

In addition to altering inflammatory gene expression profiles, mediators of stress may dysregulate immunological processes by inducing the apoptosis of immune cells (147, 148). As a consequence of psychological stress arising from physical restraint, the cellular contents of lymphoid tissues such as the spleen and thymus undergo sudden and dramatic reductions in size (149, 150). The pro-apoptotic effects of stress appear to be mediated at least in part by the GR, which can trigger the activation of caspases among other cell death-related molecules (93, 151). Indeed, glucocorticoids have been shown to induce apoptosis in eosinophils (152), DCs (153), NK cells (154), B cells (155), $CD4^+CD8^+$ double positive (DP) thymocytes (150), and mature T cells (151, 156). However, a role for endogenous opioids in facilitating stress-induced apoptosis of lymphocytes has also been posited (149, 157).

To conclude this subchapter, the studies cited above convey a prominent dogma in the literature that mediators of psychological stress promote T_H2-type responses and facilitate a broad contraction of the immune cell repertoire (144, 147). Ultimately, the immunological effects of stress may have profound implications for the development of diseases related to immunity in humans (30). Thus, continuing to elucidate the underlying cellular and molecular mechanisms by which stress modulates immune responses is critical for determining whether deleterious neuroimmune interactions can be targeted to mitigate these pathological outcomes.

1.2 Invariant natural killer T cells

Natural killer T (NKT) cells are a heterogeneous group of unconventional T cells that, in addition to the T cell receptor (TCR), express NK cell-associated markers such as NK1.1 and DX5 (in mice), CD161 (in humans), and NKG2D (158-160). Unlike the majority of T cells, which react to peptide Ags presented by major histocompatibility complex (MHC), NKT cells react to lipid Ags presented by the non-polymorphic, MHC class I-like molecule CD1d (161) which is expressed by granulocytes, DCs, macrophages, B cells, and other cell types (162). Type 1 NKT cells express a semi-invariant TCR (*i*TCR) dominated by a TRAV11-TRAJ18 (Vα14-Jα18) TCRα chain rearrangement paired with one of TRBV1 (Vβ2), TRBV13 (Vβ8), or TRBV29 (Vβ7) in mice and a TRAV10-TRAJ18 (Vα24-Jα18) TCRα chain rearrangement paired with TRBV25 (Vβ11) in humans (163-165). Consequently, type 1 NKT cells are commonly referred to as invariant natural killer T (*i*NKT) cells (166). In addition to the *i*TCR, most *i*NKT cells bear the CD4 co-receptor while the remainder, at least in mice, are $CD4^{-}CD8^{-}$ double negative (DN) (164). Of note, a minute but detectable subpopulation of $CD8^{+}$ *i*NKT cells has been reported in humans (167). In contrast with *i*NKT cells, type 2 NKT cells exhibit greater TCRα and TCRβ chain diversity and thus are also known as variant NKT (*v*NKT) cells (168). However, *v*NKT cells remain poorly characterized relative to *i*NKT cells.

The most well-studied cognate Ag recognized by *i*NKT cells is α-galactosylceramide (αGC), a glycolipid compound originally derived from the marine sponge *Agelas mauritanius* (169, 170). Importantly, *v*NKT cells do not react to the presence of αGC (171). The specificity displayed by αGC towards the *i*TCR of *i*NKT cells not only allows for the selective activation of *i*NKT cells in laboratory settings, but also enables their precise detection via the formulation of αGC-loaded CD1d tetramers (172, 173). Indeed, αGC has been exploited as the principle experimental tool used for delineating the development, phenotype, function, and therapeutic potential of *i*NKT cells since its initial discovery (170, 172).

Mature *i*NKT cells originate in the thymus where their precursors are positively selected for by DP thymocytes (174). *i*TCR-expressing DP thymocytes receive relatively strong stimulatory signals from other DP thymocytes presenting endogenous self-lipids in the

context of CD1d (175, 176). In addition, homophilic interactions between signaling lymphocytic activated molecules (SLAM) receptors provide co-stimulatory signals for *i*NKT cell precursors, which, together with *i*TCR engagement, drive their expression of early growth response protein (EGR)1 and EGR2 (177-179). In turn, these precursor cells begin to express promyelocytic leukemia zinc finger (PLZF), the "master" transcriptional regulator of *i*NKT cell development and function, thus becoming committed to the *i*NKT cell lineage (180). After positive selection, *i*NKT cells proliferate and progress through four distinct stages of development, and many emigrate into the periphery before completing the maturation process (181, 182). Throughout development, *i*NKT cells progressively acquire a pre-activated, effector memory-like phenotype characterized in part by their constitutive expression of CD44, CD69, and pre-formed cytokine mRNA (183, 184). Importantly, this pre-activated state confers upon them the innate-like quality of being able to react incredibly rapidly to the presence of danger signals, even within hours of exposure (166). Additionally, developing *i*NKT cells differentiate into discrete effector lineages with unique transcriptional signatures, giving rise to mature $PLZF^{lo}$T-bet^{+} NKT1, $PLZF^{hi}GATA\text{-}3^{+}$ NKT2, and $PLZF^{int}ROR\gamma t^{+}$ NKT17 subsets analogous to the T_H1, T_H2, and T_H17 subsets established during conventional $CD4^{+}$ T cell activation (185, 186).

Another defining feature of mature *i*NKT cells is their constitutive expression of several receptors for cytokines including IL-12, IL-18, IL-23, and IL-25, allowing the corresponding cytokine to profoundly influence the functional profile of *i*NKT cells upon *i*TCR engagement (187-189). Moreover, certain cytokines are capable of triggering *i*NKT cell activation in a CD1d-independent fashion, enabling *i*NKT cells' participation in diverse inflammatory settings in which cognate lipid Ags are completely absent. As a prime example, *i*NKT cells have been demonstrated in numerous publications to respond in an innate-like manner to the combination of IL-12 and IL-18 independently of Ag presentation by CD1d (187, 190-192).

By absolute number, mouse *i*NKT cells in the periphery preferentially home to the liver and spleen (193). There, *i*NKT cells typically comprise between 10-30% and 0.5-1.5% of hepatic and splenic lymphocytes, respectively (172, 173, 194), the majority of which are

NKT1 cells (193). In addition, mouse *i*NKT cells represent a small fraction of the lymphocytes in the lungs and lymph nodes, where NKT2 and NKT17 cell reside, the intestines, where NKT2 cells reside, and the skin, where NKT17 cells reside (166, 193). In humans, *i*NKT cells are not as abundant as they are in mice. Their presence within the human liver usually ranges from low to absent, but can account for up to 5% of intrahepatic T cells in certain cases (195, 196). Among human PBMCs, *i*NKT cells are typically detectable but only represent 0.01-0.1% of T cells, although they can comprise >1% of T cells in some individuals (197, 198). Nevertheless, *i*NKT cells are particularly abundant in the human omentum where they account for ~10% of all T cells (199).

Depending on the specific circumstances at play (e.g., stimulus, tissue location, presence of cytokines, etc.), activated *i*NKT cells can release a diverse array of immunomodulatory cytokines at large quantities (200). Consistent with their NKT1, NKT2, and NKT17 polarization states, activated *i*NKT can produce the T_H1-type cytokine IFN-γ, the T_H2-type cytokines IL-4, IL-5, and IL-13, and the T_H17-type cytokines IL-17A and IL-22 (166). IL-2, IL-6, IL-10, IL-21, TNF-α, TGF-β, and GM-CSF are among other cytokines that *i*NKT cells can produce directly (184). Given their involvement at the early stages of an immune response, activated *i*NKT cells have a highly influential role in shaping the downstream inflammatory milieu through the cytokines they secrete.

By promptly expressing a number of different cytokines and surface molecules upon stimulation, *i*NKT cells function to transactive many other types of effector cells (184). A notable example is the *i*NKT-DC-NK cell axis that was discovered at the early stages of the immune response towards αGC (166). Upon *i*TCR engagement through DC-mediated presentation of αGC in the context of CD1d, rapid IFN-γ production and CD40L upregulation by *i*NKT cells drives the full maturation of DCs (201, 202). In turn, DCs upregulate NK cell-activating ligands such as Rae-1, CD70, and CD86 and generate the T_H1-polarizing cytokine IL-12 (203), thus provoking *i*NKT cells to release even more IFN-γ, an NK cell-activating cytokine (201, 204, 205). This sequence of events culminates in the potent transactivation of NK cells, which can then carry out their own effector functions including cytotoxic activity and cytokine secretion (206, 207).

Neutrophils, macrophages, B cells, and conventional T (T_{conv}) cells are among other effector cell types that can be transactivated downstream of *i*NKT cell stimulation (166). Therefore, *i*NKT cells serve as conductors of several immunological processes as well as a bridge between the innate and adaptive arms of the immune system.

1.2.1 *i*NKT cells in infectious diseases and cancer

*i*NKT cells have long been appreciated as frontline responders to microbial infection (208). Many microbial lipids can be loaded onto CD1d and presented to *i*NKT cells, including those derived from *Streptococcus pneumoniae*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Leishmania donovani*, and others (209-212). Furthermore, pattern recognition receptor-mediated activation of APCs can skew their endogenous self lipid profile and/or instigate the production of cytokines such as IL-12 and IL-18, prompting a robust response from *i*NKT cells (213). Consistent with their ability to react to the presence of microbial or self lipids presented by CD1d and/or inflammatory cues, mouse models of bacterial infection have established *i*NKT cells as being protective against *S. pneumoniae*, *Pseudomonas aeruginosa*, and *B. burgdorferi* infections to name a few (214-216). In addition, the antibacterial effects of exogenous αGC administration have been demonstrated in the context of *Mycobacterium tuberculosis*, *Chlamydia muridarum*, *P. aeruginosa*, and *S. pneumoniae* infections among others (215, 217-219).

In addition to their participation in antibacterial immunity, *i*NKT cells serve meaningful roles in antiviral defense. Although viruses do not directly carry ligands that can be loaded onto CD1d, viral infections have been shown to induce the production of antigenic lipids in host cells that provide danger signals to *i*NKT cells in a CD1d-dependent fashion (220). While some reports suggest that *i*NKT cells contribute to the pathogenesis of select viral diseases (e.g., hepatitis C), they are generally considered to be beneficial for the resolution of viral infection (221). For instance, *i*NKT cells have been shown to prevent IAV-induced pathology, in part by inhibiting the immunosuppressive functions of MDSCs (222, 223). Exogenous αGC treatment has also been shown to confer protection against IAV infection (224). Perhaps unsurprisingly, NK cell transactivation has been deemed critical for the ability of *i*NKT cells to control several viral infections, including those caused by murine cytomegalovirus, hepatitis B virus, and others (225-227). Given

the antiviral activity of *i*NKT cells *in vivo*, αGC has been tested in clinical trials as a treatment for chronic viral infections such as hepatitis B, but has largely failed to demonstrate therapeutic promise in these settings (228, 229).

*i*NKT cells have been deemed capable of participating in antitumor immune surveillance in numerous experimental tumor systems (230, 231). Compared to wild-type (WT) mice, Jα18$^{-/-}$ mice lacking *i*NKT cells exhibit poorer control over methylcholanthrene-induced sarcomas (232, 233), spontaneous hematopoietic malignancies and sarcomas due to p53 deficiency (234), and spontaneous prostate carcinoma (235). The majority of their antitumor properties have been demonstrated through the ability of αGC to limit lung metastases of B16-F10 melanoma, RM-1 prostate carcinoma, and DA-3 mammary carcinoma, liver metastases of 3LL Lewis lung carcinoma and EL4 lymphoma, and tumor growth in other models (206, 207). Instead of free-floating αGC, injection of autologous DCs pulsed with αGC further enhances these protective effects (236). In addition, the T_H1-polarizing analog of αGC known as α-C-galactosylceramide (αCGC) confers superior antimetastatic effects over αGC (237). IFN-γ production and NK cell transactivation have in many cases been found to be critical for the ability of *i*NKT cells to restrict tumor growth *in vivo* (206, 207). CD8$^+$ T cells (238, 239), γδ T cells (240), and precursors to mature NK (pre-MNK) cells (241) also contribute to the antimetastatic effects of αGC. Positive correlations between the number of tumor infiltrating *i*NKT cells and favorable prognosis in cancer patients provide some evidence that *i*NKT cells also promote antitumor immunity in humans (242, 243).

The antitumor properties exhibited by *i*NKT cells have led to several early phase clinical trials investigating the therapeutic potential of αGC in human cancers as previously summarized (228, 230, 244). Generally, these have resulted in the conclusion that αGC-based immunotherapies are well-tolerated and can provide positive, albeit variable, clinical responses in certain instances. Other oncology trials assessing the utility of autologous αGC-loaded DCs, αGC as a vaccine adjuvant, and chimeric antigen receptor-expressing *i*NKT cells are ongoing (244). Therefore, understanding the factors that influence the efficacy of *i*NKT cell-based immunotherapies should assist in the rational design and successful implementation of such strategies in the clinic.

1.2.2 Regulation of *i*NKT cell function by mediators of stress

Anatomical evidence suggests that *i*NKT cells in their native physiological environments are regularly exposed to signals derived from the SNS. As previously mentioned, *i*NKT cells are particularly abundant within the liver and the spleen, organs which constantly filter peripheral blood containing circulating hormones and which receive innervation by post-ganglionic sympathetic nerve fibers (2, 245).

A handful of prior studies provide evidence that $CD3^+NK1.1^+$ NKT cells, a population which includes both *i*NKT cells and *v*NKT cells, are sensitive to regulation by adrenergic receptor agonists *in vivo*. In a publication by Minagawa et al., partial hepatectomy was shown to rapidly induce an expansion in the frequency of NKT cells among lymphocytes in the liver, which could be prevented by pretreating mice with the pan β-adrenergic receptor antagonist propranolol (246). Since the levels of both epinephrine and NE were shown to be significantly elevated quickly after hepatectomy (246), the catecholamine responsible for exerting this effect on NKT cell frequencies remains unclear. Nevertheless, in an earlier study by the same group, repeated systemic administration of epinephrine was found to dramatically increase the proportion of NKT cells among lymphocytes in the liver and bone marrow (247). Yet, a similar effect was not observed using only a single dose of epinephrine in a later study (248). Using leptin-deficient mice in which circulating NE levels are low, Li et al. demonstrated that hepatic NKT cell frequencies are reduced compared to those of control mice (249). Moreover, as determined by annexin V staining, the NKT cells that persisted exhibited increased early apoptotic activity. Importantly, the differences in the NKT cell compartment between these cohorts could be reversed by treatment with NE (249). In addition, reduced NKT cell frequencies could be recapitulated using WT mice treated with 6-hydroxydopamine (OHDA) to induce chemical sympathectomy or with the α_1-adrenergic receptor antagonist prazosin, suggesting that adrenergic receptor agonists are critical for NKT cell homeostasis. These studies thus demonstrate that the relative numerical abundance of NKT cells (and, by extension, assumably *i*NKT cells) is influenced by adrenergic receptor signaling in several physiological settings, particularly in the liver.

In a recent report by Nissen et al., chronic administration of the pan β-adrenergic receptor agonist isoprenaline did not affect the antitumor efficacy of an αGC-based vaccine against Eμ-myc B cell lymphoma (250). However, while isoprenaline treatment enhanced the upregulation of the activation marker CD69 and the production of IFN-γ by *i*NKT cells responding to the vaccine, it diminished the associated NK cell and $CD8^+$ T cell responses. Moreover, daily isoprenaline treatment suppressed $CD8^+$ T cell-mediated immunity against lymphoma after exposure to an agonistic monoclonal antibody (mAb) towards the co-stimulatory receptor 4-1BB or an antagonistic mAb towards the checkpoint receptor PD-1 (250). Therefore, while continuous β-adrenergic receptor signaling does not appear to directly affect *i*NKT cell activation in this system, it significantly impairs the function of effector cells which *i*NKT cells rely on to implement their antitumor activity (166).

A pair of related publications by Wong et al. describe perhaps the most renowned studies revealing the regulatory impacts of the SNS on *i*NKT cell function to date. In the original, mouse-based study, mid-cerebral artery occlusion-induced stroke was shown to trigger hepatic *i*NKT cell activation as determined by the cessation of their intra-sinusoidal crawling behavior and their upregulation of CD69 (251). Phenotypically, *i*NKT cells exhibited a greater propensity to generate IL-10 post-stroke, contributing to a systemic switch towards increased anti-inflammatory cytokine production that is accompanied by greater susceptibility to secondary and often lethal bacterial infections. Importantly, the above effects could be prevented by pretreating mice with OHDA or the β-blocker propranolol, and the crawling behaviors associated with *i*NKT cell activation could be recapitulated via localized superfusion of the liver with NE (251). Treating mice with αGC was also sufficient to revert *i*NKT cells back to a pro-inflammatory state and prevent lethal bacterial burden. However, the ability of propranolol to prevent secondary infections was lost in $CD1d^{-/-}$ mice lacking *i*NKT cells. Therefore, in a manner that depended on NE release from postganglionic sympathetic nerve termini, *i*NKT cells were implicated in inducing a state of systemic immunosuppression shortly after stroke, rendering mice vulnerable to death due to bacterial infection (251). As a caveat, $CD1d^{-/-}$ mice lack *v*NKT cells in addition to *i*NKT cells, and thus the potential contribution of *v*NKT cells to overall immunosuppression following stroke was not directly ruled out in

this study. In a follow-up, human-based study, Wong et al. demonstrated that PB *i*NKT cells from stroke patients at admission express higher levels of CD69 than hospitalized or healthy controls (252). The degree of *i*NKT cell activation was found to be correlated with patients' serum levels of IL-10. Of note, serum IL-10 also correlated with the severity of stroke, and patients with an accompanying infection exhibited greater IL-10 levels than non-infected patients. Thus, in the context of human stroke, circulating *i*NKT cells appear to be activated and may contribute to a potentially detrimental systemic anti-inflammatory state (252), resembling some of the key findings the group made earlier in a mouse model of stroke (251).

A study by Tamada et al. indicated that NKT cells are also responsive to the effects of glucocorticoids, namely the synthetic drug DEX (253). After a single injection of DEX, the frequencies of NKT cells in the mouse liver and spleen were found to be increased. Of note, these results differ slightly from those obtained in another study in which DEX was found not to affect the frequency of NKT cells in the spleen (254). Tamada et al. also characterized NKT cell counts in both organs, reporting that while hepatic and splenic NKT cell numbers were increased and unchanged, respectively, $NK1.1^-$ T cell numbers were reduced in both organs as a consequence of DEX treatment (253). Accordingly, as opposed to $NK1.1^-$ T cells, NKT cells were found to resist glucocorticoid-induced apoptosis, perhaps due to greater baseline Bcl-2 expression which also increases in response to DEX. Lastly, the authors argue that, due to a slight increase in the frequency of IL-4-producing NKT cells in the liver and spleen after stimulation with an anti-CD3 mAb, DEX selects for T_H2-polarized NKT cells, thereby providing a potential mechanism for the T_H2-type bias typically induced by glucocorticoids (148).

One group of researchers has published multiple studies characterizing the changes to $CD3^+NK1.1^+$ NKT cell frequencies resulting directly from psychological stress. These researchers repeatedly found that prolonged restraint stress induces a noticeable expansion in the frequency of NKT cells in the liver, but not the thymus or spleen (255, 256), at least in young adult mice (248). Since this effect can be prevented by adrenalectomy (256) and recapitulated by exogenous cortisol treatment (248), it is likely mediated by the release of endogenous glucocorticoids. Furthermore, as opposed to

hepatic NK1.1$^-$ T cells whose counts are reduced during stress, the total number of hepatic NKT cells remains stable during restraint (255). In a sleep deprivation-induced stress model from another group, the absolute number of splenic CD3$^+$NKp46$^+$ NKT cells was found to be significantly decreased after 1-3 days, which was not mediated by β-adrenergic receptor signaling (257). Of note, total splenic NKT cell counts were not provided in the former studies, and splenic or hepatic NKT cell frequencies or total hepatic NKT cell counts were not provided in the latter study. Thus, directly comparing the findings from both groups to attempt to draw a unified conclusion about how stress generally affects the numerical abundance of NKT cells at different tissue locations would be rather difficult with these studies alone. It is also important to note that it remains unclear whether the reduced counts of some populations in these studies were due to apoptotic death, cell migration to other tissue locations, receptor internalization preventing the detection of certain subpopulations, or a combination of these factors. Lastly, whether an active stress response ultimately impacts the functional characteristics of NKT cells was not assessed in these studies.

1.3 Mucosa-associated invariant T cells

Mucosa-associated invariant T (MAIT) cells are an evolutionarily conserved subset of innate-like T lymphocytes that recognize a unique array of Ags bound to the nonpolymorphic MHC-related protein 1 (MR1) (258-260). MAIT cell characterization commenced when an invariant TCR was found to be overrepresented among circulating DN T cells (165). We now know that human MAIT cells bear a semi-invariant TCR with a canonical TRAV1-2/TRAJ33 (Vα7.2-Jα33) rearrangement. Atypical but appreciable subpopulations of MAIT cells expressing TRAJ12 (Jα12) or TRAJ20 (Jα20) also exist in the human T cell repertoire (261). In addition, ~1-4% of circulating MAIT cells are TRAV1-2-negative and instead express other TRAV genes including TRAV12-2 (262). Human MAIT cells' *i*TCRα can pair with *i*TCRβ chains of limited diversity, most predominantly with TRBV20 (Vβ2) or TRBV6 (Vβ13) (261). In mice, MAIT cells primarily express an *i*TCR that is composed of a TRAV1/TRAJ33 (Vα19-Jα33) α chain paired with TRBV19 (Vβ6) or TRBV13 (Vβ8) (263, 264).

MAIT cells are aptly deemed "mucosa-associated" due to their abundance within the gut, lungs and female genital mucosae, representing up to 10% of T cells at these sites (265-267). MAIT cells' presence in the human liver is remarkably heavy as they comprise up to 50% of all T lymphocytes in this organ (268). They also constitute 1-10% of total T cells in the PB. In stark contrast, MAIT cells are infrequent in laboratory mouse strains with the notable exception, so far, of CAST/EiJ mice that harbor ~20 times more MAIT cells than C57BL/6 (B6) mice by carrying a single trait mapping to the TCR-α gene locus (269). Backcrossing this locus onto the B6 background generates a congenic, MAIT cell-rich strain of mice with minimal genetic diversity known as B6-MAIT^{CAST} (269).

MAIT cells develop in the thymus where they are positively selected by MR1-expressing DP thymocytes (260, 270). Thymic egress is followed by peripheral expansion, progressive expression of the PLZF transcription factor, reactivity towards microbes, and the acquisition of an effector memory-like phenotype (CD45RA^{-}CD45RO^{+}CD62L^{low}C-C chemokine receptor [CCR]7^{low}) (264, 271, 272). In the periphery, the surface expression of C-X-C motif chemokine receptor (CXCR)1, CCR2, CCR3, CXCR3, CXCR4, CCR5,

CCR6, CXCR6 and CCR9 by MAIT cells likely enable them to home to specific tissue locations and sites of inflammation (265, 273, 274).

MAIT cells are commonly defined as Vα7.2^{+} T cells co-expressing high levels of the NK cell-associated marker CD161 (NKR-P1A). They also comprise >80% of CD8^{+}CD161^{++} T cells in the circulation (265, 275, 276). However, defining MAIT cells based on the above markers neglects the minor DN and CD4^{+} fractions (275, 276). MAIT cells rapidly react with unstable, microbe-derived vitamin B metabolites that are captured and presented by MR1 (277-279). A typical example of such compounds is 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) from the riboflavin synthesis pathway (277, 278). This feature provided the basis for the invention of Ag-loaded MR1 tetramer reagents that allow for relatively precise identification of MAIT cells (262, 263, 276).

MAIT cells constitutively express several cytokine receptor components such as CD127 (IL-7Rα), CD212 (IL-12Rβ1), CD218a (IL-18Rα) and CD218b (IL-18Rβ) (265, 273, 280, 281). Therefore, acting alone or in additive/synergistic combinations, IL-2, IL-7, IL-12, IL-15, IL-18, IFN-α and IFN-β can promote robust inflammatory and/or cytotoxic responses by MAIT cells (282-284). MAIT cells' disposition to respond to cytokines, independently of TCR engagement, likely permits their participation in various scenarios in which the presence or even the existence of cognate MR1-restricted ligands is not apparent, for example during viral infections and in the context of certain autoimmune inflammatory conditions (284-286). It is also noteworthy that MAIT cells express high levels of the IL-23 receptor (both IL-23R and IL-12Rβ1 subunits), which is known to promote the T_H17 differentiation program (268, 273).

It is well recognized that MAIT cells can launch T_H1-type responses as they readily produce IFN-γ and TNF-α upon activation (287). In addition, MAIT cells express RORγt and can adopt a T_H17-like phenotype in several tissue locations, thus predominantly synthesizing IL-17 in response to select stimuli and inflammatory conditions (265, 267, 268, 288, 289). IL-2, IL-10, and IL-22 as well as several T_H2-type cytokines including IL-4, IL-5, and IL-13 are among other cytokines produced by MAIT cells (265, 267, 290,

291), which is an additional testament to MAIT cells' plasticity and their overall ability to shape the nature of ensuing immune responses.

Being potent and prompt producers of IFN-γ (261, 283), activated MAIT cells can contribute to the resolution of certain infections or possibly even the eradication of spontaneously arising neoplastic cells (see Chapter 1.3.1). IFN-γ promotes MHC class I and Fas (CD95) expression on target cells, which could facilitate their detection by $CD8^+$ cytotoxic T lymphocytes and their FasL-mediated clearance, respectively (292, 293). Furthermore, MAIT cell-derived IFN-γ likely instigates the activation of other effector cell types such as NK cells and macrophages. In fact, MAIT cells have been demonstrated to inhibit the intracellular growth of *Mycobacterium bovis* bacillus Calmette-Guérin in macrophages via their production of IFN-γ (294). Salio *et al.* recently reported that MAIT cells activated by 5-OP-RU upregulate CD40 ligand (CD40L, CD154) and/or soluble factors that promote DC maturation and production of T_H1-polarizing cytokines such as IL-12 (295). In turn, NK cells were consequently activated to produce IFN-γ. It is conceivable that MAIT cells could also enhance NK cell-mediated cytolysis against infected and cancerous cells via the above or similar interactions.

Consistent with their phenotypic resemblance to other non-phagocytic killer cell types such as NK cells, MAIT cells harbor the necessary machinery for direct targeted cytolysis. They carry pre-formed granules containing cytotoxic mediators that can be rapidly mobilized (282, 296). These include multiple granzymes (GZMs), including GZM A and GZM K, and the pore-forming proteins perforin and granulysin (282, 297). In contrast with GZM A and GZM K, GZM B expression is low or absent in MAIT cells at baseline, but is swiftly induced by TCR-dependent and -independent stimuli, including bacterial ligands, phorbol 12-myristate 13-acetate (PMA) plus ionomycin, and cytokines (265, 280, 282, 298). Exocytosis of cytotoxic granules can be tracked by the appearance of lysosome-associated membrane protein 1 (LAMP-1) (*aka*., CD107a) on the cell surface, which is at best present in negligible levels on resting MAIT cells (282, 296), but becomes rapidly detectable on activated MAIT cells (298). Degranulation of MAIT cells, for example in response to *Escherichia coli*- or *Helicobacter pylori*-infected cells, has been found to be largely MR1-dependent, but not cytokine-dependent, suggesting that

MAIT cells can polarize their cytotoxic granules towards infected cells that display cognate Ags (282, 299). Since MAIT cells display LAMP-1 on their surface quickly after exposure to diverse stimuli and infected cell types, including both professional APCs and epithelial cells (282, 296, 300), granule exocytosis appears to be a major pathway of MAIT cell-mediated cytolysis.

1.3.1 MAIT cells in infectious diseases and cancer

The ability of MAIT cells to respond to microbial insults *in vivo* has been reviewed extensively (287, 301, 302). *i*Vα19 TCRα-transgenic and MR1-deficient mice have revealed that MAIT cells participate in controlling numerous bacterial infections, including but not limited to those caused by *E. coli*, *Klebsiella pneumoniae*, *Francisella tularensis*, and *Mycobacterium* spp. (294, 303-306). Recently, using MR1-deficient mice and T, B, and NK cell-deficient $Rag2^{-/-}\gamma C^{-/-}$ mice reconstituted with MAIT cells, IFN-γ production by MAIT cells was deemed to be critical for their ability to limit *Legionella longbeachae* infection *in vivo* (307). Moreover, circulating human MAIT cell frequencies are lowered during certain infections (303) and sepsis (308), a phenomenon that appears to be exclusive to MAIT cells as opposed to all innate-like T cell types. Furthermore, in human subjects who had responded to oral vaccination with attenuated *Shigella dysenteriae* serotype 1, reduced frequencies and upregulation of the activation marker HLA-DR in the PB MAIT cell compartment has been reported (296).

Although activated MAIT cells have been known for some time to possess cytotoxic effector molecules (265, 309), their lytic activities have come to light fairly recently. In 2013, Le Bourhis *et al.* were the first to document MAIT cell-mediated cytotoxicity by demonstrating their ability to lyse *E. coli* and *Shigella flexneri*-infected HeLa cells, which was MR1-dependent and coincided with elevated LAMP-1 expression on MAIT cells (296). In a report by Kurioka *et al.*, PB MAIT cells killed *E. coli*-infected, Epstein-Barr virus-transformed B lymphoblasts in an MR1-dependent fashion via the granule exocytosis pathway (282). Several other studies have demonstrated the ability of MAIT cells to directly lyse cells infected with *Mycobacterium* spp. (261), *H. pylori* (299), and nontypeable *Haemophilus influenzae* (310). As noted above, MAIT cells are activated during *in vivo* infections with some of the microbes against which they can also mount

cytotoxic responses *in vitro*. Therefore, although MAIT cells' ability to clear microbial infections has been commonly linked to their inflammatory cytokine release (294, 306, 307), it is reasonable to assume that MAIT cell-induced cytotoxicity too may contribute to their *in vivo* antimicrobial functions. Of note, pretreatment of MAIT cells with IL-7 potently augments their production of inflammatory cytokines and their MR1-dependent ability to kill *E. coli*-infected cells (298, 311). IL-7 can therefore be viewed as a powerful regulator of MAIT cells' antibacterial (and likely other) functions.

Viruses lack the vitamin B synthesis machinery of their own and activate MAIT cells only indirectly (303, 309, 312). Using co-cultures containing human PBMCs and A549 lung epithelial cells that were infected with either an H1N1 or H3N2 strain of IAV, Loh *et al.* found MAIT cells to rapidly express GZM B and IFN-γ (313). In this study, IFN-γ synthesis by MAIT cells occurred in an MR1- and IL-12-independent fashion, but required endogenous IL-18. van Wilgenburg *et al.* independently demonstrated that MAIT cells can be primed by IAV-exposed autologous monocyte-derived macrophages (MDMs) to produce GZM B, IFN-γ and low levels of TNF-α *in vitro* (284). IFN-γ production in this work was also similarly dependent on IL-18, which was produced by IAV-exposed MDMs, but not on MR1, IL-12 or IL-15. Further, MAIT cells expressed GZM B and IFN-γ upon co-culture with dengue virus-exposed monocyte-derived DCs or hepatitis C virus (HCV)-exposed MDMs. As expected, IFN-γ production following exposure to either virus did not rely on MR1. Rather, it was dependent on IL-12 or IL-18 in the case of dengue virus, and on IL-18 in cooperation with IL-15 in the case of HCV. Interestingly, the GZM B response to HCV was type I IFN-dependent, and HCV replication in hepatocytes was dramatically inhibited by IFN-γ of MAIT cell origin (284). In a recent mouse-based study from the same group, MAIT cells were found to rapidly accumulate in the lungs following intranasal IAV infection where they exhibited an activated state and upregulated their expression of GZM B (314). The accumulation and activation of MAIT cells was found to be partially mediated by cytokines such as IL-12, IL-18, IL-15, and type I IFN. Using MR1-deficient mice and adoptive transfer approaches, MAIT cells were ultimately deemed to be protective against IAV in a manner that was somewhat dependent on their ability to produce IFN-γ (314). Taken

together, the above findings suggest that MAIT cells participate in host responses to viral pathogens in a largely cytokine-dependent but cognate Ag-independent fashion.

It is becoming increasingly evident that MAIT cell responses extend beyond antimicrobial host defense. For instance, they are a component of tumor-infiltrating leukocytes within certain TMEs (315). Peterfalvi *et al.* first reported the presence of MAIT cell *i*TCRα chain and MR1 transcripts in several kidney and brain tumor samples (316). Since then, MAIT cells have been frequently detected in colorectal tumor masses (317-320). They appear with greater frequencies within colon cancer-afflicted tissues than in adjacent, unaffected tissue samples (317). Won *et al.* found lower circulating MAIT cell percentages in patients with mucosa-associated cancers including colon cancers (317). The authors suggested that PB MAIT cells may migrate towards the colon cancer TME, in which CCL20 and CXCL16 are heavily present, by virtue of their ability to express the corresponding receptors for these chemokines, namely CCR6 and CXCR6, respectively. Sundström and coworkers found that intratumoral MAIT cells from colon adenocarcinoma patients produce IFN-γ suboptimally in response to PMA and ionomycin (318). The authors ascribed the suppressed IFN-γ response to soluble factors released in the TME because PB MAIT cells that were exposed to tumor-conditioned medium were inefficient producers of IFN-γ in response to IL-12 and IL-18.

Our lab has found grave functional impairments in MAIT cells isolated from hepatic metastases of colorectal carcinoma (321). Tumor-infiltrating MAIT cells failed to produce IFN-γ in response to a panel of TCR-dependent and -independent stimuli, including *K. pneumoniae* lysate, staphylococcal enterotoxin B, and the combination of IL-12 and IL-18. Furthermore, MAIT cells residing in the metastatic tumor margins were partially unresponsive to the above stimuli when they were compared with those isolated from the distant, unaffected hepatic tissue (321). Consistent with these findings, MAIT cells from tumor lesions also tended to produce less GZM B. Our results suggest that the physical proximity of MAIT cells to the TME may affect their effector functions, likely including cytotoxic responses, at least for some forms of malignancy.

Although MAIT cells exhibit tropism for certain TMEs, their exact functions remain far from clearly understood and may be highly context-dependent (322). MAIT cells can potentially contribute to the generation of T_H1-type anticancer effector responses. In addition, they can express or upregulate lytic molecules, which may help eliminate tumor cells directly. On the flip side, these molecules might cause undesirable collateral damage. The propensity of MAIT cells to produce IL-17A under certain conditions may potentiate tumorigenesis and neoangiogenesis, or perpetuate the state of local immunosuppression within TMEs by facilitating the recruitment of various regulatory T cell types and MDSCs (323-325). This may in turn promote the progressive escape of neoplastic cells from otherwise protective immune surveillance. Indeed, Yan et al. recently reported that MAIT cells promote the establishment and growth of lung metastases and fibrosarcoma by suppressing NK cell function in an MR1 and IL-17A dependent manner (326). However, it is important to note that human and mouse MAIT cells may differ widely in the effector functions they elicit. Namely, while activated mouse MAIT cells preferentially produce IL-17A in B6 mice, human MAIT cells exhibit a greater relative proclivity to produce IFN-γ upon stimulation (327). Therefore, assessing the participation of MAIT cells in tumor immunity in mice may not accurately reflect the roles they play in the pathogenesis of human cancers.

1.4 References

1. Dantzer R. Neuroimmune Interactions: From the Brain to the Immune System and Vice Versa. *Physiol Rev.* 2018;98(1):477-504.

2. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev.* 2000;52(4):595-638.

3. Pavlov VA, Tracey KJ. Neural regulation of immunity: molecular mechanisms and clinical translation. *Nat Neurosci.* 2017;20(2):156-166.

4. Schiller M, Ben-Shaanan TL, Rolls A. Neuronal regulation of immunity: why, how and where? *Nat Rev Immunol.* 2021;21(1):20-36.

5. Elefteriou F. Impact of the Autonomic Nervous System on the Skeleton. *Physiol Rev.* 2018;98(3):1083-1112.

6. McCorry LK. Physiology of the autonomic nervous system. *Am J Pharm Educ.* 2007;71(4):78.

7. Taub DD. Neuroendocrine interactions in the immune system. *Cell Immunol.* 2008;252(1-2):1-6.

8. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci.* 2009;10(6):397-409.

9. Capuron L, Miller AH. Immune system to brain signaling: neuropsychopharmacological implications. *Pharmacol Ther.* 2011;130(2):226-238.

10. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci.* 2008;9(1):46-56.

11. Raison CL, et al. Depression during pegylated interferon-alpha plus ribavirin therapy: prevalence and prediction. *J Clin Psychiatry.* 2005;66(1):41-48.

12. Filiano AJ, et al. Unexpected role of interferon-γ in regulating neuronal connectivity and social behaviour. *Nature.* 2016;535(7612):425-429.

13. Evans SS, Repasky EA, Fisher DT. Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat Rev Immunol.* 2015;15(6):335-349.

14. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol.* 2009;78(6):539-552.

15. Tracey KJ. Reflex control of immunity. *Nat Rev Immunol.* 2009;9(6):418-428.

16. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* 2006;6(4):318-328.

17. Cosentino M, et al. Human CD4+CD25+ regulatory T cells selectively express tyrosine hydroxylase and contain endogenous catecholamines subserving an autocrine/paracrine inhibitory functional loop. *Blood.* 2007;109(2):632-642.

18. León-Ponte M, Ahern GP, O'Connell PJ. Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor. *Blood.* 2007;109(8):3139-3146.

19. Prado C, et al. Stimulation of dopamine receptor D5 expressed on dendritic cells potentiates Th17-mediated immunity. *J Immunol.* 2012;188(7):3062-3070.

20. Ramirez VT, et al. T-cell derived acetylcholine aids host defenses during enteric bacterial infection with Citrobacter rodentium. *PLoS Pathog.* 2019;15(4):e1007719.

21. Trakhtenberg EF, Goldberg JL. Immunology. Neuroimmune communication. *Science.* 2011;334(6052):47-48.

22. Levite M. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr Opin Pharmacol.* 2008;8(4):460-471.

23. Rosas-Ballina M, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* 2011;334(6052):98-101.

24. Cox MA, et al. Choline acetyltransferase-expressing T cells are required to control chronic viral infection. *Science.* 2019;363(6427):639-644.

25. Kioussis D, Pachnis V. Immune and nervous systems: more than just a superficial similarity? *Immunity.* 2009;31(5):705-710.

26. Tada T. The immune system as a supersystem. *Annu Rev Immunol.* 1997;15(1-13.

27. Pavlov VA, Tracey KJ. The cholinergic anti-inflammatory pathway. *Brain Behav Immun.* 2005;19(6):493-499.

28. Terrando N, Pavlov VA. Editorial: Neuro-Immune Interactions in Inflammation and Autoimmunity. *Front Immunol.* 2018;9(772.

29. Pruett SB. Stress and the immune system. *Pathophysiology.* 2003;9(3):133-153.

30. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol.* 2005;5(3):243-251.

31. Reilly FD, McCuskey RS, Meineke HA. Studies of the hemopoietic microenvironment. VIII. Andrenergic and cholinergic innervation of the murine spleen. *Anat Rec.* 1976;185(1):109-117.

32. Felten SY, Olschowka J. Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. *J Neurosci Res.* 1987;18(1):37-48.

33. Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987-2007). *Brain Behav Immun.* 2007;21(6):736-745.

34. Hu D, et al. Distribution of nerve fibers and nerve-immune cell association in mouse spleen revealed by immunofluorescent staining. *Sci Rep.* 2020;10(1):9850.

35. Gilman AG. G proteins: transducers of receptor-generated signals. *Annu Rev Biochem.* 1987;56(615-649.

36. Sanders VM. The beta2-adrenergic receptor on T and B lymphocytes: do we understand it yet? *Brain Behav Immun.* 2012;26(2):195-200.

37. Sharma D, Farrar JD. Adrenergic regulation of immune cell function and inflammation. *Semin Immunopathol.* 2020;42(6):709-717.

38. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. *Pharmacol Rev.* 2001;53(4):487-525.

39. Takenaka MC, et al. Norepinephrine Controls Effector T Cell Differentiation through β2-Adrenergic Receptor-Mediated Inhibition of NF-κB and AP-1 in Dendritic Cells. *J Immunol.* 2016;196(2):637-644.

40. Takamoto T, Hori Y, Koga Y, Toshima H, Hara A, Yokoyama MM. Norepinephrine inhibits human natural killer cell activity in vitro. *Int J Neurosci.* 1991;58(1-2):127-131.

41. Wieduwild E, et al. β2-adrenergic signals downregulate the innate immune response and reduce host resistance to viral infection. *J Exp Med.* 2020;217(4).

42. Slota C, Shi A, Chen G, Bevans M, Weng NP. Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation. *Brain Behav Immun.* 2015;46(168-179.

43. Grebe KM, Hickman HD, Irvine KR, Takeda K, Bennink JR, Yewdell JW. Sympathetic nervous system control of anti-influenza CD8+ T cell responses. *Proc Natl Acad Sci U S A.* 2009;106(13):5300-5305.

44. Estrada LD, Ağaç D, Farrar JD. Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8(+) T-cell effector function. *Eur J Immunol.* 2016;46(8):1948-1958.

45. Bucsek MJ, et al. β-Adrenergic Signaling in Mice Housed at Standard Temperatures Suppresses an Effector Phenotype in CD8(+) T Cells and Undermines Checkpoint Inhibitor Therapy. *Cancer Res.* 2017;77(20):5639-5651.

46. Araujo LP, et al. The Sympathetic Nervous System Mitigates CNS Autoimmunity via β2-Adrenergic Receptor Signaling in Immune Cells. *Cell Rep.* 2019;28(12):3120-3130.e3125.

47. Mohammadpour H, et al. β2 adrenergic receptor-mediated signaling regulates the immunosuppressive potential of myeloid-derived suppressor cells. *J Clin Invest.* 2019;129(12):5537-5552.

48. Dimitrijević M, Stanojević S. The intriguing mission of neuropeptide Y in the immune system. *Amino Acids.* 2013;45(1):41-53.

49. Straub RH, et al. Neuropeptide Y cotransmission with norepinephrine in the sympathetic nerve-macrophage interplay. *J Neurochem.* 2000;75(6):2464-2471.

50. Nair MP, Schwartz SA, Wu K, Kronfol Z. Effect of neuropeptide Y on natural killer activity of normal human lymphocytes. *Brain Behav Immun.* 1993;7(1):70-78.

51. Saurer TB, Ijames SG, Lysle DT. Neuropeptide Y Y1 receptors mediate morphine-induced reductions of natural killer cell activity. *J Neuroimmunol.* 2006;177(1-2):18-26.

52. Kawamura N, et al. Differential effects of neuropeptides on cytokine production by mouse helper T cell subsets. *Neuroimmunomodulation.* 1998;5(1-2):9-15.

53. Wheway J, et al. A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. *J Exp Med.* 2005;202(11):1527-1538.

54. Prod'homme T, Weber MS, Steinman L, Zamvil SS. A neuropeptide in immune-mediated inflammation, Y? *Trends Immunol.* 2006;27(4):164-167.

55. Hassani H, Lucas G, Rozell B, Ernfors P. Attenuation of acute experimental colitis by preventing NPY Y1 receptor signaling. *Am J Physiol Gastrointest Liver Physiol.* 2005;288(3):G550-556.

56. Bedoui S, et al. Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY1 receptor-specific inhibition of autoreactive Th1 responses in vivo. *J Immunol.* 2003;171(7):3451-3458.

57. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med.* 2007;13(2):139-145.

58. Lumb R, et al. Neuropilins guide preganglionic sympathetic axons and chromaffin cell precursors to establish the adrenal medulla. *Development.* 2018;145(21).

59. Kim BJ, Jones HP. Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-γ by CD4+ T cells. *Brain Behav Immun.* 2010;24(7):1126-1136.

60. Ferguson AV, Latchford KJ, Samson WK. The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. *Expert Opin Ther Targets.* 2008;12(6):717-727.

61. Benarroch EE. Paraventricular nucleus, stress response, and cardiovascular disease. *Clin Auton Res.* 2005;15(4):254-263.

62. Bains JS, Wamsteeker Cusulin JI, Inoue W. Stress-related synaptic plasticity in the hypothalamus. *Nat Rev Neurosci.* 2015;16(7):377-388.

63. Stephens MA, Wand G. Stress and the HPA axis: role of glucocorticoids in alcohol dependence. *Alcohol Res.* 2012;34(4):468-483.

64. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *J Allergy Clin Immunol.* 2013;132(5):1033-1044.

65. Lee HC, Shibata H, Ogawa S, Maki K, Ikuta K. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *J Immunol.* 2005;174(12):7800-7806.

66. Scheschowitsch K, Leite JA, Assreuy J. New Insights in Glucocorticoid Receptor Signaling-More Than Just a Ligand-Binding Receptor. *Front Endocrinol (Lausanne).* 2017;8(16.

67. Miller AH, Spencer RL, Stein M, McEwen BS. Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone. *Am J Physiol.* 1990;259(3 Pt 1):E405-412.

68. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet.* 2009;373(9678):1905-1917.

69. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science.* 1995;270(5234):286-290.

70. Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A.* 1994;91(2):752-756.

71. Yang-Yen HF, et al. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell.* 1990;62(6):1205-1215.

72. Jonat C, et al. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell.* 1990;62(6):1189-1204.

73. Brattsand R, Linden M. Cytokine modulation by glucocorticoids: mechanisms and actions in cellular studies. *Aliment Pharmacol Ther.* 1996;10 Suppl 2(81-90; discussion 91-82.

74. Gatti G, et al. Inhibition by cortisol of human natural killer (NK) cell activity. *J Steroid Biochem.* 1987;26(1):49-58.

75. Morgan DJ, Davis DM. Distinct Effects of Dexamethasone on Human Natural Killer Cell Responses Dependent on Cytokines. *Front Immunol.* 2017;8(432.

76. Krukowski K, Eddy J, Kosik KL, Konley T, Janusek LW, Mathews HL. Glucocorticoid dysregulation of natural killer cell function through epigenetic modification. *Brain Behav Immun.* 2011;25(2):239-249.

77. Tokunaga A, et al. Selective inhibition of low-affinity memory CD8(+) T cells by corticosteroids. *J Exp Med.* 2019;216(12):2701-2713.

78. Acharya N, et al. Endogenous Glucocorticoid Signaling Regulates CD8(+) T Cell Differentiation and Development of Dysfunction in the Tumor Microenvironment. *Immunity.* 2020;53(3):658-671.e656.

79. Lu Y, et al. Glucocorticoid receptor promotes the function of myeloid-derived suppressor cells by suppressing HIF1α-dependent glycolysis. *Cell Mol Immunol.* 2018;15(6):618-629.

80. Taves MD, Ashwell JD. Glucocorticoids in T cell development, differentiation and function. *Nat Rev Immunol.* 2020.

81. Agarwal SK, Marshall GD, Jr. Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines. *J Interferon Cytokine Res.* 2001;21(3):147-155.

82. Li CC, Munitic I, Mittelstadt PR, Castro E, Ashwell JD. Suppression of Dendritic Cell-Derived IL-12 by Endogenous Glucocorticoids Is Protective in LPS-Induced Sepsis. *PLoS Biol.* 2015;13(10):e1002269.

83. Liberman AC, et al. The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction. *Faseb j.* 2007;21(4):1177-1188.

84. Franchimont D, et al. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *J Immunol.* 2000;164(4):1768-1774.

85. Ramírez F, Fowell DJ, Puklavec M, Simmonds S, Mason D. Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. *J Immunol.* 1996;156(7):2406-2412.

86. Liberman AC, Druker J, Refojo D, Holsboer F, Arzt E. Glucocorticoids inhibit GATA-3 phosphorylation and activity in T cells. *Faseb j.* 2009;23(5):1558-1571.

87. de Castro Kroner J, Knoke K, Kofler DM, Steiger J, Fabri M. Glucocorticoids promote intrinsic human T(H)17 differentiation. *J Allergy Clin Immunol.* 2018;142(5):1669-1673.e1611.

88. Schewitz-Bowers LP, et al. Glucocorticoid-resistant Th17 cells are selectively attenuated by cyclosporine A. *Proc Natl Acad Sci U S A.* 2015;112(13):4080-4085.

89. Karagiannidis C, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol.* 2004;114(6):1425-1433.

90. Peek EJ, et al. Interleukin-10-secreting "regulatory" T cells induced by glucocorticoids and beta2-agonists. *Am J Respir Cell Mol Biol.* 2005;33(1):105-111.

91. Kim D, et al. Anti-inflammatory Roles of Glucocorticoids Are Mediated by Foxp3(+) Regulatory T Cells via a miR-342-Dependent Mechanism. *Immunity.* 2020;53(3):581-596.e585.

92. Quatrini L, et al. Endogenous glucocorticoids control host resistance to viral infection through the tissue-specific regulation of PD-1 expression on NK cells. *Nat Immunol.* 2018;19(9):954-962.

93. Galon J, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *Faseb j.* 2002;16(1):61-71.

94. Xing K, Gu B, Zhang P, Wu X. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC Immunol.* 2015;16(39.

95. Xia M, Gasser J, Feige U. Dexamethasone enhances CTLA-4 expression during T cell activation. *Cell Mol Life Sci.* 1999;55(12):1649-1656.

96. Monczor F, Chatzopoulou A, Zappia CD, Houtman R, Meijer OC, Fitzsimons CP. A Model of Glucocorticoid Receptor Interaction With Coregulators Predicts Transcriptional Regulation of Target Genes. *Front Pharmacol.* 2019;10(214.

97. Ronchetti S, Migliorati G, Riccardi C. GILZ as a Mediator of the Anti-Inflammatory Effects of Glucocorticoids. *Front Endocrinol (Lausanne).* 2015;6(170.

98. Ayroldi E, et al. Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood.* 2001;98(3):743-753.

99. Mittelstadt PR, Ashwell JD. Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem.* 2001;276(31):29603-29610.

100. Yosef N, et al. Dynamic regulatory network controlling TH17 cell differentiation. *Nature.* 2013;496(7446):461-468.

101. Bereshchenko O, et al. GILZ promotes production of peripherally induced Treg cells and mediates the crosstalk between glucocorticoids and TGF-β signaling. *Cell Rep.* 2014;7(2):464-475.

102. Yang H, et al. Stress-glucocorticoid-TSC22D3 axis compromises therapy-induced antitumor immunity. *Nat Med.* 2019;25(9):1428-1441.

103. Schneiderman N, Ironson G, Siegel SD. Stress and health: psychological, behavioral, and biological determinants. *Annu Rev Clin Psychol.* 2005;1(607-628.

104. Sood P, Priyadarshini S, Aich P. Estimation of psychological stress in humans: a combination of theory and practice. *PLoS One.* 2013;8(5):e63044.

105. Spruill TM. Chronic psychosocial stress and hypertension. *Curr Hypertens Rep.* 2010;12(1):10-16.

106. Cohen S, Murphy MLM, Prather AA. Ten Surprising Facts About Stressful Life Events and Disease Risk. *Annu Rev Psychol.* 2019;70(577-597.

107. Herman JP, et al. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol.* 2003;24(3):151-180.

108. Karl JP, et al. Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota. *Front Microbiol.* 2018;9(2013.

109. Flint MS, Tinkle SS. C57BL/6 mice are resistant to acute restraint modulation of cutaneous hypersensitivity. *Toxicol Sci.* 2001;62(2):250-256.

110. Dhabhar FS. Stress, leukocyte trafficking, and the augmentation of skin immune function. *Ann N Y Acad Sci.* 2003;992(205-217.

111. Schedlowski M, et al. Changes of natural killer cells during acute psychological stress. *J Clin Immunol.* 1993;13(2):119-126.

112. Dhabhar FS, Malarkey WB, Neri E, McEwen BS. Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. *Psychoneuroendocrinology.* 2012;37(9):1345-1368.

113. Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav Immun.* 1997;11(4):286-306.

114. Brenner S, Jones JP, Rutanen-Whaley RH, Parker W, Flinn M, Muehlenbein MP. 2015.

115. Cohen S, Tyrrell DA, Smith AP. Psychological stress and susceptibility to the common cold. *N Engl J Med.* 1991;325(9):606-612.

116. Leserman J, et al. Progression to AIDS: the effects of stress, depressive symptoms, and social support. *Psychosom Med.* 1999;61(3):397-406.

117. Cohen F, Kemeny ME, Kearney KA, Zegans LS, Neuhaus JM, Conant MA. Persistent stress as a predictor of genital herpes recurrence. *Arch Intern Med.* 1999;159(20):2430-2436.

118. Deng Y, Jing Y, Campbell AE, Gravenstein S. Age-related impaired type 1 T cell responses to influenza: reduced activation ex vivo, decreased expansion in CTL culture in vitro, and blunted response to influenza vaccination in vivo in the elderly. *J Immunol.* 2004;172(6):3437-3446.

119. Glaser R, Kiecolt-Glaser JK, Bonneau RH, Malarkey W, Kennedy S, Hughes J. Stress-induced modulation of the immune response to recombinant hepatitis B vaccine. *Psychosom Med.* 1992;54(1):22-29.

120. Morag M, Morag A, Reichenberg A, Lerer B, Yirmiya R. Psychological variables as predictors of rubella antibody titers and fatigue--a prospective, double blind study. *J Psychiatr Res.* 1999;33(5):389-395.

121. Glaser R, Sheridan J, Malarkey WB, MacCallum RC, Kiecolt-Glaser JK. Chronic stress modulates the immune response to a pneumococcal pneumonia vaccine. *Psychosom Med.* 2000;62(6):804-807.

122. Glavin GB, Paré WP, Sandbak T, Bakke HK, Murison R. Restraint stress in biomedical research: an update. *Neurosci Biobehav Rev.* 1994;18(2):223-249.

123. Elftman MD, Hunzeker JT, Mellinger JC, Bonneau RH, Norbury CC, Truckenmiller ME. Stress-induced glucocorticoids at the earliest stages of herpes simplex virus-1 infection suppress subsequent antiviral immunity, implicating impaired dendritic cell function. *J Immunol.* 2010;184(4):1867-1875.

124. Padgett DA, MacCallum RC, Sheridan JF. Stress exacerbates age-related decrements in the immune response to an experimental influenza viral infection. *J Gerontol A Biol Sci Med Sci.* 1998;53(5):B347-353.

125. Sieve AN, et al. Chronic restraint stress during early Theiler's virus infection exacerbates the subsequent demyelinating disease in SJL mice. *J Neuroimmunol.* 2004;155(1-2):103-118.

126. Cao L, Filipov NM, Lawrence DA. Sympathetic nervous system plays a major role in acute cold/restraint stress inhibition of host resistance to Listeria monocytogenes. *J Neuroimmunol.* 2002;125(1-2):94-102.

127. Batty GD, Russ TC, Stamatakis E, Kivimäki M. Psychological distress in relation to site specific cancer mortality: pooling of unpublished data from 16 prospective cohort studies. *Bmj.* 2017;356(j108.

128. Chida Y, Hamer M, Wardle J, Steptoe A. Do stress-related psychosocial factors contribute to cancer incidence and survival? *Nat Clin Pract Oncol.* 2008;5(8):466-475.

129. Levav I, Kohn R, Iscovich J, Abramson JH, Tsai WY, Vigdorovich D. Cancer incidence and survival following bereavement. *Am J Public Health.* 2000;90(10):1601-1607.

130. Jansen L, Hoffmeister M, Arndt V, Chang-Claude J, Brenner H. Stage-specific associations between beta blocker use and prognosis after colorectal cancer. *Cancer.* 2014;120(8):1178-1186.

131. Renz BW, et al. β2 Adrenergic-Neurotrophin Feedforward Loop Promotes Pancreatic Cancer. *Cancer Cell.* 2018;34(5):863-867.

132. Barron TI, Connolly RM, Sharp L, Bennett K, Visvanathan K. Beta blockers and breast cancer mortality: a population- based study. *J Clin Oncol.* 2011;29(19):2635-2644.

133. Watkins JL, et al. Clinical impact of selective and nonselective beta-blockers on survival in patients with ovarian cancer. *Cancer.* 2015;121(19):3444-3451.

134. Lutgendorf SK, et al. Social support, psychological distress, and natural killer cell activity in ovarian cancer. *J Clin Oncol.* 2005;23(28):7105-7113.

135. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-674.

136. Kamiya A, et al. Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat Neurosci.* 2019;22(8):1289-1305.

137. Partecke LI, et al. Chronic stress increases experimental pancreatic cancer growth, reduces survival and can be antagonised by beta-adrenergic receptor blockade. *Pancreatology.* 2016;16(3):423-433.

138. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol.* 2003;24(8):444-448.

139. Hu D, et al. Essential role of IL-10/STAT3 in chronic stress-induced immune suppression. *Brain Behav Immun.* 2014;36(118-127.

140. Hou N, et al. A novel chronic stress-induced shift in the Th1 to Th2 response promotes colon cancer growth. *Biochem Biophys Res Commun.* 2013;439(4):471-476.

141. Maestroni GJ. Short exposure of maturing, bone marrow-derived dendritic cells to norepinephrine: impact on kinetics of cytokine production and Th development. *J Neuroimmunol.* 2002;129(1-2):106-114.

142. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci.* 2004;1024(138-146.

143. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology.* 2008;123(3):326-338.

144. Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Ann N Y Acad Sci.* 2006;1069(62-76.

145. Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol.* 2004;5(10):617-625.

146. Dai S, et al. Chronic Stress Promotes Cancer Development. *Front Oncol.* 2020;10(1492.

147. Shi Y, Devadas S, Greeneltch KM, Yin D, Allan Mufson R, Zhou JN. Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain Behav Immun.* 2003;17 Suppl 1(S18-26.

148. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol.* 2017;17(4):233-247.

149. Yin D, Tuthill D, Mufson RA, Shi Y. Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J Exp Med.* 2000;191(8):1423-1428.

150. Tarcic N, Ovadia H, Weiss DW, Weidenfeld J. Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. *J Neuroimmunol.* 1998;82(1):40-46.

151. Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci.* 2006;63(1):60-72.

152. Druilhe A, Létuvé S, Pretolani M. Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. *Apoptosis.* 2003;8(5):481-495.

153. Kim KD, Choe YK, Choe IS, Lim JS. Inhibition of glucocorticoid-mediated, caspase-independent dendritic cell death by CD40 activation. *J Leukoc Biol.* 2001;69(3):426-434.

154. Tseng RJ, Padgett DA, Dhabhar FS, Engler H, Sheridan JF. Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. *Brain Behav Immun.* 2005;19(2):153-164.

155. Garvy BA, King LE, Telford WG, Morford LA, Fraker PJ. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. *Immunology.* 1993;80(4):587-592.

156. Wang D, Müller N, McPherson KG, Reichardt HM. Glucocorticoids engage different signal transduction pathways to induce apoptosis in thymocytes and mature T cells. *J Immunol.* 2006;176(3):1695-1702.

157. Wang J, Charboneau R, Barke RA, Loh HH, Roy S. Mu-opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. *J Immunol.* 2002;169(7):3630-3636.

158. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol.* 2004;4(3):231-237.

159. Werner JM, Busl E, Farkas SA, Schlitt HJ, Geissler EK, Hornung M. DX5+NKT cells display phenotypical and functional differences between spleen and liver as well as NK1.1-Balb/c and NK1.1+ C57Bl/6 mice. *BMC Immunol.* 2011;12(26.

160. Kuylenstierna C, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *Eur J Immunol.* 2011;41(7):1913-1923.

161. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science.* 1995;268(5212):863-865.

162. Brossay L, et al. Mouse CD1 is mainly expressed on hemopoietic-derived cells. *J Immunol.* 1997;159(3):1216-1224.

163. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med.* 1994;180(3):1171-1176.

164. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med.* 1994;180(3):1097-1106.

165. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med.* 1993;178(1):1-16.

166. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol.* 2013;13(2):101-117.

167. Ishihara S, et al. CD8(+)NKR-P1A (+)T cells preferentially accumulate in human liver. *Eur J Immunol.* 1999;29(8):2406-2413.

168. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol.* 2010;11(3):197-206.

169. Morita M, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem.* 1995;38(12):2176-2187.

170. Kawano T, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997;278(5343):1626-1629.

171. Jahng A, Maricic I, Aguilera C, Cardell S, Halder RC, Kumar V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med.* 2004;199(7):947-957.

172. Berzins SP, Smyth MJ, Godfrey DI. Working with NKT cells--pitfalls and practicalities. *Curr Opin Immunol.* 2005;17(4):448-454.

173. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M. Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc.* 2008;3(1):70-78.

174. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med.* 1995;182(6):2091-2096.

175. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol.* 2000;164(5):2412-2418.

176. Moran AE, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med.* 2011;208(6):1279-1289.

177. Griewank K, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity.* 2007;27(5):751-762.

178. Dutta M, et al. A role for Ly108 in the induction of promyelocytic zinc finger transcription factor in developing thymocytes. *J Immunol.* 2013;190(5):2121-2128.

179. Seiler MP, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat Immunol.* 2012;13(3):264-271.

180. Savage AK, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity.* 2008;29(3):391-403.

181. Xu Y, et al. Regulation of the terminal maturation of iNKT cells by mediator complex subunit 23. *Nat Commun.* 2018;9(1):3875.

182. Godfrey DI, Berzins SP. Control points in NKT-cell development. *Nat Rev Immunol.* 2007;7(7):505-518.

183. Stetson DB, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med.* 2003;198(7):1069-1076.

184. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol.* 2008;20(3):358-368.

185. Krovi SH, Gapin L. Invariant Natural Killer T Cell Subsets-More Than Just Developmental Intermediates. *Front Immunol.* 2018;9(1393.

186. Engel I, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol.* 2016;17(6):728-739.

187. Leite-De-Moraes MC, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol.* 1999;163(11):5871-5876.

188. Rachitskaya AV, et al. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J Immunol.* 2008;180(8):5167-5171.

189. Watarai H, et al. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* 2012;10(2):e1001255.

190. Baxevanis CN, Gritzapis AD, Papamichail M. In vivo antitumor activity of NKT cells activated by the combination of IL-12 and IL-18. *J Immunol.* 2003;171(6):2953-2959.

191. Nagarajan NA, Kronenberg M. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol.* 2007;178(5):2706-2713.

192. Velazquez P, Cameron TO, Kinjo Y, Nagarajan N, Kronenberg M, Dustin ML. Cutting edge: activation by innate cytokines or microbial antigens can cause arrest of natural killer T cell patrolling of liver sinusoids. *J Immunol.* 2008;180(4):2024-2028.

193. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. *Immunity.* 2015;43(3):566-578.

194. Tefit JN, Davies G, Serra V. NKT cell responses to glycolipid activation. *Methods Mol Biol.* 2010;626(149-167.

195. Kenna T, et al. NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells. *J Immunol.* 2003;171(4):1775-1779.

196. Syn WK, et al. Accumulation of natural killer T cells in progressive nonalcoholic fatty liver disease. *Hepatology.* 2010;51(6):1998-2007.

197. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med.* 2002;195(5):625-636.

198. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med.* 2002;195(5):637-641.

199. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur J Immunol.* 2009;39(7):1893-1901.

200. Szabo PA, Anantha RV, Shaler CR, McCormick JK, Haeryfar SM. CD1d- and MR1-Restricted T Cells in Sepsis. *Front Immunol.* 2015;6(401.

201. Kitamura H, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med.* 1999;189(7):1121-1128.

202. Tomura M, et al. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol.* 1999;163(1):93-101.

203. Arora P, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity.* 2014;40(1):105-116.

204. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J Immunol.* 2001;166(10):6012-6018.

205. Carnaud C, et al. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol.* 1999;163(9):4647-4650.

206. Hayakawa Y, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol.* 2001;31(6):1720-1727.

207. Smyth MJ, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* 2002;99(4):1259-1266.

208. Yu KO, Porcelli SA. The diverse functions of CD1d-restricted NKT cells and their potential for immunotherapy. *Immunol Lett.* 2005;100(1):42-55.

209. Kinjo Y, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol.* 2011;12(10):966-974.

210. Chang YJ, et al. Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. *J Clin Invest.* 2011;121(1):57-69.

211. Kinjo Y, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol.* 2006;7(9):978-986.

212. Amprey JL, et al. A subset of liver NK T cells is activated during Leishmania donovani infection by CD1d-bound lipophosphoglycan. *J Exp Med.* 2004;200(7):895-904.

213. Slauenwhite D, Johnston B. Regulation of NKT Cell Localization in Homeostasis and Infection. *Front Immunol.* 2015;6(255.

214. Kawakami K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *Eur J Immunol.* 2003;33(12):3322-3330.

215. Nieuwenhuis EE, et al. CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med.* 2002;8(6):588-593.

216. Tupin E, et al. NKT cells prevent chronic joint inflammation after infection with Borrelia burgdorferi. *Proc Natl Acad Sci U S A.* 2008;105(50):19863-19868.

217. Chackerian A, Alt J, Perera V, Behar SM. Activation of NKT cells protects mice from tuberculosis. *Infect Immun.* 2002;70(11):6302-6309.

218. Wang H, et al. Protective role of α-galactosylceramide-stimulated natural killer T cells in genital tract infection with Chlamydia muridarum. *FEMS Immunol Med Microbiol.* 2012;65(1):43-54.

219. Ivanov S, et al. Key role for respiratory CD103(+) dendritic cells, IFN-γ, and IL-17 in protection against Streptococcus pneumoniae infection in response to α-galactosylceramide. *J Infect Dis.* 2012;206(5):723-734.

220. Zeissig S, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med.* 2012;18(7):1060-1068.

221. Kulkarni RR, Haeryfar SM, Sharif S. The invariant NKT cell subset in anti-viral defenses: a dark horse in anti-influenza immunity? *J Leukoc Biol.* 2010;88(4):635-643.

222. Paget C, et al. Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia. *J Immunol.* 2011;186(10):5590-5602.

223. De Santo C, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest.* 2008;118(12):4036-4048.

224. Ho LP, Denney L, Luhn K, Teoh D, Clelland C, McMichael AJ. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. *Eur J Immunol.* 2008;38(7):1913-1922.

225. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med.* 2000;192(7):921-930.

226. van Dommelen SL, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. *J Virol.* 2003;77(3):1877-1884.

227. Van Dommelen SL, Degli-Esposti MA. NKT cells and viral immunity. *Immunol Cell Biol.* 2004;82(3):332-341.

228. van den Heuvel MJ, Garg N, Van Kaer L, Haeryfar SM. NKT cell costimulation: experimental progress and therapeutic promise. *Trends Mol Med.* 2011;17(2):65-77.

229. Woltman AM, et al. Alpha-galactosylceramide in chronic hepatitis B infection: results from a randomized placebo-controlled Phase I/II trial. *Antivir Ther.* 2009;14(6):809-818.

230. Pilones KA, Aryankalayil J, Demaria S. Invariant NKT cells as novel targets for immunotherapy in solid tumors. *Clin Dev Immunol.* 2012;2012(720803.

231. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res.* 2015;3(5):425-435.

232. Smyth MJ, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med.* 2000;191(4):661-668.

233. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med.* 2002;196(1):119-127.

234. Swann JB, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood.* 2009;113(25):6382-6385.

235. Bellone M, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One.* 2010;5(1):e8646.

236. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol.* 2002;3(9):867-874.

237. Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med.* 2003;198(11):1631-1641.

238. Nishimura T, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol.* 2000;12(7):987-994.

239. Nakagawa R, et al. Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by alpha-galactosylceramide. *J Immunol.* 2004;172(11):6550-6557.

240. Paget C, Chow MT, Duret H, Mattarollo SR, Smyth MJ. Role of γδ T cells in α-galactosylceramide-mediated immunity. *J Immunol.* 2012;188(8):3928-3939.

241. Choi J, Rudak PT, Lesage S, Haeryfar SMM. Glycolipid Stimulation of Invariant NKT Cells Expands a Unique Tissue-Resident Population of Precursors to Mature NK Cells Endowed with Oncolytic and Antimetastatic Properties. *J Immunol.* 2019;203(7):1808-1819.

242. Tachibana T, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res.* 2005;11(20):7322-7327.

243. Song L, et al. Oncogene MYCN regulates localization of NKT cells to the site of disease in neuroblastoma. *J Clin Invest.* 2007;117(9):2702-2712.

244. Fujii SI, Shimizu K. Immune Networks and Therapeutic Targeting of iNKT Cells in Cancer. *Trends Immunol.* 2019;40(11):984-997.

245. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol.* 2013;14(10):996-1006.

246. Minagawa M, et al. Intensive expansion of natural killer T cells in the early phase of hepatocyte regeneration after partial hepatectomy in mice and its association with sympathetic nerve activation. *Hepatology.* 2000;31(4):907-915.

247. Tsukahara A, et al. ADRENERGIC STIMULATION SIMULTANEOUSLY INDUCES THE EXPANSION OF GRANULOCYTES AND EXTRATHYMIC T CELLS IN MICE . *Biomedical Research.* 1997;18(3):237-246.

248. Sagiyama K, et al. Age-related bias in function of natural killer T cells and granulocytes after stress: reciprocal association of steroid hormones and sympathetic nerves. *Clin Exp Immunol.* 2004;135(1):56-63.

249. Li Z, et al. Norepinephrine regulates hepatic innate immune system in leptin-deficient mice with nonalcoholic steatohepatitis. *Hepatology.* 2004;40(2):434-441.

250. Nissen MD, Sloan EK, Mattarollo SR. β-Adrenergic Signaling Impairs Antitumor CD8(+) T-cell Responses to B-cell Lymphoma Immunotherapy. *Cancer Immunol Res.* 2018;6(1):98-109.

251. Wong CH, Jenne CN, Lee WY, Léger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science.* 2011;334(6052):101-105.

252. Wong CH, et al. Prolonged Activation of Invariant Natural Killer T Cells and T(H)2-Skewed Immunity in Stroke Patients. *Front Neurol.* 2017;8(6.

253. Tamada K, Harada M, Abe K, Li T, Nomoto K. IL-4-producing NK1.1+ T cells are resistant to glucocorticoid-induced apoptosis: implications for the Th1/Th2 balance. *J Immunol.* 1998;161(3):1239-1247.

254. Chen L, Jondal M, Yakimchuk K. Regulatory effects of dexamethasone on NK and T cell immunity. *Inflammopharmacology.* 2018;26(5):1331-1338.

255. Oya H, et al. The differential effect of stress on natural killer T (NKT) and NK cell function. *Clin Exp Immunol.* 2000;121(2):384-390.

256. Shimizu T, et al. Resistance of extrathymic T cells to stress and the role of endogenous glucocorticoids in stress associated immunosuppression. *Scand J Immunol.* 2000;51(3):285-292.

257. De Lorenzo BH, de Oliveira Marchioro L, Greco CR, Suchecki D. Sleep-deprivation reduces NK cell number and function mediated by β-adrenergic signalling. *Psychoneuroendocrinology.* 2015;57(134-143.

258. Yamaguchi H, Hirai M, Kurosawa Y, Hashimoto K. A highly conserved major histocompatibility complex class I-related gene in mammals. *Biochem Biophys Res Commun.* 1997;238(3):697-702.

259. Tilloy F, et al. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med.* 1999;189(12):1907-1921.

260. Treiner E, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* 2003;422(6928):164-169.

261. Lepore M, et al. Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. *Nat Commun.* 2014;5(3866.

262. Meermeier EW, et al. Human TRAV1-2-negative MR1-restricted T cells detect S. pyogenes and alternatives to MAIT riboflavin-based antigens. *Nat Commun.* 2016;7(12506.

263. Rahimpour A, et al. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med.* 2015;212(7):1095-1108.

264. Martin E, et al. Stepwise development of MAIT cells in mouse and human. *PLoS Biol.* 2009;7(3):e54.

265. Dusseaux M, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood.* 2011;117(4):1250-1259.

266. Hinks TS, Wallington JC, Williams AP, Djukanovic R, Staples KJ, Wilkinson TM. Steroid-induced Deficiency of Mucosal-associated Invariant T Cells in the Chronic Obstructive Pulmonary Disease Lung. Implications for Nontypeable Haemophilus influenzae Infection. *Am J Respir Crit Care Med.* 2016;194(10):1208-1218.

267. Gibbs A, et al. MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation. *Mucosal Immunol.* 2017;10(1):35-45.

268. Tang XZ, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol.* 2013;190(7):3142-3152.

269. Cui Y, et al. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J Clin Invest.* 2015;125(11):4171-4185.

270. Seach N, et al. Double-positive thymocytes select mucosal-associated invariant T cells. *J Immunol.* 2013;191(12):6002-6009.

271. Leeansyah E, Loh L, Nixon DF, Sandberg JK. Acquisition of innate-like microbial reactivity in mucosal tissues during human fetal MAIT-cell development. *Nat Commun.* 2014;5(3143.

272. Le Bourhis L, Mburu YK, Lantz O. MAIT cells, surveyors of a new class of antigen: development and functions. *Curr Opin Immunol.* 2013;25(2):174-180.

273. Billerbeck E, et al. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A.* 2010;107(7):3006-3011.

274. Dias J, Leeansyah E, Sandberg JK. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines. *Proc Natl Acad Sci U S A.* 2017;114(27):E5434-E5443.

275. Walker LJ, et al. Human MAIT and CD8alphaalpha cells develop from a pool of type-17 precommitted CD8+ T cells. *Blood.* 2012;119(2):422-433.

276. Reantragoon R, et al. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med.* 2013;210(11):2305-2320.

277. Kjer-Nielsen L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* 2012;491(7426):717-723.

278. Corbett AJ, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* 2014;509(7500):361-365.

279. Soudais C, et al. In Vitro and In Vivo Analysis of the Gram-Negative Bacteria-Derived Riboflavin Precursor Derivatives Activating Mouse MAIT Cells. *J Immunol.* 2015;194(10):4641-4649.

280. Shaler CR, et al. MAIT cells launch a rapid, robust and distinct hyperinflammatory response 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 Biol.* 2017;15(6):e2001930.

281. Ussher JE, et al. CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol.* 2014;44(1):195-203.

282. Kurioka A, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* 2015;8(2):429-440.

283. Slichter CK, et al. Distinct activation thresholds of human conventional and innate-like memory T cells. *JCI Insight.* 2016;1(8).

284. van Wilgenburg B, et al. MAIT cells are activated during human viral infections. *Nat Commun.* 2016;7(11653.

285. Chiba A, et al. Activation status of mucosal-associated invariant T cells reflects disease activity and pathology of systemic lupus erythematosus. *Arthritis Res Ther.* 2017;19(1):58.

286. Willing A, et al. CD8(+) MAIT cells infiltrate into the CNS and alterations in their blood frequencies correlate with IL-18 serum levels in multiple sclerosis. *Eur J Immunol.* 2014;44(10):3119-3128.

287. Wong EB, Ndung'u T, Kasprowicz VO. The role of mucosal-associated invariant T cells in infectious diseases. *Immunology.* 2017;150(1):45-54.

288. Carolan E, et al. Altered distribution and increased IL-17 production by mucosal-associated invariant T cells in adult and childhood obesity. *J Immunol.* 2015;194(12):5775-5780.

289. Magalhaes I, et al. Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. *J Clin Invest.* 2015;125(4):1752-1762.

290. Kelly J, et al. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol Cell Biol.* 2019;97(8):689-699.

291. Kurioka A, et al. Shared and Distinct Phenotypes and Functions of Human CD161++ Vα7.2+ T Cell Subsets. *Front Immunol.* 2017;8(1031.

292. Mullbacher A, Lobigs M, Hla RT, Tran T, Stehle T, Simon MM. Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. *J Immunol.* 2002;169(1):145-150.

293. Reeves E, James E. Antigen processing and immune regulation in the response to tumours. *Immunology.* 2017;150(1):16-24.

294. Chua WJ, Truscott SM, Eickhoff CS, Blazevic A, Hoft DF, Hansen TH. Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection. *Infect Immun.* 2012;80(9):3256-3267.

295. Salio M, et al. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J Immunol.* 2017;199(8):2631-2638.

296. Le Bourhis L, et al. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog.* 2013;9(10):e1003681.

297. Havenith SH, et al. Analysis of stem-cell-like properties of human CD161++IL-18Ralpha+ memory CD8+ T cells. *Int Immunol.* 2012;24(10):625-636.

298. Leeansyah E, et al. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. *PLoS Pathog.* 2015;11(8):e1005072.

299. Booth JS, et al. Mucosal-Associated Invariant T Cells in the Human Gastric Mucosa and Blood: Role in Helicobacter pylori Infection. *Front Immunol.* 2015;6(466.

300. Jeffery HC, et al. Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J Hepatol.* 2016;64(5):1118-1127.

301. Ussher JE, Klenerman P, Willberg CB. Mucosal-associated invariant T-cells: new players in anti-bacterial immunity. *Front Immunol.* 2014;5(450.

302. Napier RJ, Adams EJ, Gold MC, Lewinsohn DM. The Role of Mucosal Associated Invariant T Cells in Antimicrobial Immunity. *Front Immunol.* 2015;6(344.

303. Le Bourhis L, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol.* 2010;11(8):701-708.

304. Georgel P, Radosavljevic M, Macquin C, Bahram S. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. *Mol Immunol.* 2011;48(5):769-775.

305. Sakala IG, et al. Functional Heterogeneity and Antimycobacterial Effects of Mouse Mucosal-Associated Invariant T Cells Specific for Riboflavin Metabolites. *J Immunol.* 2015;195(2):587-601.

306. Meierovics A, Yankelevich WJ, Cowley SC. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proc Natl Acad Sci U S A.* 2013;110(33):E3119-3128.

307. Wang H, et al. MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun.* 2018;9(1):3350.

308. Grimaldi D, et al. Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections. *Intensive Care Med.* 2014;40(2):192-201.

309. Gold MC, et al. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol.* 2010;8(6):e1000407.

310. Wallington JC, Williams AP, Staples KJ, Wilkinson TMA. IL-12 and IL-7 synergize to control mucosal-associated invariant T-cell cytotoxic responses to bacterial infection. *J Allergy Clin Immunol.* 2017.

311. Dias J, Sobkowiak MJ, Sandberg JK, Leeansyah E. Human MAIT-cell responses to Escherichia coli: activation, cytokine production, proliferation, and cytotoxicity. *J Leukoc Biol.* 2016;100(1):233-240.

312. Howson LJ, Salio M, Cerundolo V. MR1-Restricted Mucosal-Associated Invariant T Cells and Their Activation during Infectious Diseases. *Front Immunol.* 2015;6(303.

313. Loh L, et al. Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc Natl Acad Sci U S A.* 2016;113(36):10133-10138.

314. van Wilgenburg B, et al. MAIT cells contribute to protection against lethal influenza infection in vivo. *Nat Commun.* 2018;9(1):4706.

315. Yao T, Shooshtari P, Haeryfar SMM. Leveraging Public Single-Cell and Bulk Transcriptomic Datasets to Delineate MAIT Cell Roles and Phenotypic Characteristics in Human Malignancies. *Front Immunol.* 2020;11(1691.

316. Peterfalvi A, et al. Invariant Valpha7.2-Jalpha33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells. *Int Immunol.* 2008;20(12):1517-1525.

317. Won EJ, et al. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget.* 2016;7(46):76274-76290.

318. Sundstrom P, et al. Human Mucosa-Associated Invariant T Cells Accumulate in Colon Adenocarcinomas but Produce Reduced Amounts of IFN-gamma. *J Immunol.* 2015;195(7):3472-3481.

319. Ling L, et al. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci Rep.* 2016;6(20358.

320. Zabijak L, et al. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol, Immunother.* 2015;64(12):1601-1608.

321. Shaler CR, et al. Mucosa-associated invariant T cells infiltrate hepatic metastases in patients with colorectal carcinoma but are rendered dysfunctional within and adjacent to tumor microenvironment. *Cancer Immunol Immunother.* 2017;66(12):1563-1575.

322. Haeryfar SMM, Shaler CR, Rudak PT. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunol Immunother.* 2018.

323. Numasaki M, et al. Interleukin-17 promotes angiogenesis and tumor growth. *Blood.* 2003;101(7):2620-2627.

324. He D, et al. IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. *J Immunol.* 2010;184(5):2281-2288.

325. Hayata K, et al. Inhibition of IL-17A in tumor microenvironment augments cytotoxicity of tumor-infiltrating lymphocytes in tumor-bearing mice. *PLoS One.* 2013;8(1):e53131.

326. Yan J, et al. MAIT Cells Promote Tumor Initiation, Growth, and Metastases via Tumor MR1. *Cancer Discov.* 2020;10(1):124-141.

327. Garner LC, Klenerman P, Provine NM. Insights Into Mucosal-Associated Invariant T Cell Biology From Studies of Invariant Natural Killer T Cells. *Front Immunol.* 2018;9(1478.

2 Chapter 2: Rationale, objectives, and hypotheses

2.1 Rationale

The immunoregulatory capabilities of the nervous system are now well-documented (1). During prolonged or chronic psychological stress, disrupted neuroimmune interactions can arise as a consequence of sustained activation of the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis (2, 3). The primary mediators released by the SNS are catecholamines which engage adrenergic receptors on target immune cells (4). The HPA axis mediates the release of glucocorticoids into circulation which engage glucocorticoid receptors expressed by distant immune cells (5). Mediators of stress have broad immunomodulatory effects that can result in dysregulated immune responses by inducing apoptosis (5-8), altering the expression of genes dictating function (e.g., co-stimulatory/co-inhibitory receptors) (6, 9, 10), and skewing the T helper-type (T_H)1/T_H2/T_H17 ratio (11-13). A particularly prevalent dogma is that sustained psychological stress establishes a T_H2 bias, thus impairing the generation of antimicrobial and antitumor immune responses that depend on T_H1-type immunity (3, 14). This has been proposed as a major underlying mechanism of increased susceptibility to infection and cancer due to psychological stress (15, 16).

Invariant natural killer T (*i*NKT) cells and mucosa-invariant T (MAIT) cells are unconventional, innate-like T lymphocytes bearing a semi-invariant T cell receptor (17, 18); collectively, they can be referred to as invariant T (*i*T) cells. *i*NKT cells and MAIT cells react to antigens (Ags) presented by cluster of differentiation (CD)1d and major histocompatibility complex (MHC)-related protein 1 (MR1), respectively (19, 20). Upon activation, *i*T cells exhibit remarkable immunomodulatory properties. Depending on the circumstance, they can rapidly produce T_H1-, T_H2-, and/or T_H17-type cytokines, thus shaping the nature of several immunological processes downstream (21, 22). In addition, they are responsible for transactivating a number of different effector cells (e.g., natural killer cells, dendritic cells, and conventional T cells), thereby initiating antimicrobial and antitumor immune responses (18, 21). Importantly, CD1d and MR1 are non-polymorphic molecules (23, 24). Therefore, *i*T cells represent attractive targets for immunotherapy since their ligands should be functional across genetically diverse patients, regardless of the MHC restriction barrier (25-27). Indeed, the utility of *i*NKT cells in cancer

immunotherapy continues to be an active area of investigation (28), and the potential utility of MAIT cells in this regard has been strongly suggested (29, 30).

Despite their wide range of immunomodulatory capabilities at the early stages of immunity and their potential as immunotherapeutic targets, the effects of sustained psychological stress on *i*T cells remain unclear. From select studies, one can infer that *i*NKT cells are responsive to adrenergic and glucocorticoid receptor signaling in various contexts (31-34) and that their frequencies significantly expand during psychological stress (35-37). However, the effects of psychological stress on the phenotype and functional characteristics of *i*NKT cells as well as the underlying mechanisms by which stress affects their relative proportions are unknown. Even less is understood about how stress impacts the survival, phenotype, and function of MAIT cells. In one study, CD161-expressing T cells, a major proportion of which are MAIT cells, were found to express relatively high levels of the β_2-adrenergic receptor (38), suggesting that MAIT cells may be sensitive to regulation by catecholamines.

Defining how sustained psychological stress influences the survival, phenotype, and function of *i*T cells is important for: i) determining whether *i*T cells contribute to the T_H2 bias induced by stress; ii) delineating whether stress increases susceptibility to certain diseases by modulating *i*T cell survival and/or function; iii) identifying novel targets that can potentially be exploited to reverse states of stress-induced immunosuppression; and iv) revealing whether the stress response impedes the efficacy of *i*T cell-based immunotherapies. Exploring these unanswered questions was the central objective of the research highlighted in the remainder of this thesis.

2.2 Objectives and hypotheses

2.2.1 Aim 1: To determine how sustained psychological stress impacts the survival and function of innate-like invariant T cells

*i*NKT cell responses are typified by the swift and robust production of T_H1- and T_H2-type cytokines (namely IFN-γ and IL-4, respectively) after their exposure to αGC (39). Although MAIT cells are best known for their production of T_H1- and/or T_H17-type cytokines upon stimulation, their capacity to produce T_H2-type cytokines has come to light recently (22, 40). Given the T_H2 bias that has frequently been reported to be brought about by psychological stress (14, 16), I sought to determine whether this also occurred in the context of *i*T cell responses, which could have profound impacts on the characteristics of many immune responses downstream. I also aimed to deduce the mechanisms by which stress altered the functions of *i*T cells and whether these ultimately led to their inability to limit tumor growth. In doing so, it was important to assess whether stress modulates *i*T cell function simply by inducing their apoptosis, as described for other cell types (5-8). Of note, prior studies demonstrated that $CD3^+NK1.1^+$ NKT cells expand by frequency during stress (35-37), which may be due to their relative resistance to stress-inflicted cell death over other populations. Therefore, I hypothesized that innate-like *i*T cells are refractory to stress-induced apoptosis but exhibit dysregulated functional characteristics and gene expression profiles during sustained psychological stress.

In Chapter 3, I used enzyme-linked immunosorbent and multiplex cytokine assays to define the types of inflammatory responses mounted by *i*T cells during chronic variable and/or prolonged restraint stress. αGC and 5-OP-RU were used as model Ags to trigger *i*NKT and MAIT cell responses, respectively. Pharmacological inhibition, conditional knockout mice, flow cytometry, and transcript profiling enabled me to determine the mechanisms by which stress impacted *i*T cell function. *In vitro* and *in vivo* cytotoxicity assays were used to characterize the ability of *i*T cells to transactivate NK cells during stress. Lastly, the B16 metastatic melanoma model was used to assess how stress affected *i*T cells' antitumor properties. This study was the first to define the pathways by which psychological stress affects innate-like *i*T cell survival, gene expression, and function.

2.2.2 Aim 2: To identify whether sustained psychological stress induces the expression of co-inhibitory receptors in innate-like invariant T cells

Throughout chapter 3, I demonstrate that sustained psychological stress results in the profound suppression of many aspects of *i*T cell function, including their capacity to promote T_H1 and T_H2-type cytokine responses, transactivate effector cells, and protect against experimental metastases. Importantly, these effects appeared to be mediated by intrinsic glucocorticoid receptor (GR) signaling within *i*T cells despite the discovery that *i*T cells are unusually resistant to glucocorticoid-induced apoptosis. Given their persistence in stressed mice, my second aim was to identify pathways induced by GR signaling in *i*T cells that may be targeted to potentially reverse their functionally impaired state, thereby bringing additional aspects of translatability to my work.

Prior studies have indicated that glucocorticoids can induce the upregulation of known co-inhibitory molecules including programmed cell death-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), T cell immunoglobulin and mucin-3 (TIM-3), and lymphocyte activation gene 3 (LAG-3) in various contexts (6, 10, 41-43). Thus, it is conceivable that stress-induced GR signaling in *i*T cells impairs their functional fitness by driving their expression of certain co-inhibitory receptors. These would represent targetable molecular pathways *a priori* since monoclonal antibody-based drugs blocking co-inhibitory receptors (*aka.*, immune checkpoint inhibitors) have demonstrated clear clinical success in cancer immunotherapy (44). I hypothesized that GR signaling in *i*T cells mediates their expression of co-inhibitory receptors during stress.

In Chapter 4, gene expression and flow cytometric analyses allowed me to uncover a selective and robust rise in the expression of T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) in *i*T cells during stress. Pharmacological inhibition, conditional knockout mice, and *in vitro* culture systems were used to validate that TIGIT upregulation was mediated by direct GR signaling in *i*T cells. Using an *in vivo* blocking antibody towards mouse TIGIT, I later discovered that poorer IFN-γ responses upon *i*NKT cell activation in stressed mice was partially mediated by increased expression of TIGIT (see also Chapter 5 Figure 5.1).

2.3 References

1. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* 2006;6(4):318-328.

2. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol.* 2003;24(8):444-448.

3. Pruett SB. Stress and the immune system. *Pathophysiology.* 2003;9(3):133-153.

4. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev.* 2000;52(4):595-638.

5. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol.* 2017;17(4):233-247.

6. Acharya N, et al. Endogenous Glucocorticoid Signaling Regulates CD8(+) T Cell Differentiation and Development of Dysfunction in the Tumor Microenvironment. *Immunity.* 2020;53(3):658-671.e656.

7. Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci.* 2006;63(1):60-72.

8. Shi Y, Devadas S, Greeneltch KM, Yin D, Allan Mufson R, Zhou JN. Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain Behav Immun.* 2003;17 Suppl 1(S18-26.

9. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. *Pharmacol Rev.* 2001;53(4):487-525.

10. Galon J, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *Faseb j.* 2002;16(1):61-71.

11. Kim BJ, Jones HP. Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-γ by CD4+ T cells. *Brain Behav Immun.* 2010;24(7):1126-1136.

12. Takenaka MC, et al. Norepinephrine Controls Effector T Cell Differentiation through β2-Adrenergic Receptor-Mediated Inhibition of NF-κB and AP-1 in Dendritic Cells. *J Immunol.* 2016;196(2):637-644.

13. Taves MD, Ashwell JD. Glucocorticoids in T cell development, differentiation and function. *Nat Rev Immunol.* 2020.

14. Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Ann N Y Acad Sci.* 2006;1069(62-76.

15. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol.* 2005;5(3):243-251.

16. Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol.* 2004;5(10):617-625.

17. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol.* 2008;20(3):358-368.

18. Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *J Leukoc Biol.* 2018;104(3):473-486.

19. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science.* 1995;268(5212):863-865.

20. Kjer-Nielsen L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* 2012;491(7426):717-723.

21. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol.* 2013;13(2):101-117.

22. Kurioka A, et al. Shared and Distinct Phenotypes and Functions of Human CD161++ Valpha7.2+ T Cell Subsets. *Front Immunol.* 2017;8(1031.

23. Kawano T, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997;278(5343):1626-1629.

24. Treiner E, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* 2003;422(6928):164-169.

25. Haeryfar SM, Mallevaey T. Editorial: CD1- and MR1-Restricted T Cells in Antimicrobial Immunity. *Front Immunol.* 2015;6(611.

26. Parrot T, et al. Expansion of donor-unrestricted MAIT cells with enhanced cytolytic function suitable for TCR-redirection. *JCI Insight.* 2021.

27. Crowther MD, et al. Genome-wide CRISPR-Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1. *Nat Immunol.* 2020;21(2):178-185.

28. Fujii SI, Shimizu K. Immune Networks and Therapeutic Targeting of iNKT Cells in Cancer. *Trends Immunol.* 2019;40(11):984-997.

29. Lukasik Z, Elewaut D, Venken K. MAIT Cells Come to the Rescue in Cancer Immunotherapy? *Cancers (Basel).* 2020;12(2).

30. Haeryfar SMM, Shaler CR, Rudak PT. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunol Immunother.* 2018.

31. Wong CH, Jenne CN, Lee WY, Léger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science.* 2011;334(6052):101-105.

32. Minagawa M, et al. Intensive expansion of natural killer T cells in the early phase of hepatocyte regeneration after partial hepatectomy in mice and its association with sympathetic nerve activation. *Hepatology.* 2000;31(4):907-915.

33. Nissen MD, Sloan EK, Mattarollo SR. β-Adrenergic Signaling Impairs Antitumor CD8(+) T-cell Responses to B-cell Lymphoma Immunotherapy. *Cancer Immunol Res.* 2018;6(1):98-109.

34. Tamada K, Harada M, Abe K, Li T, Nomoto K. IL-4-producing NK1.1+ T cells are resistant to glucocorticoid-induced apoptosis: implications for the Th1/Th2 balance. *J Immunol.* 1998;161(3):1239-1247.

35. Oya H, et al. The differential effect of stress on natural killer T (NKT) and NK cell function. *Clin Exp Immunol.* 2000;121(2):384-390.

36. Shimizu T, et al. Resistance of extrathymic T cells to stress and the role of endogenous glucocorticoids in stress associated immunosuppression. *Scand J Immunol.* 2000;51(3):285-292.

37. Sagiyama K, et al. Age-related bias in function of natural killer T cells and granulocytes after stress: reciprocal association of steroid hormones and sympathetic nerves. *Clin Exp Immunol.* 2004;135(1):56-63.

38. Fergusson JR, et al. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep.* 2014;9(3):1075-1088.

39. Tefit JN, Davies G, Serra V. NKT cell responses to glycolipid activation. *Methods Mol Biol.* 2010;626(149-167.

40. Kelly J, et al. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol Cell Biol.* 2019;97(8):689-699.

41. Quatrini L, et al. Endogenous glucocorticoids control host resistance to viral infection through the tissue-specific regulation of PD-1 expression on NK cells. *Nat Immunol.* 2018;19(9):954-962.

42. Xia M, Gasser J, Feige U. Dexamethasone enhances CTLA-4 expression during T cell activation. *Cell Mol Life Sci.* 1999;55(12):1649-1656.

43. Xing K, Gu B, Zhang P, Wu X. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC Immunol.* 2015;16(39.

44. Robert C. A decade of immune-checkpoint inhibitors in cancer therapy. *Nat Commun.* 2020;11(1):3801.

3 Chapter 3: Chronic stress physically spares but functionally impairs innate-like invariant T cells

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3.1 Introduction

Long-term stress due to persistent negative emotions or repeated exposure to environmental stressors carries adverse immunoregulatory consequences (1). Individuals experiencing greater relative levels of perceived stress mount weaker responses to vaccination and infection (1). Stress has also been linked to cancer progression in animal models, often in a manner that implicates diminished or dysregulated antitumor immunity (2-6). According to prospective epidemiological studies, a higher degree of stress is associated with elevated incidences of neoplasia and cancer mortality in previously healthy individuals, and with poorer prognosis in cancer patients (7, 8). Despite such and similar findings, the mechanisms underlying stress-induced immunological abnormalities remain ill-defined.

A stress response is typically launched via the coordinated activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (1). Dense innervation of virtually all organs by the SNS, including lymphoid tissues, enables targeted release of specific neurotransmitters into the extracellular milieu in which immune cells reside and operate (9). Norepinephrine (NE), the most dominant neurotransmitter utilized by post-ganglionic sympathetic nerve termini, signals through α- and/or β-adrenergic receptors (9). Under profound sympathetic stimulation, NE is co-transmitted from large intraneuronal storage vesicles with neuropeptide Y (NPY) (10), which utilizes Y1, Y2, Y4, Y5 and Y6 as its receptors (11). The activation of the HPA axis raises the circulating levels of glucocorticoids, which exert broad anti-inflammatory effects by binding to the glucocorticoid receptor (GR) encoded by *Nr3c1* (12). Stress-elicited defects in antimicrobial and antitumor immunity are often attributable to the operation of the SNS and/or the HPA axis (13).

Previous studies on the immunological ramifications of stress have focused heavily on conventional T (T_{conv}) cells (14). By inducing apoptosis in $CD4^+CD8^+$ thymocytes and mature T_{conv} cells, glucocorticoids shrink the size of T cell pools (15, 16). Moreover, stress is believed to create a T_H2 bias (14), for instance by favoring IL-4 production in the context of *ex vivo* T_{conv} cell stimulation (17) or within tumor microenvironments (18). NE

(9), NPY (19) and glucocorticoids (20) have each been reported to permit or promote T_H2-skewed phenotypes while inhibiting T_H1-type responses.

To date, how psychological stress and its mediators affect innate-like invariant T (*i*T) cells, including invariant natural killer T (*i*NKT) and mucosa-associated invariant T (MAIT) cells, has been essentially unexplored. This is a critical question in light of the remarkable immunomodulatory, cytotoxic, antitumor, antibacterial and antiviral properties of these unconventional T cells (21-25).

*i*NKT cells bear a CD1d-restricted, glycolipid-reactive, semi-invariant T cell receptor (*i*TCR), which contains a canonically rearranged α chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) paired with one of only few Vβ chain choices (26). Activated *i*NKT cells swiftly produce copious quantities of T_H1, T_H2 and/or T_H17 cytokines that shape ensuing immune responses (27). The most commonly studied glycolipid ligand of *i*NKT cells is α-galactosylceramide (αGC) (28), which prompts the transactivation of several effector cell types, including but not limited to NK cells (29), precursors to mature NK (pre-mNK) cells (30), and $CD8^+$ T_{conv} cells (31). Therefore, *i*NKT cells display potent antimicrobial and antitumor properties *in vivo*, and αGC and its analogs continue to be pursued as potential therapeutics (27, 32).

MAIT cells too harbor *i*TCRs with an invariant α chain (typically Vα19-Jα33 in mice and Vα7.2-Jα33 in humans) and minimal Vβ chain diversity (33, 34). They are abundant in human peripheral blood and strategically poised in the human liver, lungs and mucosal layers (35-37), the ports of entry for many pathogens and common sites of neoplastic transformation and metastatic growth. Upon stimulation with vitamin B intermediates of bacterial and fungal origin [*e.g.*, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)] (38), select drugs and drug-like metabolites (39, 40), and perhaps other compounds presented by MHC-related protein 1 (MR1), MAIT cells quickly produce a wide array of inflammatory mediators, including T_H1-, T_H2- and T_H17-type cytokines (41, 42), and transactivate key downstream effectors (43). Although best known for their antimicrobial activities, MAIT cells participate in tissue repair (44-48) and may play significant roles within various tumor microenvironments (25, 49). Both *i*NKT and

MAIT cells are also capable of responding to viral infections in a TCR-independent fashion, primarily through cytokines such as IL-12 and IL-18 (50, 51).

Here, we leveraged multiple mouse models of psychological stress as well as human cell culture systems to comprehensively investigate the impact of stress on *i*NKT and MAIT cell responses. Our findings reveal a novel mechanism of stress-induced immunosuppression with important implications for future *i*NKT cell- and MAIT cell-based immunotherapies.

3.2 Materials and Methods

3.2.1 Mice

Adult WT B6 and BALB/c mice, between 8 and 16 weeks of age, were purchased from Charles River Canada (Saint-Constant, QC). B6(Cg)-Tyr^{c-2J}/J (B6 albino) mice (#000058) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-MAIT^{CAST} mice, a congenic strain harboring a larger MAIT cell compartment relative to WT B6 mice, and MAIT cell-deficient MR1$^{-/-}$ B6-MAIT^{CAST} mice have been previously described (52). β2M$^{-/-}$ mice were provided by Dr. Anthony Jevnikar (Western University, London, ON). Nr3c1^{fl}Lck^{cre} mice, whose T cells lack the GR, were generated by crossing B6.Nr3c1$^{fl/fl}$ (Jackson #021021) with B6.Lck$^{cre/cre}$ mice (Jackson #003802), followed by back-crossing the offspring with Nr3c1^{fl} mice. Lck^{cre} mice were generated by crossing offspring with WT B6 mice. PCR-based genotyping was conducted throughout breeding. Animals were housed in an institutional barrier facility with constant light/dark cycles. Prior to any experiment, mice were randomly assigned to treatment groups. Cohorts were always age- and sex-matched. Mouse experiments were conducted following Animal Use Protocols 2010-241, 2018-093 and 2018-130, which were approved by the Animal Care Committee of Animal Care and Veterinary Services at Western University.

3.2.2 Human specimens

HMNCs were obtained from tumor-free liver samples from patients undergoing surgical resection, without prior neoadjuvant therapy, at University Hospital (London Health Sciences Centre, London, ON). Three patients had undergone surgery for colorectal liver metastasis, one for ampullary cancer, one for ampullary adenoma, and one for pancreatic cancer. Patients had a mean age of 64 (range: 37-80) and were all male.

PBMCs were isolated from 4 healthy blood donors, two males and two females, with a mean age of 37 (range: 29-51). Human specimens were collected after obtaining written, informed consent from participants as per study protocols 5545, 2597 and 113362 approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects.

3.2.3 Cell lines

The mouse lymphoma cell line YAC-1 (ATCC TIB-160) was grown at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX-I, 0.1 mM MEM nonessential amino acids, 1mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES, which will hereafter be referred to as complete medium. The mouse melanoma line B16-F10 was provided by Dr. Ann Chambers (Western University, London, ON) and maintained in MEM Alpha medium supplemented with 10% FBS. Luciferase-expressing B16-FLuc cells (PerkinElmer #BW124734) were grown in RPMI 1640 containing 10% FBS. DN32.D3, a $CD4^{-}CD8^{-}$ *i*NKT hybridoma cell line from Dr. Albert Bendelac (University of Chicago, Chicago, IL), was cultured in RPMI 1640 containing 10% FBS, 2 mM GlutaMAX-I, and 0.1 mM MEM nonessential amino acids.

3.2.4 Models of psychological stress

To induce prolonged confinement stress, mice were held horizontally for 12 h inside well-ventilated 50-mL conical tubes. This immobilization procedure prompts psychological stress in rodents without causing pain or physical compression and activates the SNS and the HPA axis (53). Cage-mate controls remained undisturbed but were deprived of access to food and water for 12 h. To model acute stress, mice were restrained for 15 minutes (54) while control animals were left in home cages without food and water.

Repeated restraint stress (RRS) was inflicted by subjecting mice to 21 days of physical immobilization for 1 h daily. The experimenter and the time at which each stressor was applied remained constant throughout the entire procedure.

The chronic variable stress (CVS) model entailed daily exposures to heterotypic psychological or physical stressors for 21 days. One brief stressor during the light cycle and one overnight (O/N) stressor during the dark cycle were introduced each day. In no particular order, brief stressors included placement in a 4°C environment for 1 h, physical restraint for 1 h, horizontal cage shaking at 80 rpm for 1 h, and placement in 30°C water

for 15 minutes. O/N stressors included water deprivation, cage tilting at a 45-degree angle, constant exposure to light, wet bedding (through pouring ~200 mL of water onto cage bedding), and food deprivation. Unlike RRS, mice fail to habituate to unpredictable stressors in the CVS model and display continuously elevated stress responses as a consequence (55). Parallel cohorts of non-stressed control mice were left undisturbed with food and water *ad libitum* for 21 days.

3.2.5 Chemical sympathectomy

Six days before mice were subjected to prolonged restraint stress, 6-hydroxydopamine (OHDA) (Sigma-Aldrich) was administered i.p. at 200 mg/kg in a vehicle containing 0.9% NaCl and 10^{-7} M ascorbic acid in phosphate-buffered saline (PBS). Successful sympathectomy was confirmed by immunoblotting for TH in splenic and brain tissues of OHDA-treated mice. We used a rabbit polyclonal Ab (ab117112 from abcam) to capture mouse TH, and an anti-mouse β-actin mAb (mAbGEa from Thermo Scientific) served as a loading control. Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG secondary Abs were from Thermo Scientific. Enzymatic reactions were initiated using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Blots were digitally imaged using a C-DiGit Blot Scanner (LI-COR Biosciences).

3.2.6 Administration and pharmacological inhibition of glucocorticoids

To simulate stress-elicited rise in glucocorticoids, corticosterone (CS) (Sigma-Aldrich) was administered orally. CS was dissolved in absolute ethanol and then diluted in standard drinking water to yield a final concentration of 25 μg/mL of water with 1% ethanol. CS-containing water was provided for 21 days with weekly replenishments. This regimen gives rise to CS serum levels that are comparable to those found in chronically stressed mice as we previously reported (56).

In several experiments, mice were injected i.p. with 200 mg/kg of the glucocorticoid synthesis inhibitor metyrapone (Sigma-Aldrich) or with 25 mg/kg of the GR antagonist RU486 (Sigma-Aldrich) 1 h prior to prolonged restraint stress.

3.2.7 *In vivo* administration of *i*T cell ligands/stimuli

Glycolipid stimulation of *i*NKT cells was achieved via i.p. administration of 100 μg/kg of KRN7000/αGC (Funakoshi, Tokyo, Japan) or 200 μg/kg of αCGC in a vehicle containing 5.6% sucrose, 0.75% L-histidine and 0.5% Tween-20, which was further diluted in PBS. αCGC was supplied by the NIH Tetramer Core Facility (Atlanta, GA). To stimulate *i*NKT cells in a cytokine-dependent manner, each animal was injected i.v. with 2 ng recombinant mouse IL-12 (Peprotech, Rocky Hill, NJ) plus 200 ng recombinant mouse IL-18 (R&D Systems) in PBS.

To activate MAIT cells, mice were injected i.p. with 200 μL of PBS containing 20 μL of a 5-OP-RU stock solution. The stock solution was prepared by mixing equal volumes of 2 mM 5-amino-6-D-ribitylaminouracil (5-ARU) and 2 mM methylglyoxal in DMSO for 24 h at room temperature. Aliquots were stored at -80°C until use. Control mice received vehicle (2 mM methylglyoxal in DMSO) diluted in PBS.

Where indicated, mice were given 200 μg of a TIGIT-blocking mAb (clone 1B4) or a mouse IgG1κ isotype control (clone MOPC-21 from BioXCell) 1 h prior to αGC administration.

3.2.8 Quantification of serum cytokines and CS

Mice were bled immediately after stress or at 2, 12 and 24 h post-treatment with αGC, αCGC, 5-OP-RU, or an appropriate vehicle. Sera were isolated, aliquoted and stored at -20°C. Mouse IFN-γ, IL-2 and IL-4 concentrations were measured using eBioscience Ready-SET-Go! ELISA Kits. Cytokine multiplex analyses were performed by Eve Technologies (Calgary, AB). CS levels were measured using a DetectX Corticosterone Enzyme Immunoassay kit (Arbor Assays, Ann Arbor, MI).

3.2.9 Cytofluorometric analyses

After cervical dislocation, mouse spleens were mechanically homogenized and depleted of erythrocytes through exposure to ACK (Ammonium-Chlorine-Potassium) lysis buffer for 3 minutes at room temperature. HMNCs were isolated from mouse livers or human tumor-free liver samples. Specimens were homogenized, and parenchymal cells were

removed by density gradient centrifugation at 700 × *g* in 33.75% Percoll PLUS (GE Healthcare). This was followed by treatment with ACK lysis buffer to eliminate erythrocytes. To isolate human PBMCs, uncoagulated blood from healthy donors was spun at 1,200 × *g* in 50 mL SepMate PBMC Isolation Tubes (STEMCELL Technologies) containing Ficoll-Paque PLUS (GE Healthcare).

Before surface staining, mouse cell suspensions were incubated for 10 minutes on ice with 5 μg/mL of an anti-mouse CD16/CD32 mAb (clone 2.4G2) to prevent non-specific binding to Fcγ receptors. Cell surface staining was conducted for 30 minutes at 4°C in PBS containing 2% FBS. Intracellular detection of cytoplasmic proteins was performed using the Intracellular Fixation & Permeabilization Buffer Set (Thermo Scientific). To stain nuclear proteins, we used the Foxp3/Transcription Factor Staining Buffer Set (Thermo Scientific). A FITC CaspaTag Pan-Caspase *In Situ* Assay Kit (EMD Millipore) was used to detect intracellular active caspases.

Mouse *i*NKT cells were defined as TCRβ$^+$PBS-57-loaded mCD1d tetramer$^+$ cells, and human *i*NKT cells as CD3$^+$PBS-57-loaded hCD1d tetramer$^+$ cells, while empty CD1d tetramers served as staining controls. Mouse MAIT cells were defined as B220$^-$TCRβ$^+$5-OP-RU-loaded mMR1 tetramer$^+$ cells, and human MAIT cells as CD3$^+$5-OP-RU-loaded hMR1$^+$ cells. 6-formylpterin (6-FP)-loaded MR1 tetramer reagents, which do not react with the *i*TCR of MAIT cells, were utilized in parallel as staining controls. We identified mouse T_{conv} cells as TCRβ$^+$PBS-57-loaded mCD1d tetramer$^-$ cells, and human T_{conv} cells as CD3$^+$PBS-57-loaded hCD1d tetramer$^-$5-OP-RU-loaded hMR1 tetramer$^-$ cells. Mouse NK cells, B cells and DCs were immunophenotyped as TCRβ$^-$NK1.1$^+$, TCRβ$^-$B220$^+$ and TCRβ$^-$CD11c$^+$ cells, respectively. Staining with isotype controls was used to draw gates as appropriate.

The fluorochrome-conjugated mAbs and tetramer reagents employed in this study are listed in Table 3.1. Cells were interrogated using a BD FACSCanto II flow cytometer equipped with BD FACSDiva version 6.1.2 software.

Table 3.1: Key resources used for the study highlighted in Chapter 3.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa 700-conjugated anti-mouse/human B220 (Clone RA3-6B2)	Thermo Fisher Scientific	Cat # 56-0452-82; RRID: AB_891458
Alexa 700-conjugated anti-human CD3 (Clone UCHT1)	Thermo Fisher Scientific	Cat # 56-0038-42; RRID: AB_10597906
PE-Cy7-conjugated anti-mouse CD3ε (Clone 145-2C11)	Thermo Fisher Scientific	Cat # 25-0031-82; RRID: AB_469572
FITC-conjugated anti-mouse CD4 (Clone GK1.5)	Thermo Fisher Scientific	Cat # 11-0041-81; RRID: AB_464891
PE-eFluor610-conjugated anti-mouse CD11c (Clone N418)	Thermo Fisher Scientific	Cat # 61-0114-80; RRID: AB_2574529
PE-conjugated anti-mouse CD25 (Clone PC61.5)	Thermo Fisher Scientific	Cat # 12-0251-81; RRID: AB_465606
PerCP-Cy5.5-conjugated anti-mouse CD28 (Clone 37.51)	Thermo Fisher Scientific	Cat # 45-0281-80; RRID: AB_925744
PE-Cy5-conjugated anti-mouse/human CD44 (Clone IM7)	Thermo Fisher Scientific	Cat # 15-0441-81; RRID: AB_468748
FITC-conjugated anti-mouse/human CD44 (Clone IM7)	Thermo Fisher Scientific	Cat # 11-0441-81; RRID: AB_465044
PE-conjugated anti-mouse CD45 (Clone 30-F11)	Thermo Fisher Scientific	Cat # 12-0451-82; RRID: AB_465668
APC-eFluor780-conjugated anti-mouse CD62L (Clone MEL-14)	Thermo Fisher Scientific	Cat # 47-0621-80; RRID: AB_1603258
PerCP-Cy5.5-conjugated anti-mouse CD69 (Clone H1.2F3)	Thermo Fisher Scientific	Cat # 45-0691-82; RRID: AB_1210703
PE-conjugated anti-mouse CD127 (Clone A7R34)	Thermo Fisher Scientific	Cat # 12-1271-82; RRID: AB_465844
PerCP-Cy5.5-conjugated anti-human CD127 (Clone eBioRDR5)	Thermo Fisher Scientific	Cat # 45-1278-41; RRID: AB_10669708
FITC-conjugated anti-mouse/rat/Rhesus monkey CD278/ICOS (Clone C398.4A)	Thermo Fisher Scientific	Cat # 11-9949-82; RRID: AB_465458
PE-Cy7-conjugated anti-mouse NK1.1 (Clone PK136)	Thermo Fisher Scientific	Cat # 25-5941-82; RRID: AB_469665
FITC-conjugated anti-mouse TCRβ (Clone H57-597)	Thermo Fisher Scientific	Cat # 11-5961-85; RRID: AB_465324
PE-Cy7-conjugated anti-mouse TCRβ (Clone H57-597)	Thermo Fisher Scientific	Cat # 25-5961-82; RRID: AB_2573507
APC-eFluor780-conjugated anti-mouse TCRβ (Clone H57-597)	Thermo Fisher Scientific	Cat # 47-5961-82; RRID: AB_1272173
PE-Cy7-conjugated anti-mouse/rat Bcl-2 (Clone 10C4)	Thermo Fisher Scientific	Cat # 25-6992-42; RRID: AB_2573516
PE-conjugated anti-mouse/human GILZ (Clone CFMKG15)	Thermo Fisher Scientific	Cat # 12-4033-80; RRID: AB_1659717
PE-conjugated anti-mouse IFN-γ (Clone XMG1.2)	Thermo Fisher Scientific	Cat # 12-7311-82; RRID: AB_466193
PE-Cy7-conjugated anti-mouse IL-2 (Clone JES6-5H4)	Thermo Fisher Scientific	Cat # 25-7021-82; RRID: AB_1235004
PE-Cy7-conjugated anti-mouse IL-4 (Clone 11B11)	BD Biosciences	Cat # 560699; RRID: AB_1727548
PE-Cy7-conjugated anti-mouse IL-4 (Clone BVD6-24G2)	Thermo Fisher Scientific	Cat # 25-7042-82; RRID: AB_469674

PE-conjugated anti-mouse/human IL-5 (Clone TRFK5)	Thermo Fisher Scientific	Cat # 12-7052-81; RRID: AB_763588
eFluor660-conjugated anti-mouse IL-12p35 (Clone 4D10p35)	Thermo Fisher Scientific	Cat # 50-7352-80; RRID: AB_2574284
APC-eFluor780-conjugated anti-mouse IL-13 (Clone eBio13A)	Thermo Fisher Scientific	Cat # 47-7133-80; RRID: AB_2716963
PerCP-eFluor710-conjugated anti-mouse/rat/human/non-human primate/cynomolgus monkey/dog Ki67 (Clone SolA15)	Thermo Fisher Scientific	Cat # 46-5698-82; RRID: AB_11040981
PE-conjugated anti-mouse/rat/human/guinea pig/rabbit/sheep/yeast glucocorticoid receptor (Clone BuGR2)	Novus Biologicals	Cat # NB300-731PE; RRID: AB_2298869
PE-Cy5-conjugated rat IgG2bκ isotype control (Clone eB149/10H5)	Thermo Fisher Scientific	Cat # 15-4031-82; RRID: AB_470133
FITC-conjugated rat IgG2bκ isotype control (Clone Eb149/10H5)	Thermo Fisher Scientific	Cat # 11-4031-81; RRID: AB_470003
APC-eFluor780-conjugated rat IgG2aκ isotype control (Clone eBR2a)	Thermo Fisher Scientific	Cat # 47-4321-80; RRID: AB_1272001
PerCP-Cy5.5-conjugated Armenian hamster IgG isotype control (Clone eBio299Arm)	Thermo Fisher Scientific	Cat # 45-4888-80; RRID: AB_906260
PE-conjugated rat IgG2aκ isotype control (Clone eBR2a)	Thermo Fisher Scientific	Cat # 12-4321-42; RRID: AB_1518773
PerCP-Cy5.5-conjugated mouse IgG1κ isotype control (Clone P3.6.2.8.1)	Thermo Fisher Scientific	Cat # 45-4714-82; RRID: AB_906257
PE-Cy7-conjugated mouse IgG1κ isotype control (Clone P3.6.2.8.1)	Thermo Fisher Scientific	Cat # 25-4714-42; RRID: AB_1548705
PE-conjugated rat IgG1κ isotype control (Clone eBRG1)	Thermo Fisher Scientific	Cat # 12-4301-82; RRID: AB_470047
PE-Cy7-conjugated rat IgG2bκ isotype control (Clone eB149/10H5)	Thermo Fisher Scientific	Cat # 25-4031-82; RRID: AB_891624
eFluor660-conjugated rat IgG2aκ isotype control (Clone eBR2a)	Thermo Fisher Scientific	Cat # 50-4321-80; RRID: AB_10598640
APC-eFluor780-conjugated rat IgG1κ isotype control (Clone eBRG1)	Thermo Fisher Scientific	Cat # 47-4301-80; RRID: AB_1271986
PE-Cy7-conjugated rat IgG1κ isotype control (Clone R3-34)	BD Biosciences	Cat # 557645; RRID: AB_396762
PE-Cy7-conjugated rat IgG1κ isotype control (Clone eBRG1)	Thermo Fisher Scientific	Cat # 25-4301-82; RRID: AB_470198
PerCP-eFluor 710-conjugated rat IgG2aκ isotype control (Clone eBR2a)	Thermo Fisher Scientific	Cat # 46-4321-82; RRID: AB_1834455
PE-conjugated mouse IgG2aκ isotype control (Clone G155-178)	BD Biosciences	Cat # 556653; RRID: AB_396517
PE-Cy7-conjugated Armenian hamster IgG isotype control (Clone eBio299Arm)	Thermo Fisher Scientific	Cat # 25-4888-82; RRID: AB_470204
FITC-conjugated Armenian hamster IgG isotype control (Clone eBio299Arm)	Thermo Fisher Scientific	Cat # 11-4888-85; RRID: AB_470038
PE-conjugated rat IgG2bκ isotype control (Clone eB149/10H5)	Thermo Fisher Scientific	Cat # 12-4031-82; RRID: AB_470042
Anti-mouse/rat tyrosine hydroxylase (polyclonal)	Abcam	Cat # ab117112
Anti-mouse/rat/human/cow/fruit fly/sheep/plant/xenopus/yeast/zebrafish β-actin (Clone mAbGEa)	Thermo Fisher Scientific	Cat # MA1-744; RRID: AB_2223496
HRP-conjugated goat anti-Rabbit IgG (H+L) (Polyclonal)	Thermo Fisher Scientific	Cat # 31460; RRID: AB_228341

HRP-conjugated goat anti-mouse IgG (H+L) (Polyclonal)	Thermo Fisher Scientific	Cat # 31430; RRID: AB_228307
Anti-mouse TIGIT (Clone 1B4)	Cell Essentials, Inc. (Boston, MA): http://www.cell-essentials.com	Lot # 111704
Mouse IgG1κ isotype control (Clone MOPC-21)	BioXCell	Cat # BE0083; RRID: AB_1107784
Biological Samples		
Human tumor-free liver samples	This paper/University Hospital at London Health Sciences Centre	N/A
Healthy human blood samples	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
APC- or PE-conjugated PBS-57-loaded mouse or human CD1d tetramer	NIH Tetramer Core Facility	N/A
APC- or PE-conjugated unloaded mouse or human CD1d tetramer	NIH Tetramer Core Facility	N/A
APC- or PE-conjugated 5-OP-RU-loaded mouse or human MR1 tetramer (Corbett et al., 2014)	NIH Tetramer Core Facility	N/A
APC- or PE-conjugated 6-FP-loaded mouse or human MR1 tetramer (Corbett et al., 2014)	NIH Tetramer Core Facility	N/A
αGC	Funakoshi	Cat # KRN7000
αCGC	NIH Tetramer Core Facility	N/A
5-amino-6-D-ribitylaminouracil (5-ARU)	Dr. Olivier Lantz	N/A
Methylglyoxal solution	Sigma-Aldrich	Cat # M0252
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat # P1585
Ionomycin	Sigma-Aldrich	Cat # I9657
Brefeldin A	Sigma-Aldrich	Cat # B7651
Recombinant mouse GM-CSF	Peprotech	Cat # 315-03
Recombinant mouse IL-4	Peprotech	Cat # 214-14
Recombinant mouse IL-12p70	Peprotech	Cat # 210-12
Recombinant mouse IL-18	R&D Systems	Cat # 9139-IL-050
6-hydroxydopamine hydrobromide	Sigma-Aldrich	Cat # 162957
Corticosterone	Sigma-Aldrich	Cat # 27840
Hydrocortisone	Sigma-Aldrich	Cat # H0888
Dexamethasone	Sigma-Aldrich	Cat # D4902
Metyrapone	Sigma-Aldrich	Cat # M2696
RU486	Sigma-Aldrich	Cat # M8046
Norepinephrine	Sigma-Aldrich	Cat # A7257
Neuropeptide Y	Sigma-Aldrich	Cat # N5017
Propranolol hydrochloride	Sigma-Aldrich	Cat # P0884
Percoll PLUS	GE Healthcare	Cat # 17-5445-01
Ficoll-Paque PLUS	GE Healthcare	Cat # 17-1440-02
$Na_2{}^{51}CrO_4$	PerkinElmer	Cat # NEZ030S001MC
Triton X-100	Sigma-Aldrich	Cat # T8787
XenoLight D-Luciferin	PerkinElmer	Cat # 127799

7-aminoactinomycin D (7-AAD) viability dye	Thermo Fisher Scientific	Cat # 00-6993-50
Critical Commercial Assays		
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare	Cat # RPN2232
eBioscience Ready-SET-Go! Mouse IFN-γ ELISA Kit	Thermo Fisher Scientific	Cat # 88-7314-88
eBioscience Ready-SET-Go! Mouse IL-2 ELISA Kit	Thermo Fisher Scientific	Cat # 88-7024-88
eBioscience Ready-SET-Go! Mouse IL-4 ELISA Kit	Thermo Fisher Scientific	Cat # 88-7044-88
DetectX Corticosterone Enzyme Immunoassay Kit	Arbor Assays	Cat # K014-H1
Intracellular Fixation & Permeabilization Buffer Set	Thermo Fisher Scientific	Cat # 88-8824-00
Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat # 00-5523-00
FITC CaspaTag Pan-Caspase In Situ Assay Kit	EMD Millipore	Cat # APT420
EasySep Mouse CD11c Positive Selection Kit II	STEMCELL Technologies	Cat # 18780A
PureLink RNA Mini Kit	Thermo Fisher Scientific	Cat # 12183018A
SuperScript VILO cDNA Synthesis Kit	Thermo Fisher Scientific	Cat # 11755-050
Taqman Fast Advanced Master Mix	Thermo Fisher Scientific	Cat # 4444557
CellTrace CFSE Cell Proliferation Kit	Thermo Fisher Scientific	Cat # C34554
Experimental Models: Cell Lines		
Mouse: YAC-1 lymphoma cells	ATCC	Cat # TIB-160; RRID: CVCL_2244
Mouse: B16-F10 melanoma cells	Dr. Ann Chambers at Western University	Available from ATCC (Cat # CRL-6475; RRID: CVCL_0159)
Mouse: B16-F10-Red-FLuc (B16-FLuc) melanoma cells	PerkinElmer	Cat # BW124734
Mouse: DN32.D3 hybridoma cells	Dr. Albert Bendelac at the University of Chicago	N/A
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	Charles River Canada	Cat # 027; RRID: IMSR_CRL:027
Mouse: BALB/c	Charles River Canada	Cat # 028; RRID: IMSR_CRL:028
Mouse: β2M$^{-/-}$: B6.129P2-B2m^{tm1Unc}/DcrJ	Dr. Anthony Jevnikar	Available from The Jackson Laboratory (Cat # 002087; RRID: IMSR_JAX:002087)
Mouse: B6 albino: B6(Cg)-Tyr^{c-2J}/J	The Jackson Laboratory	Cat # 000058; RRID: IMSR_JAX:000058
Mouse: B6.Nr3c1$^{fl/fl}$: B6.Cg-Nr3c1$^{tm1.1Jda}$/J	The Jackson Laboratory	Cat # 021021; RRID: IMSR_JAX:021021
Mouse: B6.Lck$^{cre/cre}$: B6.Cg-Tg(Lck-cre)548Jxm/J	The Jackson Laboratory	Cat # 003802; RRID: IMSR_JAX:003802
Mouse: Nr3c1^{fl}Lck^{cre}: B6.Nr3c1$^{fl/fl}$Lck$^{cre/wt}$	This paper	N/A

Mouse: Lck^{cre}: B6.$Lck^{cre/wt}$	This paper	N/A
Mouse: B6-$MAIT^{CAST}$ (Cui et al., 2015)	Dr. Olivier Lantz	N/A
Mouse: $MR1^{-/-}$ B6-$MAIT^{CAST}$ (Cui et al., 2015)	Dr. Olivier Lantz	N/A
Oligonucleotides		
See Table 3.2 for qPCR primer/probe sets	Thermo Fisher Scientific	N/A
Software and Algorithms		
FlowJo version 10.0.7 software	Tree Star	www.flowjo.com/
Image Studio version 3.1.4 software	LI-COR Biosciences	www.licor.com/bio/image-studio/
LivingImage software	PerkinElmer	www.perkinelmer.com/product/spectrum-200-living-image-v4series-1-128113
GraphPad Prism version 6.0 software	GraphPad	www.graphpad.com/

3.2.10 Quantitative PCR analyses

Hepatic *i*NKT and/or T_{conv} cells from ≥5 stressed or control mice were sorted to 100% purity using a BD FACSAria III Cell Sorter. Total RNA was isolated using a PureLink RNA Mini Kit (Thermo Scientific), and cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Thermo Scientific). Taqman Fast Advanced Master Mix (Applied Biosystems) was added to each cDNA sample, and the resulting mixture was plated in Custom Taqman Array Fast Plates (Thermo Scientific) containing probe/primer sets listed in Table 3.2. Cycle threshold (Ct) values from amplified transcripts were generated using a StepOne Plus Real-Time PCR instrument (Applied Biosystems). Normalized Ct (ΔCt) values were calculated by subtracting each Ct value by that of *Actb* and/or *Tbp*. The following formula was used to determine the relative mRNA content of the cells: Fold Change = $2^{-(\Delta\Delta Ct)}$.

3.2.11 Generation of bone marrow-derived dendritic cells (BMDCs)

Marrow cells were flushed out of femurs and tibias of B6 mice and depleted of erythrocytes. Cells were then washed, filtered and placed inside a T75 polystyrene flask at a density of 1×10^6 cells/mL of complete medium supplemented with 10 ng/mL each of recombinant mouse GM-CSF and IL-4 (Peprotech, Rocky Hill, NJ). Cultures were maintained for 6 days at 37°C in a humidified atmosphere containing 6% CO_2. Every other day, non-adherent cells were discarded, and cultures were replenished with fresh medium, GM-CSF and IL-4. Upon completion of the culture, cells were harvested using a cell scraper and $CD11c^+$ BMDCs were magnetically enriched using an EasySep Mouse CD11c Positive Selection Kit II (STEMCELL Technologies).

Table 3.2: Taqman-based qPCR primer/probe sets used in the study highlighted in Chapter 3.

Target	Assay Identification
Abl1	Mm00802029_m1
Actb	Mm00607939_s1
Adra1a	Mm00442668_m1
Adra1b	Mm00431685_m1
Adra1d	Mm01328600_m1
Adra2a	Mm00845383_s1
Adra2b	Mm00477390_s1
Adra2c	Mm00431686_s1
Adrb1	Mm00431701_s1
Adrb2	Mm02524224_s1
Adrb3	Mm02601819_g1
Aifm1	Mm00442540_m1
Anxa5	Mm01293059_m1
Apaf1	Mm01223702_m1
Api5	Mm00500189_m1
Atf5	Mm04179654_m1
Bad	Mm00432042_m1
Bag1	Mm01208593_m1
Bag3	Mm00443474_m1
Bak1	Mm00432045_m1
Bax	Mm00432051_m1
Bbc3	Mm00519268_m1
Bcl2	Mm00477631_m1
Bcl2a1a	Mm03646861_mH
Bcl2l1	Mm00437783_m1
Bcl2l11	Mm00437796_m1
Bid	Mm00432073_m1
Bik	Mm00476123_m1
Birc2	Mm00431811_m1
Birc3	Mm01168413_m1
Birc5	Mm00599749_m1
Bmf	Mm00506773_m1
Bnip2	Mm00443990_m1
Card10	Mm00459941_m1
Card6	Mm01297056_m1
Casp1	Mm00438023_m1
Casp2	Mm00432314_m1
Casp3	Mm01195085_m1
Casp4	Mm00432307_m1
Casp6	Mm00438053_m1

Casp7	Mm00432324_m1
Casp8	Mm00802247_m1
Casp9	Mm00516563_m1
Cblb	Mm01343092_m1
Cd27	Mm01185212_g1
Cd28	Mm00483137_m1
Cd40lg	Mm00441911_m1
Cd44	Mm01277161_m1
Cd69	Mm01183378_m1
Cd274	Mm03048248_m1
Cdk2	Mm00443947_m1
Cdk4	Mm00726334_s1
Cflar	Mm01255578_m1
Cradd	Mm01226172_m1
Csf1	Mm00432686_m1
Dad1	Mm01319221_m1
Dffa	Mm00438410_m1
Dffb	Mm00432822_m1
Diablo	Mm01194441_m1
Egr2	Mm00456650_m1
Egr3	Mm00516979_m1
Fadd	Mm00438861_m1
Fas	Mm01204974_m1 Mm00433237_m1
Fasl	Mm00438864_m1
Fos	Mm00487425_m1
Foxo3	Mm01185722_m1
Foxp1	Mm00474848_m1
Gata3	Mm00484683_m1
Gzma	Mm01304452_m1
Gzmb	Mm00442834_m1
Hells	Mm00468580_m1
Icam1	Mm00516023_m1
Icos	Mm00497600_m1
Ifng	Mm01168134_m1
Ifnar1	Mm00439544_m1
Ifnar2	Mm00494916_m1
Igf1r	Mm00802831_m1
Il10ra	Mm00434151_m1
Il12rb1	Mm00434189_m1
Il18rap	Mm00516053_m1
Il2	Mm00434256_m1
Il2ra	Mm01340213_m1
Il2rb	Mm00434268_m1

Il4	Mm00445259_m1
Il7ra/Cd127	Mm00434295_m1
Irf4	Mm00516431_m1
Itch	Mm01246513_m1
Jak1	Mm00600614_m1
Jak3	Mm00439962_m1
Jun	Mm00495062_s1
Lat	Mm00456761_m1
Lta	Mm00440228_gH
Myb	Mm00501741_m1
Naip2	Mm00440446_m1
Nfatc1	Mm00479445_m1
Nfatc2	Mm00477776_m1
Nfatc3	Mm01249200_m1
Nfkb1	Mm00476361_m1
Notch1	Mm00435249_m1
Npy1r	Mm04208490_m1
Npy2r	Mm01218209_m1
Npy4r	Mm01220859_m1
Npy5r	Mm00443855_m1
Npy6r	Mm00627550_m1
Nr3c1	Mm00433832_m1
Nr4a1	Mm01300401_m1
Pim2	Mm00454579_m1
Pmaip1	Mm00451763_m1
Polb	Mm00448234_m1
Prf1	Mm00812512_m1
Ptger2	Mm00436051_m1
Ripk1	Mm00436354_m1
Rnf128	Mm00480990_m1
Sell	Mm00441291_m1
Sphk2	Mm00445021_m1
Stat3	Mm01219775_m1
Stat6	Mm01160477_m1
Tbp	Mm00446973_m1
Tbx21	Mm00450960_m1
Tgfb1	Mm01178820_m1
Tnfa	Mm00443258_m1
Tnfrsf10b	Mm00457866_m1
Tnfrsf14	Mm00619239_m1
Tnfrsf4	Mm00442039_m1
Tnfrsf9	Mm00441899_m1
Tnfsf10	Mm01283606_m1
Tnfsf14	Mm00444567_m1

Tnfsf8	Mm00437153_m1
Traf1	Mm00493827_m1
Traf2	Mm00801978_m1
Traf3	Mm00495752_m1
Trp53bp2	Mm00557629_m1
Xiap	Mm01311594_mH
Zbtb16	Mm01176868_m1
Zc3hc1	Mm01168068_m1

3.2.12 *Ex vivo* and *in vitro* treatments and stimulations

DN32.D3 cells were seeded at 1×10^5 cells/well of a U-bottom microplate and treated for 20 minutes with NE (0.1-10 μM) or NPY (10^{-6}-1 μM), both from Sigma-Aldrich, before they were stimulated with 100 ng/mL of αGC. Where indicated, cells were pretreated with 10 μM propranolol hydrochloride (Sigma-Aldrich) for 20 minutes before they were exposed to NE. After 24 h at 37°C, cell viability was assessed by 7-AAD staining, and the IL-2 content of supernatants was measured by ELISA.

Hepatic mouse *i*NKT cells were FACS-sorted from ≥5 stressed animals or controls. In a U-bottom microplate, *i*NKT cells were mixed with purified $CD11c^+$ BMDCs at an *i*NKT:DC ratio of 2:1. Co-cultures were stimulated with 100 ng/mL of αGC for 24 h at 37°C, after which cell-free supernatants were stored at -20°C for subsequent cytokine measurements.

In indicated experiments, HMNCs and splenocytes were seeded at 5×10^5 cells/well in a microplate and stimulated with 15 ng/mL of phorbol 12-myristate 13-acetate (PMA) plus 500 ng/mL of ionomycin in the presence of 10 μg/mL brefeldin A, all of which were purchased from Sigma-Aldrich. After 2 h at 37°C, cells were washed and stained for intracellular cytokines.

Hydrocortisone (HC) and dexamethasone (DEX) were purchased from Sigma-Aldrich, dissolved in absolute ethanol and diluted in RPMI 1640. In a U-bottom microplate, 1×10^5 human HMNCs/well or 5×10^5 human PBMCs/well were incubated for 24 h at 37°C in complete medium containing 0.01-10 μM HC or DEX.

3.2.13 51-Chromium (^{51}Cr) release assay

YAC-1 target cells were incubated for 90 minutes at 37°C with 100 μCi $Na_2{}^{51}CrO_4$ (PerkinElmer). Labelled target cells were washed and then co-cultured at indicated effector:target ratios with splenocytes from stressed or control B6 mice that had received αGC or vehicle 24 hours before cytotoxicity assays. Cell-free supernatants were collected 4 h later, in which the ^{51}Cr activity was quantified using a PerkinElmer Wizard 1470 Automatic Gamma Counter. Experimental release (ER) values were obtained from wells

in which effector and target cells were both present. Spontaneous release (SR) and total release (TR) were measured from wells containing medium alone or 1% Triton X-100, respectively. Cytotoxicity was calculated using the following formula: % specific lysis = [(ER-SR) ÷ (TR-SR)] × 100.

3.2.14 *In vivo* killing assay

Donor splenocytes from WT B6 and $\beta 2M^{-/-}$ mice were labelled with 0.2 µM and 2 µM CFSE, respectively, and used as target cells. Cells were washed, mixed at a 1:1 ratio, and analyzed by flow cytometry before injection. Approximately 1×10^7 cells from the resulting suspension were injected i.v. into stressed recipients that were treated with αGC or vehicle. Two h later, animals were sacrificed for their spleen in which the remaining $CFSE^{hi}$ and $CFSE^{lo}$ target cells were traced by flow cytometry. *In vivo* killing was calculated using the following formula: % specific killing = (1 - [($CFSE^{hi}$ events in recipient ÷ $CFSE^{lo}$ events in recipient) ÷ ($CFSE^{hi}$ events pre-injection ÷ $CFSE^{lo}$ events pre-injection)]) × 100 as we previously described (30).

3.2.15 Metastatic melanoma model

Six h after administration of αGC or vehicle, stressed and control B6 mice received between $2.5 \times 10^5 – 1 \times 10^6$ B16-F10 cells in 200 µL PBS i.v. Fourteen days later, lungs were harvested for digital imaging, and distinct tumor nodules on each lung were visually counted. Lungs harboring >400 nodules were deemed to carry too many nodules for accurate enumeration. Therefore, they were conservatively represented as containing at least 400 nodules.

As an alternative method of measuring the metastatic tumor burden, B6 albino mice were injected with 5×10^5 B16-FLuc cells. After 21 days, they were anaesthetized with isoflurane and injected i.p. with 3 mg XenoLight D-Luciferin (PerkinElmer) in PBS. For up to 35 minutes thereafter, whole body bioluminescence imaging was conducted in an IVIS Lumina XRMS In Vivo Imaging System (PerkinElmer).

3.2.16 Quantification and statistical analysis

Flow cytometry results were analyzed using FlowJo version 10.0.7 software (Tree Star, Ashland, OR). The relative pixel intensities of bands in Western blot images were quantified using Image Studio version 3.1.4 software. For bioluminescence imaging, total signal (photons/second/cm^2/steradian) was quantified by region-of-interest analysis using LivingImage software (PerkinElmer).

Throughout this investigation, objective quantification methods, as opposed to subjective scoring, were used. Therefore, blinding was not necessary. Sufficient sample sizes were not statistically predetermined but were consistent with those from comparable studies and based on our prior experience.

Student's *t*-tests or one- or two-way ANOVA were employed, as appropriate, using GraphPad Prism version 6.0 software (La Jolla, CA). *, **, *** and **** denote statistically significant differences with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. Details related to sample sizes, measures of dispersion and the specific statistical tests used can be found in figure legends.

3.3 Results

3.3.1 Psychological stress impairs the ability of *i*NKT cells to trigger IL-4 and IFN-γ production and forces them to promote an atypical systemic inflammatory signature

Mediators of stress are known to induce T_H2 bias in T_{conv} cell responses (14, 17, 18). *i*NKT cells are unconventional, innate-like T cells with profound immunomodulatory properties and emergency response roles in antitumor and antimicrobial immunity (32); yet, how stress shapes *i*NKT cell responses has remained largely unknown.

We compared wild-type (WT) C57BL/6 (B6) mice that were left undisturbed or subjected to prolonged physical restraint (Figure 3.1A) for their *in vivo* IL-4 and IFN-γ responses to αGC, the prototypic glycolipid agonist of *i*NKT cells (27) (Figure 3.1A-C). The primary, if not the exclusive, source of IL-4 in this model are *i*NKT cells – hence the rapidity with which they release this cytokine (57). To our surprise, confinement stress resulted in dramatically reduced IL-4 levels (Figure 3.1B), which goes against the T_H2 paradigm of stress and immunity (14, 17, 18). Peak IFN-γ levels were also similarly decreased in stressed animals (Figure 3.1C).

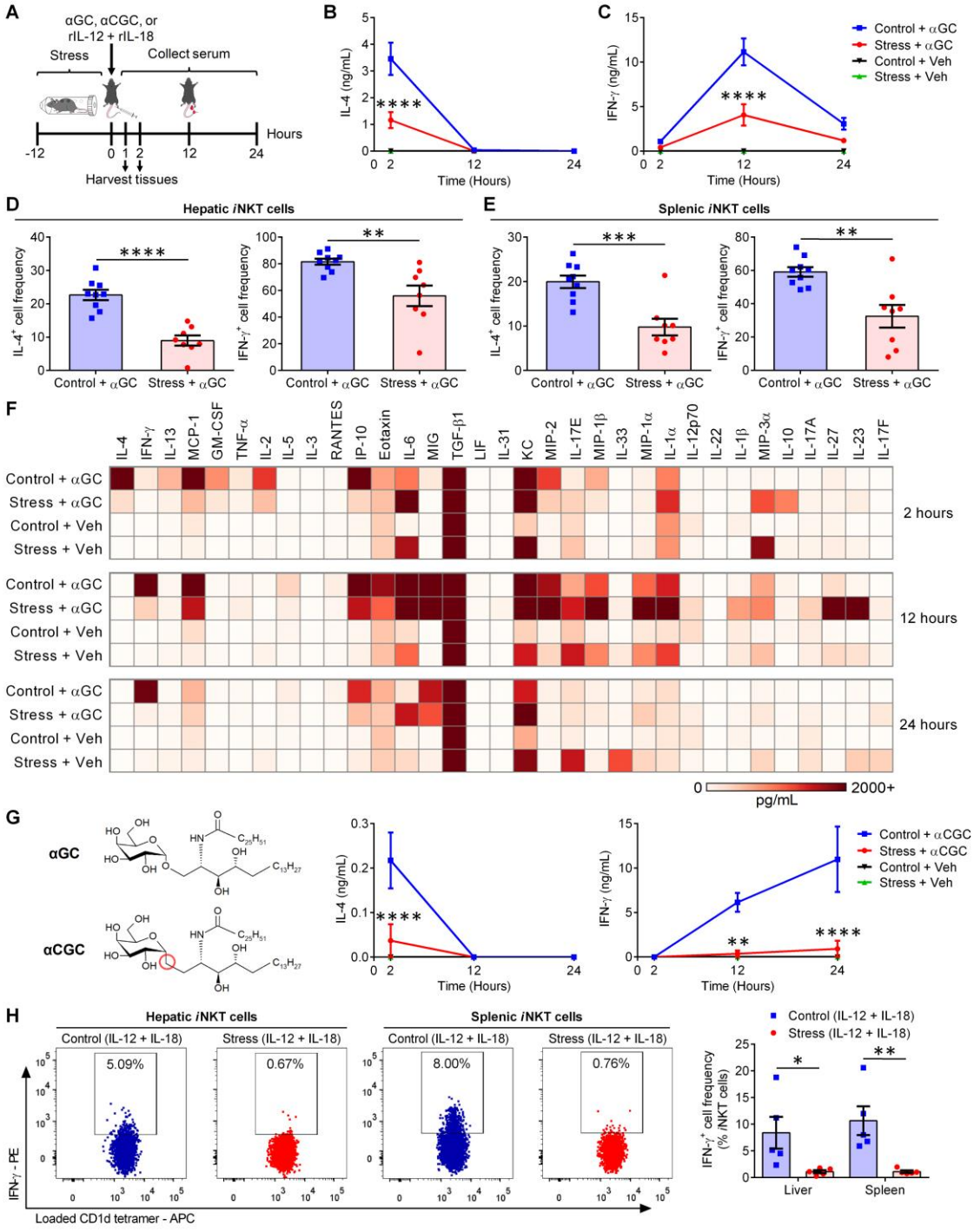

Figure 3.1: Prolonged stress impairs *i*NKT cells' capacity to trigger IL-4 and IFN-γ production and potentiates an abnormal inflammatory response to glycolipid Ags.

(A) WT B6 mice were physically restrained for 12 h. Control animals remained undisturbed but were deprived of food and water. Mice subsequently received αGC,

αCGC, or a corresponding vehicle (Veh) i.p. or a combination of IL-12 and IL-18 i.v. At indicated time points post-αGC administration, serum IL-4 **(B)** and IFN-γ **(C)** concentrations were quantified by ELISA (n=10/group). Two hours after αGC injection, the frequencies of IL-4$^+$ and IFN-γ$^+$ cells among hepatic **(D)** and splenic **(E)** TCRβ$^+$PBS-57-loaded CD1d tetramer$^+$ *i*NKT cells were determined by flow cytometry. **(F)** Two, 12 and 24 h after αGC (or Veh) administration, serum cytokine levels were measured via multiplex assays, and average values (n=3/cohort) were used to generate a heat map. **(G)** Separate cohorts (n=4) were injected with αCGC or Veh, and blood IL-4 and IFN-γ levels were measured by ELISA. **(H)** Parallel cohorts of stressed and control B6 mice were injected with IL-12 and IL-18 and sacrificed 1 h later for their livers and spleens, in which the percentages of IFN-γ$^+$ *i*NKT cells were determined. Representative cytofluorometric plots and summary data are illustrated. Each symbol in **D**-**E** and **H** represents an individual mouse. Error bars represent SEM. *, **, *** and **** denote statistical differences with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, by two-way ANOVA with Dunnett's correction (**B**-**C** and **G**) or unpaired Student's *t*-tests (**D**-**E** and **H**).

*i*NKT cells are not the only source of serum IFN-γ, which is also secreted by transactivated secondary effectors such as NK cells, following αGC administration (29, 58). Therefore, it was important to assess the impact of stress on IFN-γ production by *i*NKT cells. To this end, 2 h after αGC treatment, hepatic (Figure 3.1D) and splenic (Figure 3.1E) *i*NKT cells were identified via CD1d tetramer staining and examined for their intracellular cytokine content. Consistent with our serum cytokine results, far fewer *i*NKT cells from stressed mice generated IL-4 or IFN-γ (Figure 3.1D-E and Figure 3.2A). Furthermore, purified hepatic *i*NKT cells that were sorted after restraint stress and exposed to αGC in co-cultures with $CD11c^{+}$ bone marrow-derived dendritic cells (BMDCs) were weak cytokine-producers (Figure 3.2B). In contrast, upon *ex vivo* stimulation with a combination of PMA and ionomycin, *i*NKT cells from stressed animals demonstrated intact IL-4 and IFN-γ production capacities (Figure 3.2C). PMA and ionomycin work synergistically to activate protein kinase C (PKC) and Ca^{++}/calmodulin-dependent kinases, resulting in T cell activation independently of TCR engagement (59). Therefore, under stress, poor *i*NKT cell responses to cognate antigens are likely due to impaired *i*TCR proximal signaling events. In a limited number of experiments, we found splenic *i*NKT cells from stressed animals to express reduced levels of *i*TCR αβ, CD28 and inducible T cell costimulator (ICOS) (Figure 3.2D). These changes were accompanied by decreased phospho-SLP76 (pY128) levels and a trend towards diminished phospho-CD3ζ (pY142) and phospho-ZAP70 (pY319)/phospho-Syk (pY352) levels, but not phospho-Lck (pY505), in hepatic *i*NKT cells (data not shown).

Intrinsic host factors dictate or contribute to skewed cytokine responses in genetically diverse mammals. For instance, B6 and BALB/c mice are traditionally considered T_H1- and T_H2-dominant, respectively (60, 61). Also importantly, previous reports have suggested differences between these strains in terms of susceptibility to stress (62). Despite these fundamental differences, similar to B6 mice, BALB/c mice that had been stressed before receiving αGC had lower blood IL-4 and IFN-γ levels (Figure 3.3A).

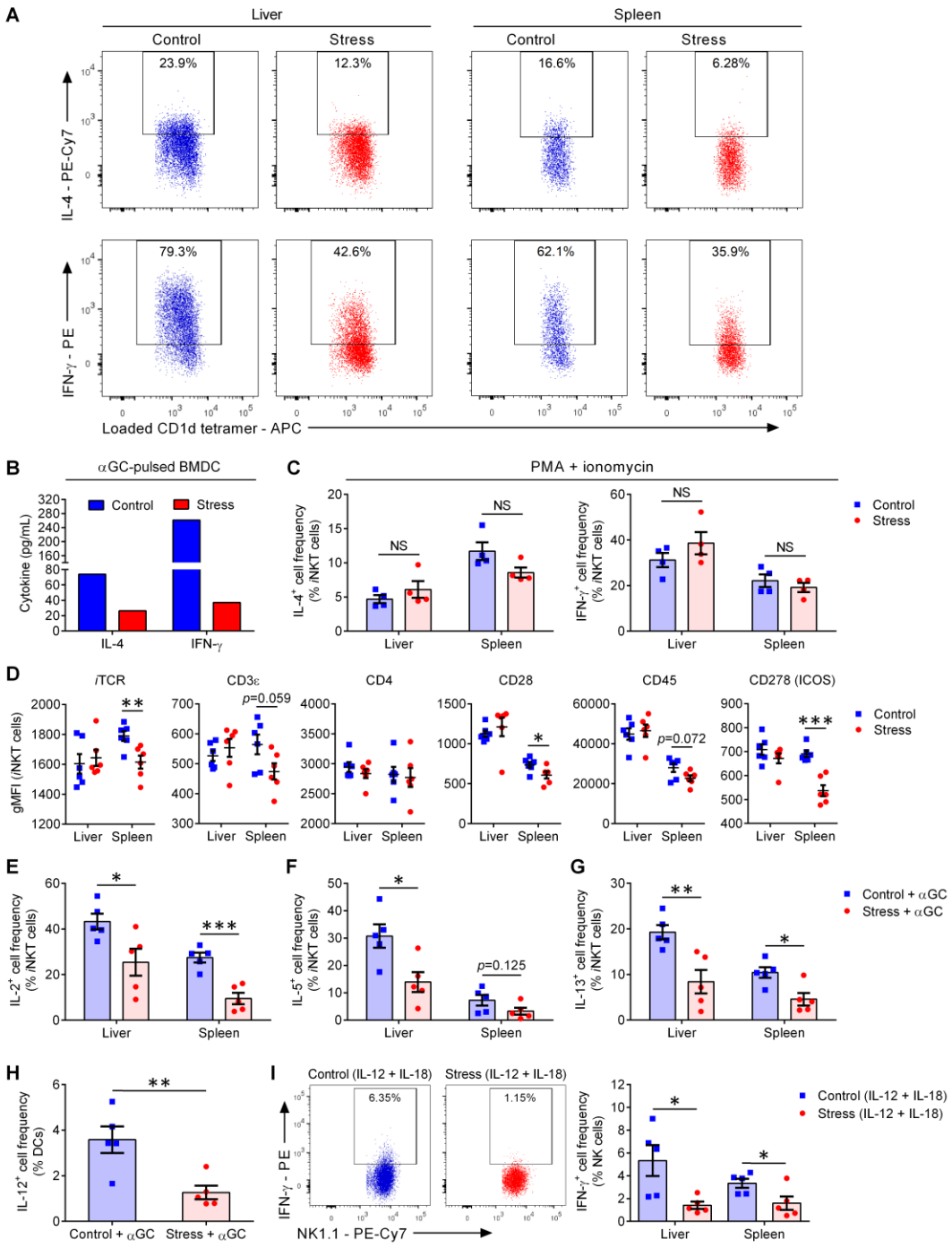

Figure 3.2: Prolonged physical restraint impairs the ability of *i*NKT cells to elicit TH1 and/or TH2-type cytokine responses to αGC or to a combination of IL-12 and IL-18, but not to a combination of PMA and ionomycin.

(A) B6 mice were physically restrained (or not) for 12 h before they were given αGC. Two hours later, HMNCs and splenocytes were stained with mAbs against IL-4 and IFN-

γ and analyzed by flow cytometry. Representative dot plots illustrate the frequencies of IL-4$^+$ and IFN-γ$^+$ *i*NKT cells after gates were set based on isotype control staining. **(B)** Sorted hepatic *i*NKT cells pooled from ≥5 stressed or control B6 mice were stimulated *ex vivo* with 100 ng/mL of αGC in the presence of CD11c$^+$ BMDCs. After 24 h, IL-4 and IFN-γ levels in culture supernatants were measured. **(C)** HMNCs and splenocytes from stressed and control B6 mice were stimulated for 2 h with 15 ng/mL of PMA and 500 ng/mL of ionomycin before intracellular levels of IL-4 and IFN-γ in *i*NKT cells were determined by flow cytometry. **(D)** HMNCs and splenocytes from restrained or control animals were stained with loaded CD1d tetramer or mAbs against indicated molecules. After gating on *i*NKT cells, the gMFI of staining for each molecule is depicted. **(E-G)** As in (**A**), but summary data indicates the frequencies of *i*NKT cells staining positively for mAbs against IL-2 **(E)**, IL-5 **(F)** and IL-13 **(G)**. **(H)** B6 mice were restrained (or not) for 12 h before they were given αGC. Six h later, HMNCs were stained with an anti-IL-12p35 mAb or a rat IgG2aκ isotype control. The frequency of IL-12$^+$ DCs was determined after gating on TCRβ$^-$CD11c$^+$ events. **(I)** Mice that had been restrained or left undisturbed were injected with IL-12 and IL-18 one h before the percentages of IFN-γ$^+$ events among TCRβ$^-$NK1.1$^+$ NK cells were determined. Representative flow plots and summary data are shown. Each symbol represents an individual mouse, and error bars represent SEM. *, ** and *** denote significant differences with $p<0.05$, $p<0.01$ and $p<0.001$, respectively, by unpaired Student's *t*-tests. NS = not significant

There is sexual dimorphism in sensitivity to various stressors (53). In addition, *i*NKT cell frequencies and cytokine profiles, including IL-4 and IFN-γ responses to αGC, can be different between males and females (63, 64). Therefore, it was important to include both male and female mice in our study. We found restraint stress to similarly reduce serum IL-4 and IFN-γ concentrations in both sexes (Figure 3.3B).

IL-4 and IFN-γ are classic T_H2- and T_H1 cytokines, respectively. However, numerous other cytokines and chemokines also participate in inflammatory responses. To more widely capture the *i*NKT cell response landscape amid prolonged restraint stress, we performed multiplex analysis on serum samples collected 2, 12 and 24 h after treatment with αGC or vehicle. A multitude of cytokines, other than IL-4 and IFN-γ, were significantly reduced at one or several time points in the serum of stressed mice. These included IL-2, IL-5, IL-13, eotaxin, GM-CSF, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and TNF-α (Figure 3.1F and Figure 3.4). In our cytofluorimetric analyses, we confirmed diminished intracellular levels of IL-2, IL-5 and IL-13 in αGC-stimulated *i*NKT cells from stressed animals (Figure 3.2E-G). In contrast with the above mediators, three cytokines, namely IL-10, IL-23 and IL-27, became notably detectable at strikingly high quantities in animals that had been restrained (Figure 3.1F and Figure 3.4). Furthermore, stress augmented the production of IL-1α, IL-1β, MIP-1α/CCL3 and MIP-3α/CCL20 in αGC-treated mice (Figure 3.1F and Figure 3.4), and also resulted in a trend towards increased IL-17A levels at the 12-hour time point (p=0.075).

There were a number of cytokines whose serum levels were not different between stressed and control cohorts, including G-CSF, IL-6, IL-7, IL-9, IL-15, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-28B/IFNL3, IL-31, IL-33, KC/CXCL1, LIF, LIX/CXCL5, M-CSF, MIG/CXCL9, MIP-1β/CCL4, MIP-2/CXCL2, TGF-β1, TGF-β2, TGF-β3 and VEGF (Figure 3.1F and data not shown).

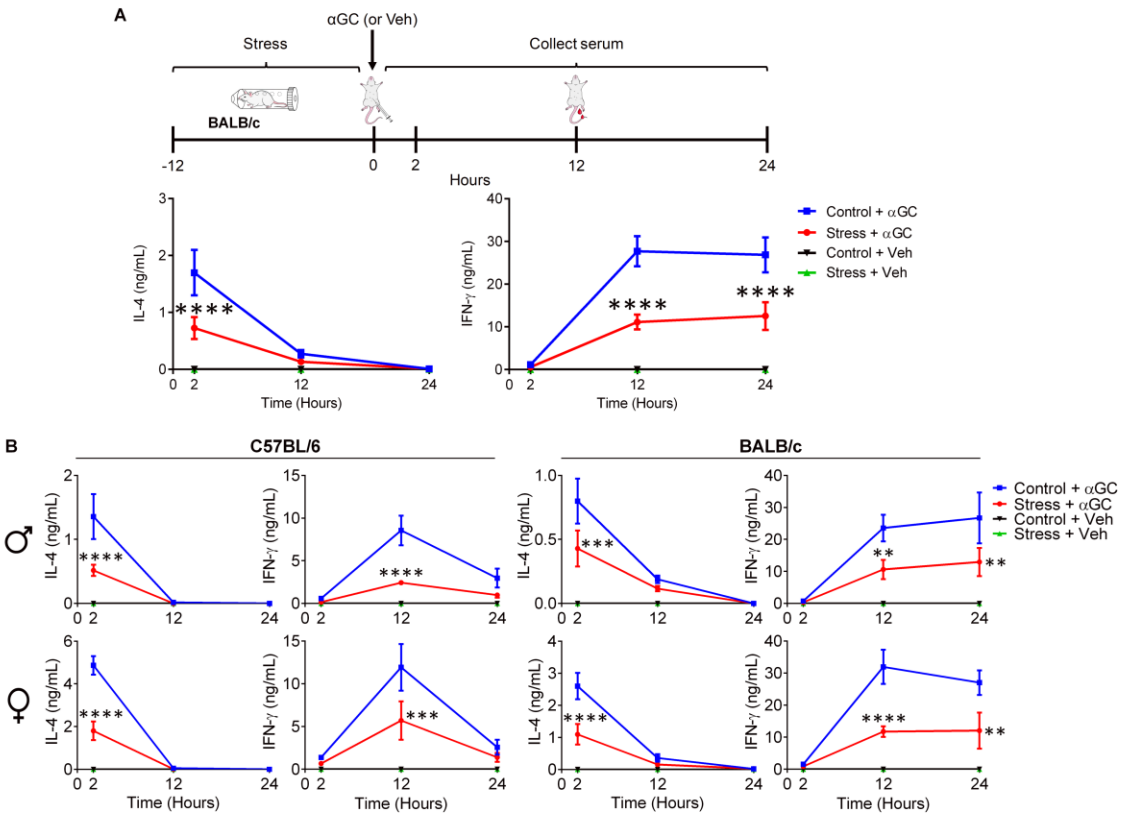

Figure 3.3: Prolonged stress impairs cognate *i*NKT cell responses in male and female B6 and BALB/c mice.

(A) WT BALB/c mice were subjected to physical restraint or left undisturbed for 12 h before they were injected i.p. with αGC or Veh. At indicated time points, IL-4 and IFN-γ in serum samples were quantified by ELISA (n=8-9/group). **(B)** Data presented in Figure 3.1B-C and Figure 3.3A were segregated by sex. The kinetics of serum cytokine levels following αGC (or Veh) administration in male (B6: n=5; BALB/c: n=5) and female (B6: n=5; BALB/c: n=3-4) mice are depicted. Error bars represent SEM. **, *** and **** denote differences with $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, using two-way ANOVA with Dunnett's post-hoc analysis.

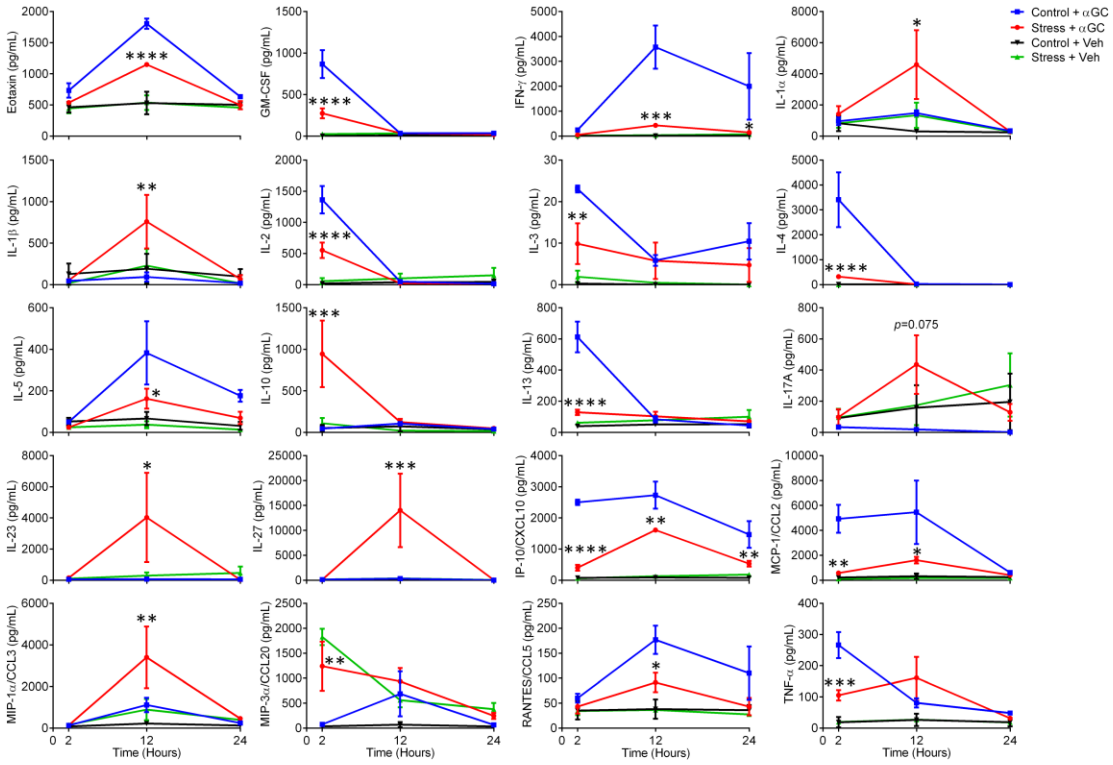

Figure 3.4: Prolonged physical restraint before αGC administration alters the serum concentrations of a wide range of inflammatory mediators.

WT B6 mice were restrained or left undisturbed for 12 h before they were injected with αGC or Veh (n=3/cohort). Two, 12 and 24 h later, mice were bled and serum levels of indicated mediators were quantified by cytokine/chemokine multiplexing. *, **, *** and **** denote significant differences between stressed and control animals receiving αGC with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, using two-way ANOVA with Tukey's post-hoc analysis.

Taken together, our cytokine analyses indicate that *i*NKT cells defy the popular belief that stress indiscriminately steers all T cell responses towards a purely or heavily T_H2-biased phenotype. Instead, *i*NKT cells display a mixed signature dominated by 'select' pro- and anti-inflammatory cytokines. Even within the same general category, atypical patterns emerged. For instance, while stress raised the levels of IL-1α and IL-1β after αGC injection, TNF-α concentrations were diminished, and IL-6 remained unaltered.

Our multiplexing analyses did not include a 6-hour time point post-αGC administration, at which IL-12 is known to reach its peak levels (65). This limitation was remedied by separate ELISA assays in which a nearly 10-fold reduction in IL-12 was evident. To be exact, serum IL-12 levels were 4355 ± 422 pg/mL and 403 ± 278 pg/mL in control and stressed animals, respectively. Given the prominent role of DCs in IL-12 production following *in vivo i*NKT cell activation (66, 67), we examined the frequency of IL-12^+ DCs, which was significantly reduced in stressed mice that had received αGC 6 h earlier (Figure 3.2H). The above results are consistent with a 'split mini-signature' even among classic pro-inflammatory cytokines.

Next, we determined whether stress alters *i*NKT cell responses to CD1d-binding glycolipids other than αGC. We found prolonged restraint stress (Figure 3.1A) to suppress cytokine responses triggered by α-C-galctosylceramide (αCGC), a T_H1-polarizing analog of αGC that carries a single glycosidic O-to-CH2 substitution (Figure 3.1G) (68). To determine whether stress compromises CD1d/*i*TCR-independent, cytokine-driven *i*NKT cell responses, we used a combination of IL-12 and IL-18, which enables NK cells and innate-like T cells to produce IFN-γ (69). Therefore, we subjected mice to restraint before injecting them with IL-12 and IL-18 (Figure 3.1A). Both hepatic and splenic *i*NKT cells from stressed mice failed to produce IFN-γ in this setting (Figure 3.1H). Similarly, NK cells from a stressed cohort were unable to mount an optimal IFN-γ response to IL-12 and IL-18 (Figure 3.2I).

Collectively, prolonged psychological stress abrogates IL-4 and IFN-γ responses that are either elicited or potentiated by αGC-exposed *i*NKT cells. This finding is reproducible irrespective of the individual's genetic background and sex, the tissue location of *i*NKT

cells, and their means and modes of stimulation via *i*TCR and IL-12/IL-18 receptor engagement. The mixed inflammatory signature observed in stressed animals represents a stark contrast with T_H2-polarized T_{conv} responses.

3.3.2 Unlike other lymphocyte subsets, *i*NKT cells are resistant to stress- and glucocorticoid-induced apoptosis

Mediators of stress disrupt certain host defense mechanisms by inducing apoptosis in lymphocytes (16, 70, 71). As expected, non-parenchymal hepatic mononuclear cell (HMNC) and splenocyte counts were significantly lower in stressed mice than in controls immediately after restraint ($2.2\times10^6 \pm 0.2\times10^6$ and $3.5\times10^6 \pm 0.3\times10^6$, respectively, for HMNCs; $54.7\times10^6 \pm 7.8\times10^6$ and $77.6\times10^6 \pm 6.3\times10^6$, respectively, for splenocytes; n=6 per group) and also 2 h after αGC administration ($1.2\times10^6 \pm 0.1\times10^6$ and $2.3\times10^6 \pm 0.1\times10^6$, respectively, for HMNCs; $33.2\times10^6 \pm 7.0\times10^6$ and $62.0\times10^6 \pm 8.3\times10^6$, respectively, for splenocytes; n=5 per group). Therefore, since physical restraint impeded IL-4 and IFN-γ responses to αGC (Figure 3.1), we asked whether cytokine-producing cells had simply died. We found significant increases, rather than decreases, in hepatic and splenic *i*NKT cell frequencies in stressed mice (Figure 3.5A). This was curious since $Ki67^+$ *i*NKT cell frequencies were similar between stressed and control animals (30.3 ± 6.7% and 27.0 ± 3.0%, respectively, for hepatic *i*NKT cells; 17.6 ± 3.3% and 21.7 ± 5.3%, respectively, for splenic *i*NKT cells; n=4 per group), dismissing the possibility that *i*NKT cells had undergone expeditious proliferation during stress. When enumerating tissue-resident cells, we found the absolute numbers of hepatic and splenic *i*NKT cells to remain stable contrasting with marked drops in T_{conv} cell numbers (Figure 3.5B), suggesting that *i*NKT cells were unusually refractory to stress-provoked apoptosis. This was validated by measuring the intracellular caspase content of *i*NKT and T_{conv} cells, which were steady and increased, respectively (Figure 3.5C).

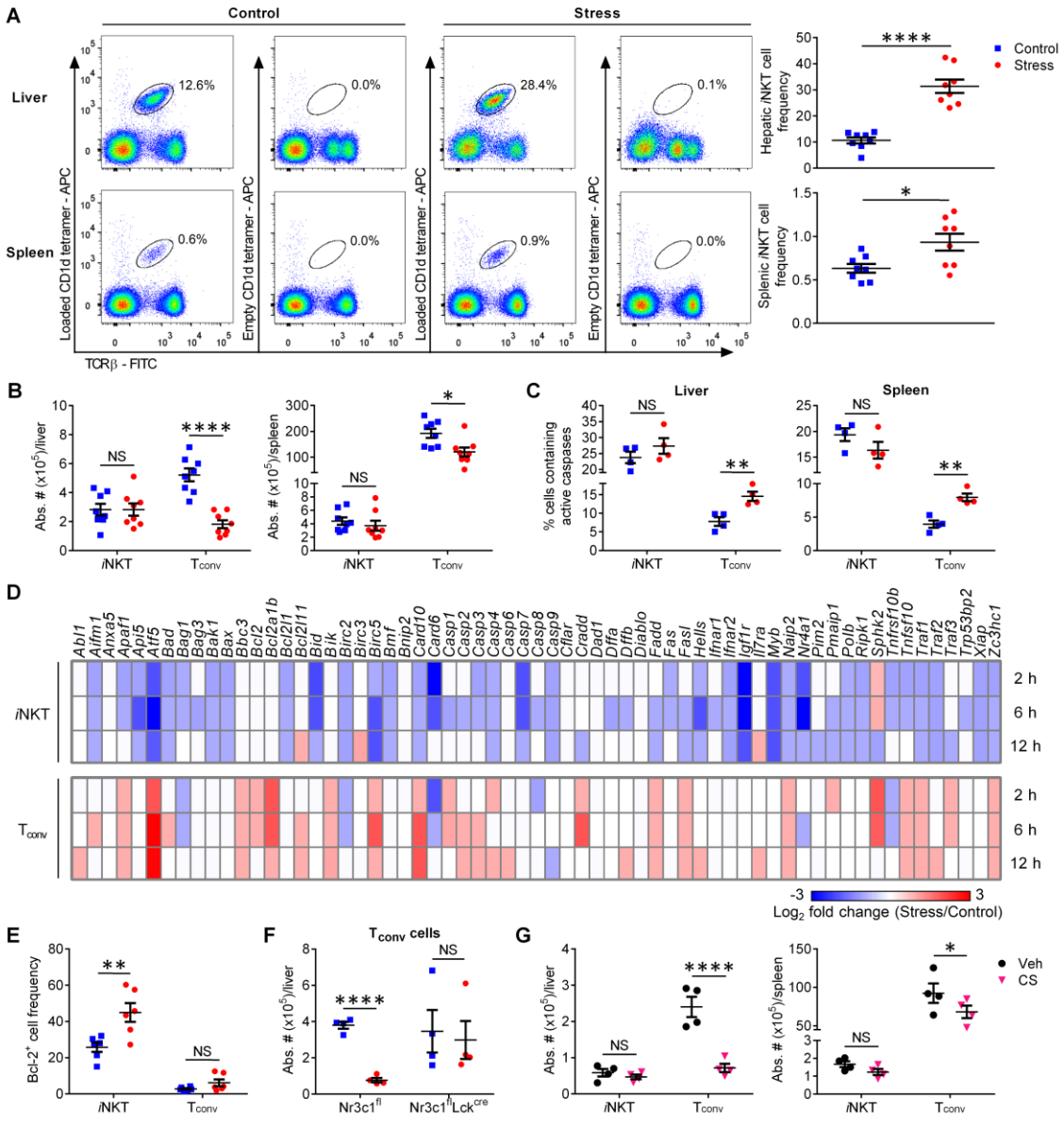

Figure 3.5: Unlike T_{conv} cells, *i*NKT cells are resistant to glucocorticoid-induced apoptosis.

(A) WT B6 mice were left undisturbed or restrained for 12 h. Immediately afterwards, HMNCs and splenocytes were harvested and stained with a mAb to TCRβ along with empty (staining control) or PBS-57-loaded CD1d tetramers to detect *i*NKT cells. Representative dot plots and summary data depicting the frequencies of hepatic and splenic *i*NKT cells in stressed and control mice are shown. **(B)** The absolute numbers of *i*NKT and TCRβ$^+$PBS-57-loaded CD1d tetramer$^-$ T_{conv} cells were also calculated. **(C)** In

addition, the percentages of *i*NKT and T_{conv} cells containing active caspases were determined by flow cytometry. **(D)** Hepatic *i*NKT and T_{conv} cells were purified from ≥5 mice that had been either subjected to 2, 6 or 12 h of restraint stress or left undisturbed. After obtaining cDNA, the indicated gene products were amplified by quantitative PCR. Gene expression fold changes in *i*NKT and T_{conv} cells isolated from stressed mice relative to corresponding cell populations from control animals were calculated using the $2^{-(\Delta\Delta Ct)}$ method and used to generate a heat map. **(E)** Hepatic *i*NKT and T_{conv} cells were analyzed for their intracellular Bcl-2 content. **(F)** Hepatic T_{conv} cells were enumerated in $Nr3c1^{fl}$ and $Nr3c1^{fl}Lck^{cre}$ mice that had been either subjected to prolonged restraint stress or left undisturbed. **(G)** Cohorts of WT B6 mice were given corticosterone (CS) or Veh in drinking water for 21 days before they were sacrificed for their livers and spleens, in which *i*NKT and T_{conv} cells were enumerated. Each symbol in **A-C** and **E-G** represents an individual mouse, and error bars represent SEM. **p*<0.05, ***p*<0.01, *****p*<0.0001 by unpaired Student's *t*-tests. NS = not significant

*i*NKT cells are 'pre-activated, memory-like' T cells (72, 73). Therefore, we asked whether their resistance to apoptosis during stress was a mere reflection of the above phenotype and therefore mimicable by the memory subset of T_{conv} cells. Naïve and memory T_{conv} pools were distinguished based on CD44 expression (Figure 3.6A). Similar to unfractionated and naïve T_{conv} cells, but unlike *i*NKT cells, $CD44^{+}$ memory T_{conv} cells were less frequent and contained more intracellular active caspases in restrained animals (Figure 3.6B). Therefore, *i*NKT cells' resistance to stress does not appear to have much, if anything, to do with their memory-like feature.

To shed more light on molecular mediators of cell death in the T cell compartment, we analyzed the transcript levels of relevant genes in sorted *i*NKT and T_{conv} cells 2, 6 and 12 h into restraint stress. Compared with baseline levels, several pro-apoptotic genes were upregulated in T_{conv} but not in *i*NKT cells. These were, among others, the apoptosome component *Apaf1*, the Bcl-2 family members *Bad*, *Bbc3*, *Bik* and *Pmaip1*, and the executioner caspases-*3* and -*6* (Figure 3.5D). In fact, a number of these genes were downregulated by *i*NKT cells. At the protein level, the pro-survival molecule Bcl-2 was elevated in *i*NKT cells, but not in the remaining T_{conv} cells, during stress (Figure 3.5E). Therefore, Bcl-2 may have a preventative or compensatory role in circumventing the apoptogenic effects of stress on *i*NKT cells.

Glucocorticoids are known culprits of T cell apoptosis under certain circumstances (15, 16). As such, we posited that the dimorphic impact of stress on *i*NKT and T_{conv} cells stems from differential glucocorticoid actions in these cell types. To mechanistically address this question, we generated mice whose T cells were devoid of GR. Indeed, unlike in B6.Nr3c1$^{fl/fl}$ (Nr3c1^{fl}) controls, T_{conv} cells were maintained in B6.Nr3c1$^{fl/fl}$Lck$^{cre/WT}$ (Nr3c1^{fl}Lck^{cre}) mice that were subjected to restraint stress (Figure 3.5F). We also observed a sharp numerical drop in hepatic and splenic NK cells and B cells during prolonged restraint stress, which was reversible by treatment with the GR antagonist RU486 (Figure 3.6C).

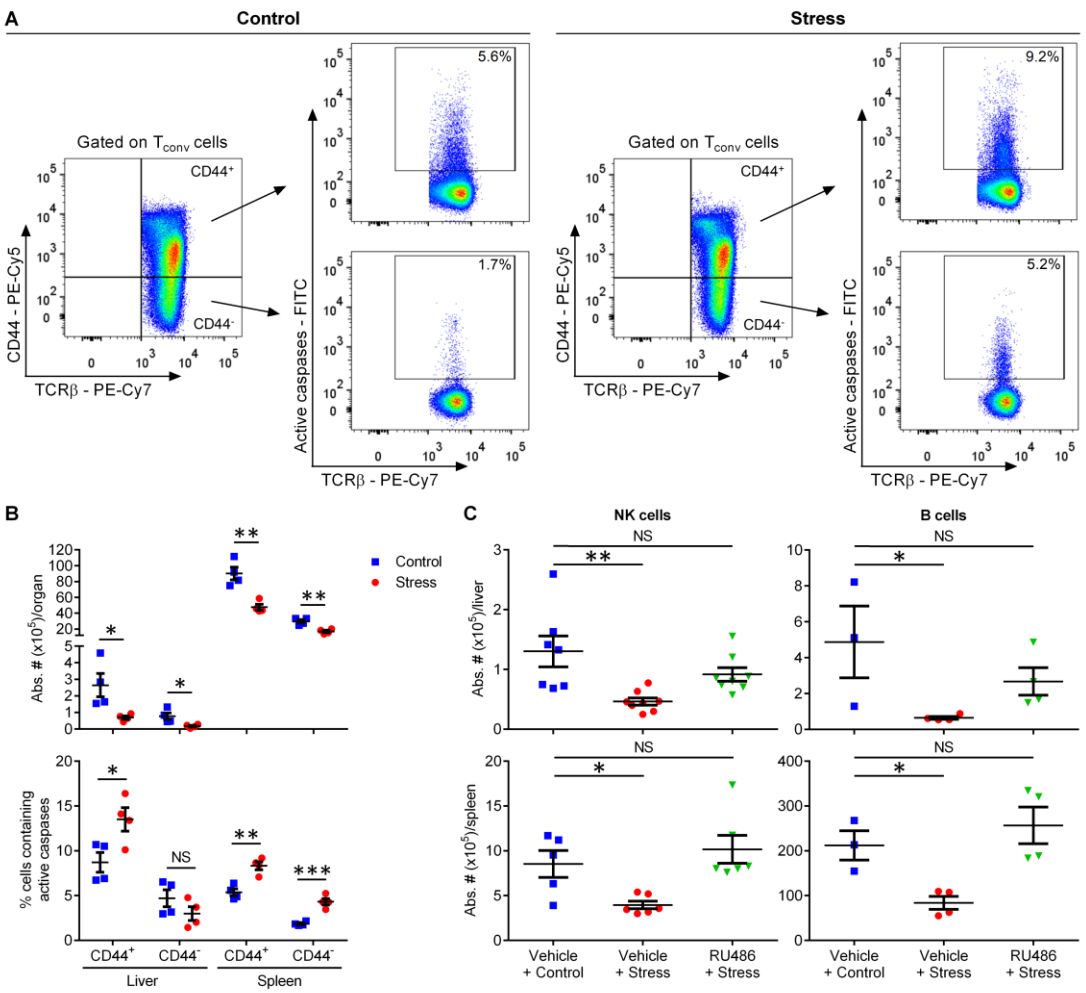

Figure 3.6: Unlike *i*NKT cells, naïve and memory T_{conv} cells, NK cells and B cells are prone to stress-induced apoptosis.

(A-B) Splenocytes and HMNCs from restrained and control B6 mice were stained for surface TCRβ, surface CD44 and intracellular active caspases. A rat IgG2bκ isotype control was used to set the gate for CD44 staining. **(A)** Representative FACS plots illustrate our gating strategy to distinguish between splenic CD44$^+$ and CD44$^-$ populations among TCRβ$^+$CD1d tetramer$^-$ events, which correspond to memory and naïve T_{conv} cells, respectively. Gates containing T_{conv} cells with intracellular active caspases are also shown. **(B)** The absolute numbers of hepatic and splenic memory and naïve T_{conv} cells and the percentages of T_{conv} cells staining positively for active caspases are depicted. **(C)** Separate cohorts of B6 mice were treated i.p. with RU486 or Veh. One hour later, animals were subjected to prolonged restraint stress or were left undisturbed for 12 h.

HMNCs and splenocytes were prepared shortly afterwards and stained with a panel of mAbs against TCRβ, NK1.1 and B220. $TCR\beta^{-}NK1.1^{+}$ NK cells and $TCR\beta^{-}B220^{+}$ B cells were identified by flow cytometry, and their absolute numbers were calculated. Each symbol represents an individual mouse, and error bars represent SEM. *, ** and *** denote differences with $p<0.05$, $p<0.01$ and $p<0.001$, respectively, using unpaired Student's *t*-tests (**B**) or one-way ANOVA with Dunnett's post-hoc analysis (**C**). NS = not significant

In the clinic, exogenous glucocorticoids are prescribed for many diseases and conditions. Therefore, we tested the effect of long-term, oral administration of corticosterone (CS), the main glucocorticoid in rodents (13), on T cell apoptosis. Similar to endogenous glucocorticoids, CS reduced T_{conv}, but not *i*NKT, cell numbers both in the liver and in the spleen (Figure 3.5G), a finding that also serves to confirm the dominant role of glucocorticoids in stress-induced T_{conv} cell demise.

To summarize the above findings, unlike other lymphocyte subsets (naïve and memory T_{conv}, B and NK cells), *i*NKT cells are uniquely refractory to stress-inflicted apoptotic death. Their resilience in the face of a stress response is mediated by glucocorticoid-GR interactions. Also importantly, these results rule out cell death as the reason behind the dwarfed IL-4 and IFN-γ responses of *i*NKT cells to glycolipid antigens (Ags) or cytokines (Figure 3.1) in stressed animals.

3.3.3 Stress suppresses αGC-elicited inflammatory responses through an *i*NKT cell-intrinsic, GR signaling-dependent mechanism independently of neurotransmission from post-ganglionic sympathetic neurons

We next attempted to elucidate the upstream neurological pathway(s) governing *i*NKT cell hyporesponsiveness in the context of prolonged restraint stress. Given the paramount role of the SNS in the fight-or-flight response (9), we first looked into the expression of SNS neurotransmitter receptors in *i*NKT cells purified from the liver of treatment-naïve B6 mice. We did not detect *Npy1r*, *Npy2r*, *Npy4r*, *Npy5r* or *Npy6r* transcripts (data not shown), and synthetic NPY failed to modulate cytokine production by the *i*NKT cell hybridoma DN32.D3 in response to αGC (data not shown). Hepatic *i*NKT cells had detectable mRNAs encoding the adrenergic receptors *Adra2a*, *Adra2b*, *Adrb1* and *Adrb2* at levels comparable to or lower than those found in matched hepatic T_{conv} cells (Figure 3.7A). In addition, norepinephrine (NE) inhibited IL-2 production by αGC-stimulated DN32.D3 cells (Figure 3.7B), which was preventable by β-adrenergic receptor antagonism with propranolol (Figure 3.7C). However, this was only an *in vitro* event since chemical sympathectomy through 6-hydroxydopamine (OHDA) administration failed to restore IL-4 and IFN-γ production in restrained mice that were subsequently

injected with αGC (Figure 3.8A). Sympathectomy was confirmed by reduced tyrosine hydroxylase (TH) staining in the spleens of OHDA-treated animals (Figure 3.8B). In these experiments, the brain served as a negative control because OHDA does not cross the blood-brain barrier when administered systemically (74).

We then shifted our focus back onto the HPA axis and glucocorticoids. Elevated serum CS levels in stressed mice reassured us that prolonged restraint stress in our hands could induce robust activation of the HPA axis (Figure 3.8C). Pretreatment with the glucocorticoid synthesis inhibitor metyrapone was able to rescue IL-4 and IFN-γ production in stressed mice receiving αGC (Figure 3.8D). To ensure that our results were not confounded by the reported buildup of circulating 11-deoxycorticosterone upon metyrapone treatment (75), we used a second pharmacological approach, namely GR antagonism by RU486. Similar to metyrapone, RU486 could prevent the suppressive effect of stress on cytokine production in restrained animals (Figure 3.8E). Of note, we used male mice in these experiments out of an abundance of caution to avoid the antagonistic action of RU486 on progesterone receptors (13).

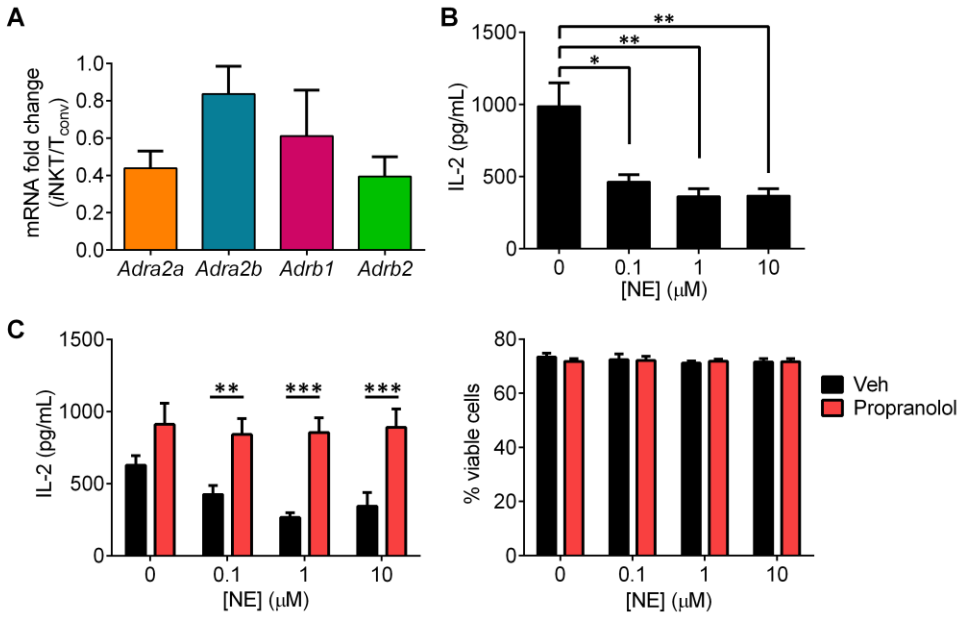

Figure 3.7: *i*NKT cells express adrenergic receptors and respond weakly to αGC in the presence of NE in an *in vitro* setting.

(A) HMNCs from 10 naïve B6 mice were pooled and co-stained with an anti-TCRβ mAb and PBS-57-loaded CD1d tetramers. *i*NKT and T_{conv} cells were FACS-purified for cDNA synthesis and gene expression analysis by quantitative PCR. The expression of indicated genes by *i*NKT cells relative to T_{conv} cells was calculated using the $2^{-(\Delta\Delta Ct)}$ method. Data from 3 independent experiments were used to determine fold change values. **(B)** DN32.D3 cells were exposed for 20 minutes to indicated concentrations of norepinephrine (NE) before they were stimulated with 100 ng/mL of αGC. The IL-2 content of culture supernatants was quantified after 24 h by ELISA (n=3-5). **(C)** DN32.D3 cells were pretreated for 20 minutes with propranolol before they were exposed to NE and subsequently stimulated with αGC (n=4). After 24 h, IL-2 was measured in supernatants (n=4) and cellular viability was assessed by 7-AAD staining (n=3). Error bars represent SEM. *, ** and *** denote differences with $p<0.05$, $p<0.01$ and $p<0.001$, respectively, by one-way ANOVA with Dunnett's post-hoc analysis (**B**) or by unpaired Student's *t*-tests (**C**).

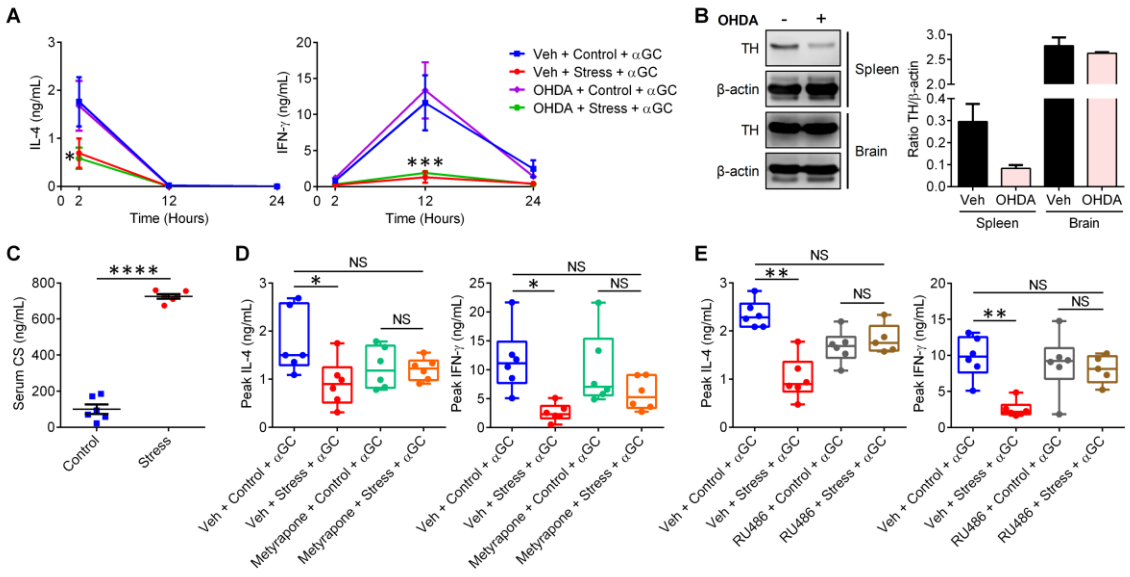

Figure 3.8: Stress-induced suppression of *i*NKT cell responses is mediated by glucocorticoids, not by SNS signals.

(A) WT B6 mice (n=3-4/group) were injected i.p. with OHDA or vehicle six days before they were subjected to prolonged physical restraint. Control cohorts were left undisturbed. Stressed and control animals were injected with αGC and subsequently bled at indicated time points. Circulating IL-4 and IFN-γ levels were measured by ELISA. **(B)** Protein extracts from flash-frozen spleen and brain samples from mice in (**A**) were loaded onto the same SDS-PAGE gel and examined for their tyrosine hydroxylase (TH) content by Western blotting. Staining for β-actin was used to ensure equal loading of protein extracted from lysates. Summary data illustrate normalized TH levels after densitometry analyses. **(C)** Separate cohorts that had been restrained for 12 h or left undisturbed were bled, and serum CS concentrations were quantified by ELISA. **(D-E)** Mice were injected i.p. with metyrapone **(D)** or RU486 **(E)** 1 h before they were subjected to physical restraint for 12 h (or not) followed by an i.p. injection of αGC. Box-and-Whisker plots show IL-4 and IFN-γ levels at 2 h and 12 h time points post-αGC administration, respectively, with each symbol representing an individual mouse. Statistical comparisons were made by two-way ANOVA with Dunnett's correction (**A**), unpaired Student's *t*-test (**C**), or one-way ANOVA with Tukey's correction (**D** and **E**). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, NS = not significant

Although the systemic ablation of GR signaling could relieve *i*NKT cell hyporesponsiveness during stress, it was unclear whether glucocorticoids were acting on *i*NKT cells directly or on other cell types such as glycolipid Ag-presenting cells. We first demonstrated that *i*NKT cells from naïve mice express the GR, at a greater level than that in matched T_{conv} cells. This was manifest at both mRNA and protein levels (Figure 3.9A-B). Second, we observed that stress instigates GR signaling in hepatic and splenic *i*NKT cells as judged by increased intracellular levels of the anti-inflammatory protein glucocorticoid-induced leucine zipper (GILZ), a known transcriptional target of the activated GR (Figure 3.9C) (76). Finally, unlike in the $Nr3c1^{fl}$ control cohort, stress failed to compromise the IFN-γ response of αGC-injected $Nr3c1^{fl}Lck^{cre}$ mice (Figure 3.9D). This was not due to the possible off-target effects of the Cre recombinase since IFN-γ levels were not rescued in stressed $B6.Lck^{cre/WT}$ (Lck^{cre}) mice (Figure 3.9E). T_{conv} cells are highly unlikely to contribute to rapid IFN-γ production in αGC-treated animals. Therefore, one may infer that direct GR signaling disrupts the ability of *i*NKT cells to generate or trigger inflammatory cytokine responses *in vivo*.

In the next series of experiments, we asked whether physical restraint *per se* alters the activation status of *i*NKT cells. Consistent with their 'pre-activated' phenotype (72, 73), hepatic and splenic *i*NKT cells expressed high surface levels of CD25, CD44 and CD69 and minimal CD62L in their steady state (Figure 3.9F). Moreover, confinement stress failed to appreciably change these baseline levels (Figure 3.9F). Therefore, we took a more comprehensive approach by comparing the transcriptional profiles of purified *i*NKT cells from stressed and control mice. While stress upregulated *Bcl2* and *CD127* (*Il7ra*), the mRNA levels of a number of genes that should support the effector functions of *i*NKT cells were reduced. These include *Cd40l*, *Il18rap*, *Egr2*, *Irf4*, *Nfatc3*, *Tbx21*, *Ifng*, *Il4*, *Gzma*, *Tnf*, *Tnfrsf9* and *TNFsf10* among others (Figure 3.9G). Therefore, wide-ranging *i*NKT cell dysfunctions, beyond select cytokine production, can be expected in the aftermath of stress-induced GR signaling.

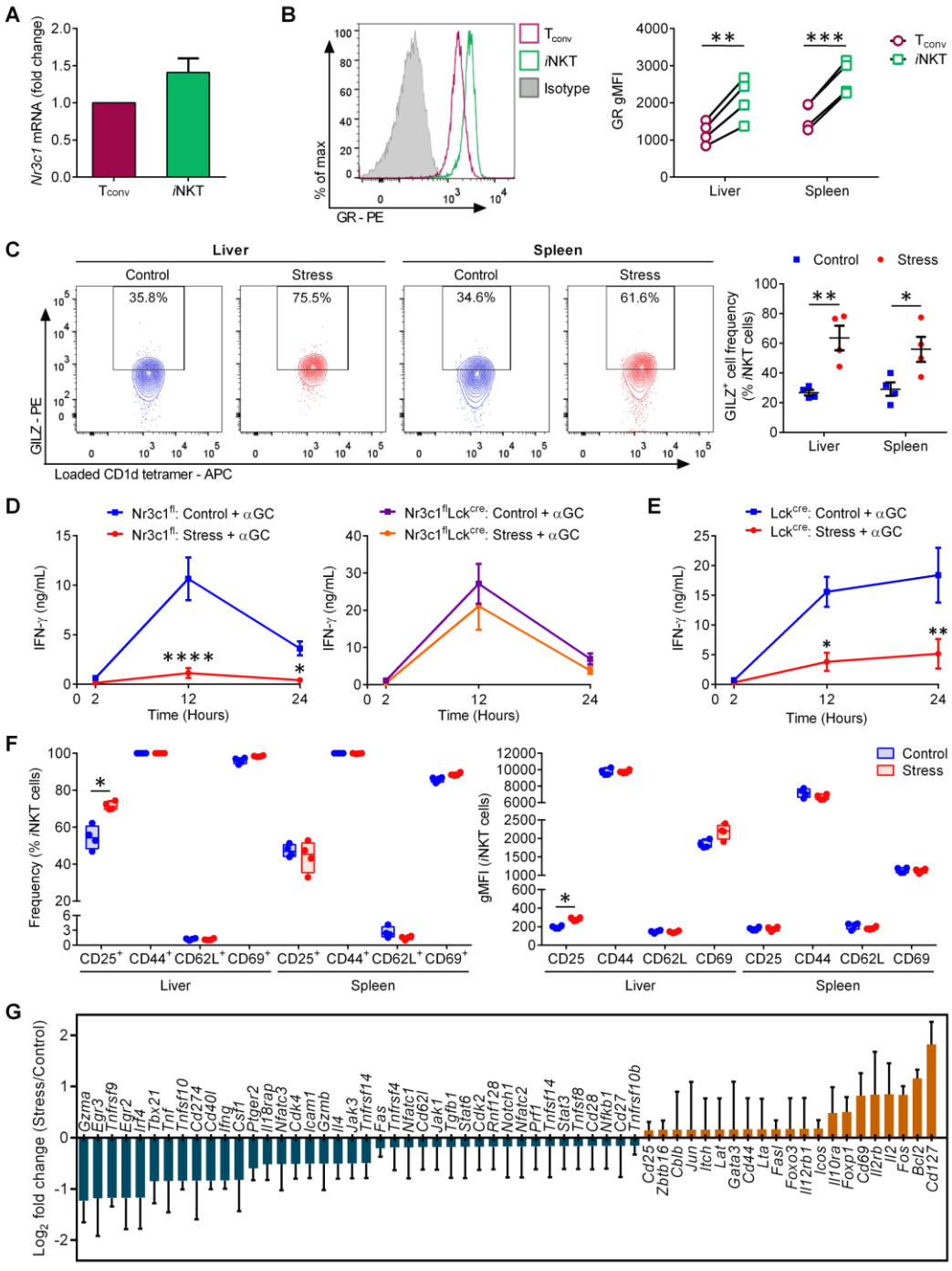

Figure 3.9: Stress impedes *i*NKT cell responses to αGC in a cell-autonomous, GR-dependent manner and creates a transcriptomic signature consistent with extensive *i*NKT cell dysfunctions.

(A) Hepatic *i*NKT and T_{conv} cells from naïve B6 mice (n=10 per experiment) were FACS-purified and the *Nr3c1* mRNA content of *i*NKT cells relative to that of T_{conv} cells

was PCR-quantitated. Fold change values were determined in 3 independent experiments. **(B)** HMNCs and splenocytes were stained with an anti-GR mAb or a mouse IgG2aκ isotype control. Open histograms illustrate the GR positivity of hepatic *i*NKT and T_{conv} cells, and the filled histogram corresponds to the staining of $TCR\beta^+$ cells with the above isotype control. Summary data depict the geometric mean fluorescence intensity (gMFI) of GR staining. **(C)** HMNCs and splenocytes from mice that had been subjected to prolonged restraint or left undisturbed were stained with an anti-GILZ mAb or a rat IgG2aκ isotype control. Representative contour plots depict the frequencies of $GILZ^+$ *i*NKT cells, which are summarized in scatter plots. **(D-E)** $Nr3c1^{fl}$ and $Nr3c1^{fl}Lck^{cre}$ mice (n=6-9) **(D)** and Lck^{cre} mice (n=4-5) **(E)** were restrained or left undisturbed before they were injected with αGC. Serum IFN-γ levels were subsequently quantified by ELISA. **(F)** HMNCs and splenocytes from stressed and control mice were stained with mAbs against CD25, CD44, CD62L and CD69 or with corresponding isotype controls. Box-and-Whisker plots illustrate the frequencies of *i*NKT cells staining positively for each marker. **(G)** Hepatic *i*NKT cells were FACS-sorted from stressed and control B6 mice ($n \geq 5$ per group per experiment). Gene expression fold changes were determined by quantitative PCR in 3 independent experiments. Each symbol in (**B-C**) and (**F**) represents an individual mouse. Error bars in (**A**), (**C-E**) and (**G**) represent SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by paired (**B**) or unpaired (**C** and **F**) Student's *t*-tests or by two-way ANOVA with Sidak's correction (**D-E**).

To assess the duration for which the inhibitory effects of stress on *i*NKT cell responses last, we injected αGC one day or one week after the mice were removed from prolonged restraint. In doing so, we found that *i*NKT cell responses to αGC remained impaired for at least one day post-stress (Figure 3.10A) but were restored to control levels after one week (Figure 3.10B). Therefore, *i*NKT cells from stressed mice do not exhibit signs of long-term dysfunction.

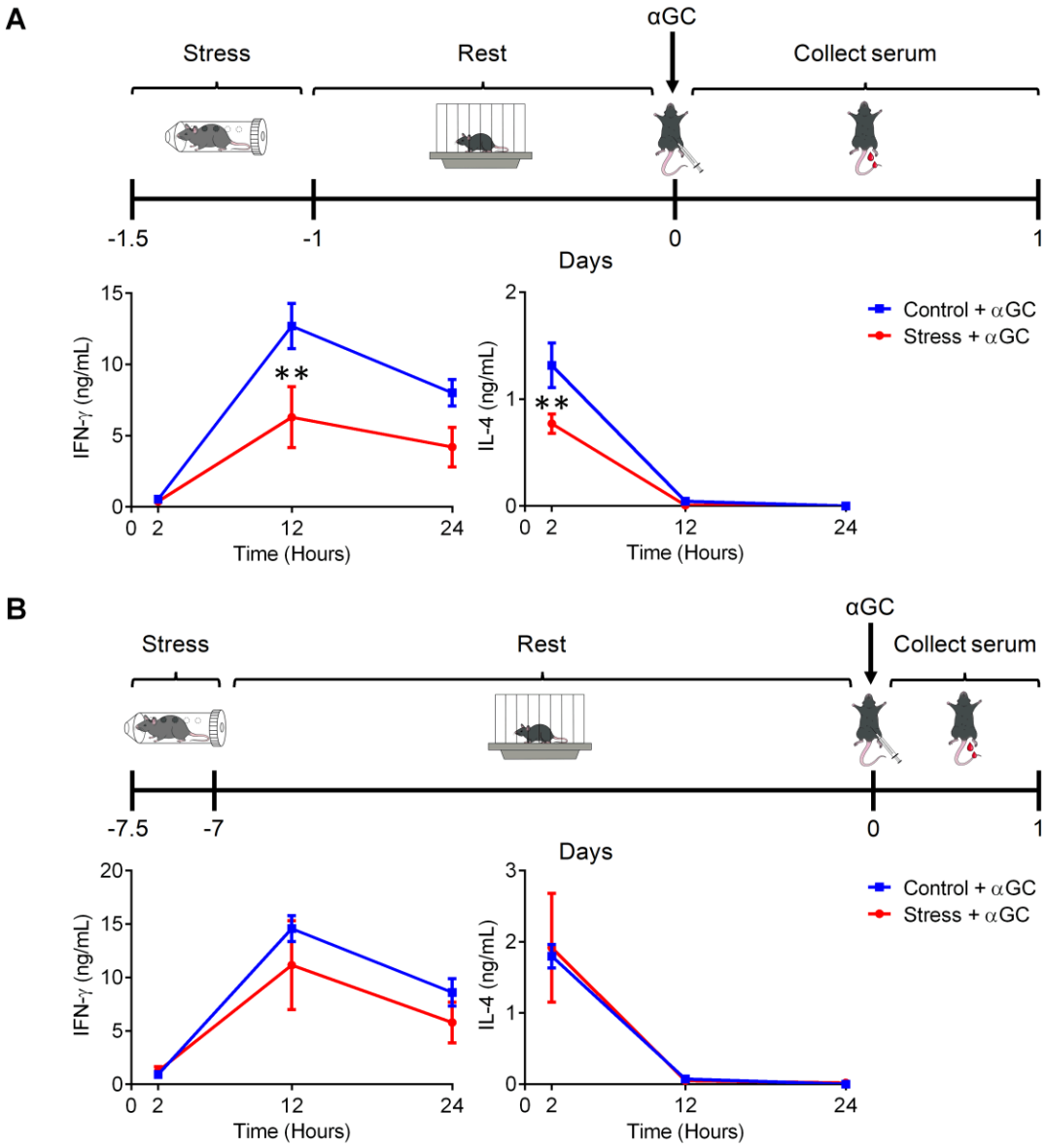

Figure 3.10: Stress-induced iNKT cell impairments do not last long after the stressor is removed.

(A-B) B6 mice were physically restrained or left undisturbed for 12 h. Mice were then returned to standard housing conditions for one day **(A)** or seven days **(B)** before they were injected i.p. with αGC. Serum IFN-γ and IL-4 levels were measured at indicated time points (n=4/group). Error bars represent SEM. ** denotes differences with $p<0.01$ using two-way ANOVA with Sidak's post-hoc analysis.

3.3.4 Long-term exposure to multiple unpredictable stressors, but not to the same stressor, hinders *i*NKT cell responses

Chronic stress can be due to long-term exposure to different stressful events or elements, resulting in sustained glucocorticoid release in the absence of organismal 'habituation'. This can be simulated by the chronic variable stress (CVS) model whereby mice are subjected to heterotypic psychological and/or physical stressors, once daily and once nightly, for 21 days (Figure 3.11A). Given the importance of GR signaling in restraint-induced *i*NKT cell impairments, we hypothesized that CVS should impede *in vivo* cytokine production in response to αGC. We first confirmed elevated blood CS levels at the conclusion of the CVS experiment (Figure 3.11B), consistent with previous reports that CVS continuously activates the HPA axis (55). In addition, hepatic and splenic *i*NKT cells maintained their absolute numbers in mice that had been subjected to CVS (Figure 3.11C). Finally and as hypothesized, animals that were injected with αGC after CVS had lower levels of IL-4, IFN-γ and IL-2 (Figure 3.11D). In these experiments, parallel cohorts of mice were repeatedly exposed to the same stressor, physical restraint, for 21 consecutive days before αGC treatment. In this repeated restraint stress (RRS) model, animals predict the stressor and habituate and adapt to it by gradually de-escalating glucocorticoid release (77). Indeed, αGC-induced cytokine production remained impeccable in mice that had undergone RRS (Figure 3.11D), a sharp contrast with the CVS model.

The above results extend our findings to another clinically relevant rodent model of chronic stress (*i.e.*, CVS). Moreover, they further enforce our conclusion that the activation of the HPA axis mediates stress-induced *i*NKT cell dysfunctions.

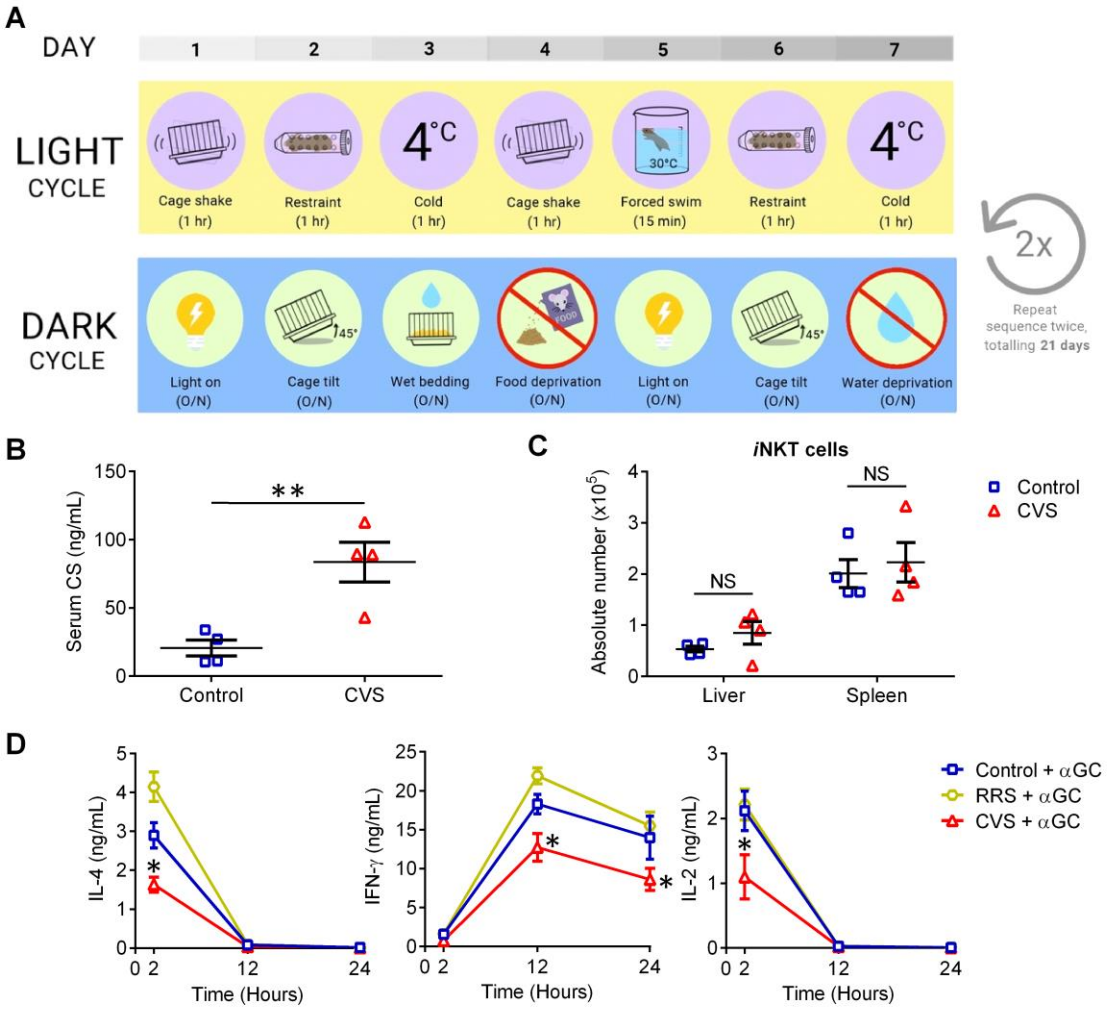

Figure 3.11: Prolonged exposure to unpredictable stressors impairs cytokine production in response to αGC.

(A) B6 mice were subjected to 21 days of chronic variable stress (CVS) involving three weekly cycles of exposure to indicated heterotypic stressors. Control mice remained undisturbed in home cages with food and water supplied *ad libitum*. At the conclusion of the third cycle, serum CS concentrations were measured by ELISA **(B)**, and hepatic and splenic *i*NKT cells were enumerated **(C)**. **(D)** In additional experiments, parallel cohorts of mice (n=4/group) were subjected to CVS, or to repeated restraint stress (RRS) involving 1 h of daily physical confinement for 21 consecutive days. Control mice were left undisturbed. At the conclusion of the 21-day period, animals were injected with αGC and assessed for their serum IL-4, IFN-γ and IL-2 levels at indicated time points. Each

symbol in (**B**) and (**C**) represents an individual mouse. Error bars denote SEM. Statistical comparisons were conducted using unpaired Student's *t*-tests (**B**-**C**) or two-way ANOVA with Tukey's correction (**D**). *p<0.05, **p<0.01, NS = not significant.

3.3.5 Glucocorticoid secretion due to prolonged psychological stress interferes with *i*NKT cells' antimetastatic function

*i*NKT cells are known to participate in anticancer immune surveillance, and their glycolipid agonists have been used in clinical trials for multiple malignancies (32, 78). However, whether psychological stress alters the antitumor property of *i*NKT cells has not been addressed.

We first assessed the oncolytic capacity of splenocytes from mice that had been subjected to prolonged restraint before they received an αGC injection. Effector cells isolated from stressed mice were less competent in killing YAC-1 lymphoma cells *in vitro* (Figure 3.12A). We then used an *in vivo* killing assay with which to test the ability of αGC-transactivated NK cells and pre-mNK cells to destroy B6.β2M$^{-/-}$ (β2M$^{-/-}$) splenocytes as we previously described (30). Consistent with our *in vitro* results, β2M$^{-/-}$ target cells were only poorly cleared in previously restrained animals (Figure 3.12B).

Next, we examined whether and how stress interferes with the ability of αGC-primed *i*NKT cells to prevent metastatic cancer *in vivo*. To this end, mice were restrained, or not, before receiving a single intraperitoneal (i.p.) injection of αGC or vehicle, followed shortly afterwards by an intravenous (i.v.) inoculum of B16-F10 melanoma cells. Visual enumeration of pulmonary metastases revealed a complete loss of αGC-mediated protection in stressed mice (Figure 3.12C). This finding was validated by bioluminescence whole body imaging of tumor-bearing B6 albino mice that showed far greater B16-F10-Red-FLuc (B16-FLuc) tumor burden in their lungs if they were previously restrained (Figure 3.12D). We then asked whether the suppressive effect of stress required GR signaling. RU486 treatment before stress reinstated the efficacy of αGC therapy against B16-F10 metastasis (Figure 3.12E). Therefore, through a GR-dependent pathway, prolonged psychological stress abolishes the capacity of *i*NKT cells to orchestrate a fruitful antimetastatic response.

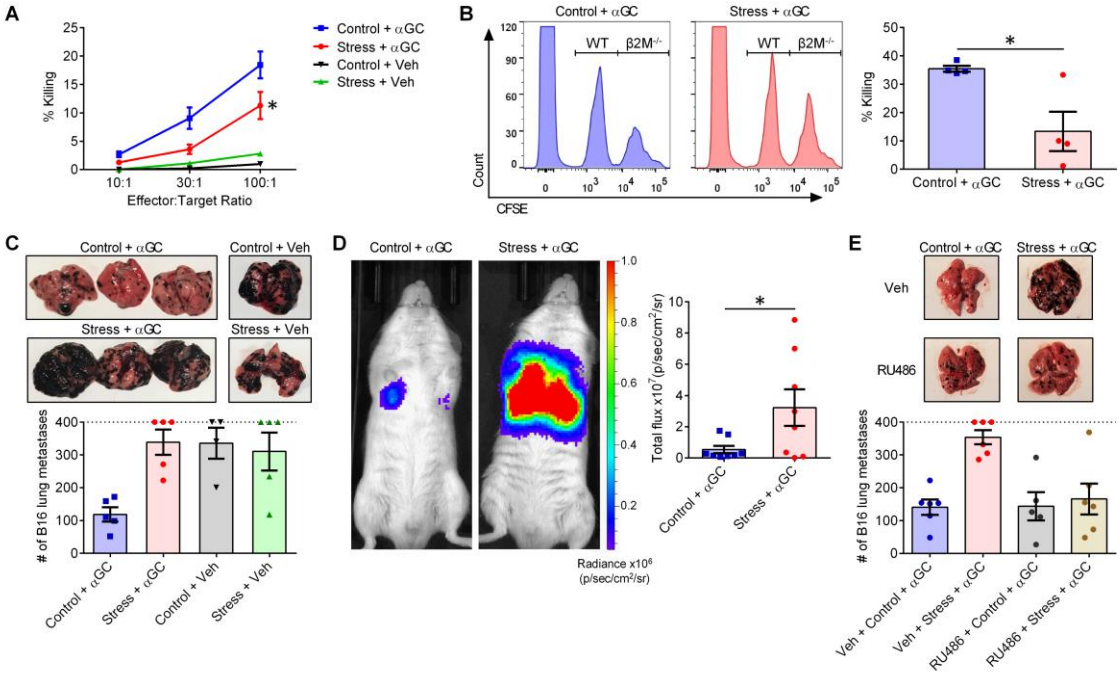

Figure 3.12: GR signaling during stress compromises the ability of *i*NKT cells to trigger oncolytic and antimetastatic responses.

B6 mice were restrained or left undisturbed before they were given αGC (n=6-7) or Veh (n=2). **(A)** Twenty-four hours later, mice were euthanized, and their splenocytes were employed at indicated effector:target ratios against ^{51}Cr-labeled YAC-1 lymphoma cells. Percent specific cytotoxicity was calculated using the formula described in Methods. **(B)** Twenty-four hours after αGC administration, previously stressed and control mice were injected i.v. with a 1:1 mixture of CFSE^{lo} WT B6 and CFSE^{hi} β2M$^{-/-}$ B6 splenocytes. After 2 h, the relative proportion of each population was determined by flow cytometry, and percent cytotoxicity against NK-susceptible β2M$^{-/-}$ target cells was calculated as described in Methods. **(C)** In separate experiments, 6 h after αGC (or Veh) administration, mice were injected i.v. with B16-F10 cells. Lungs were photographed 14 days later for representative images, and metastatic lung nodules were visually counted as a measure of tumor burden. Mice bearing too many nodules to count (>400) are conservatively represented on the dotted line. **(D)** In similar experiments, B6 albino mice received B16-FLuc cells. Twenty-one days later, they received an i.p. injection of luciferin and subjected to whole body bioluminescence imaging. **(E)** In additional

experiments, B6 mice were used as in (**C**) except they were injected i.p. with RU486 (or Veh) 1 h before they were restrained (or not). Each symbol in (**B-E**) represents an individual mouse. Error bars denote SEM. * denotes a significant difference with $p<0.05$ by two-way ANOVA with Tukey's correction (**A**) or by unpaired Student's *t*-tests (**B** and **D**).

3.3.6 MAIT cells are resistant to glucocorticoid-induced apoptosis but fail to mount optimal cognate responses under psychological stress

MAIT cells are MR1-restricted *i*T cells with powerful effector and regulatory functions. They have been dubbed human "cousins" of mouse *i*NKT cells due to the developmental, phenotypic and functional characteristics they share (79, 80). MAIT cells are scarce in conventional strains of laboratory mice (52) but frequent in the human peripheral blood, mucosal layers, liver and lungs (35-37). However, how stress affects MAIT cell frequencies and functions has not been explored before.

We first asked whether similar to mouse *i*NKT cells, human *i*T cells survive exposure to glucocorticoids. We incubated human peripheral blood mononuclear cells (PBMCs) (Figure 3.13A) or HMNCs (Figure 3.13B) for 24 h with hydrocortisone (HC), the main stress-induced glucocorticoid produced by humans, or with dexamethasone (DEX), a commonly prescribed synthetic glucocorticoid. Exposure to either HC or DEX elevated the intracellular active caspase levels of peripheral blood T_{conv} cells (Figure 3.13A). By contrast, matched blood *i*NKT and MAIT cells contained very low and stable levels of active caspases. A similar pattern was evident among hepatic MAIT cells (Figure 3.13B). Moreover, in two liver samples in which *i*NKT cells were detectable, there was no evidence of increased caspase activity after incubation with HC or DEX (data not shown).

Similar to mouse *i*NKT and T_{conv} cells from stressed animals, which upregulate CD127 via a cell-autonomous, GR-dependent manner, to respond to the pro-survival cytokine IL-7 (Figure 3.14A-E), human MAIT, *i*NKT and T_{conv} cells increased their surface CD127 levels upon exposure to glucocorticoids (Figure 3.14F). These GR-mediated effects likely serve as a defense mechanism at the cellular level.

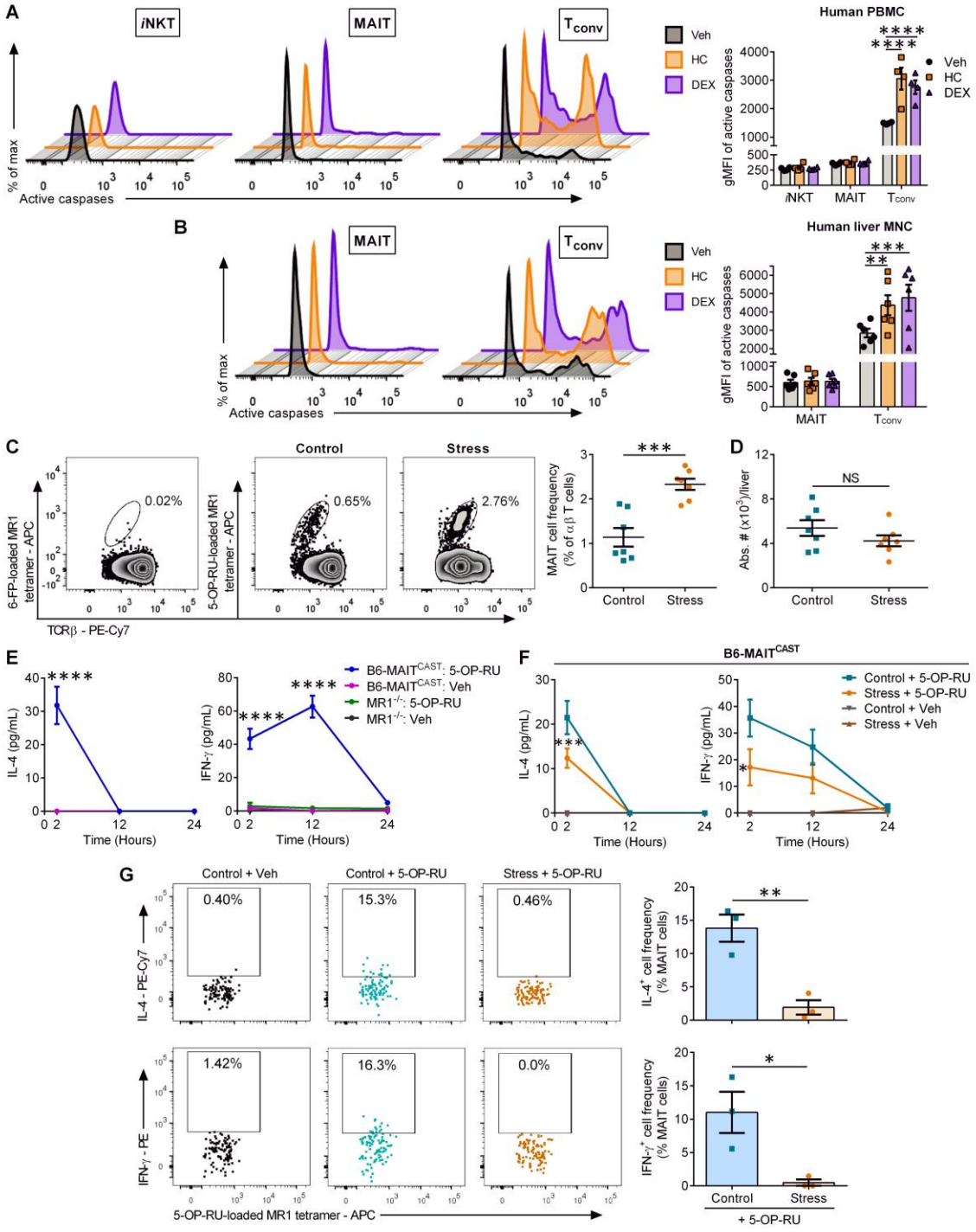

Figure 3.13: Stress and glucocorticoids fail to program MAIT cells for apoptosis but render them hyporesponsive to 5-OP-RU.

Human PBMCs **(A)** and HMNCs **(B)** were exposed to hydrocortisone (HC), dexamethasone (DEX), or Veh for 24 h before they were analyzed by flow cytometry to

detect intracellular active caspases in indicated T cell subsets. gMFI values are summarized in bar graphs. **(C)** Immediately after prolonged restraint stress (or not), HMNCs from B6-MAIT^{CAST} mice were stained with 6-FP- (staining control) or 5-OP-RU-loaded MR1 tetramers along with anti-TCRβ and -B220 mAbs. Representative zebra plots illustrate MAIT cell populations after gating on TCRβ^{+}B220^{-} events, and scatter plots summarize MAIT cell frequencies among total αβ T cells. **(D)** The absolute numbers of hepatic MAIT cells were also calculated. **(E)** B6-MAIT^{CAST} and MR1$^{-/-}$ mice (n≥3) were injected i.p. with 5-OP-RU or Veh, and serum IL-4 and IFN-γ levels were quantified at indicated time points. **(F)** B6-MAIT^{CAST} mice were restrained (or left undisturbed), injected with 5-OP-RU (n=8) or Veh (n=3-4), and bled at indicated time points to quantify serum IL-4 and IFN-γ levels. **(G)** Stressed and control B6-MAIT^{CAST} mice were injected with 5-OP-RU (or Veh) 30 minutes before they were sacrificed for their liver. Hepatic MAIT cells were identified by MR1 tetramers, and IL-4^{+} and IFN-γ^{+} MAIT cell frequencies were determined via staining with anti-cytokine mAbs or isotype controls. Representative dot plots and summary data are depicted. Each symbol in (**A**-**D**) and (**G**) represents an individual sample or mouse. Error bars represent SEM. *, **, *** and **** denote differences with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, by two-way ANOVA with Dunnett's (**A** and **B**) or Tukey's (**E** and **F**) correction or by unpaired Student's *t*-tests (**C**-**D** and **G**). NS = not significant

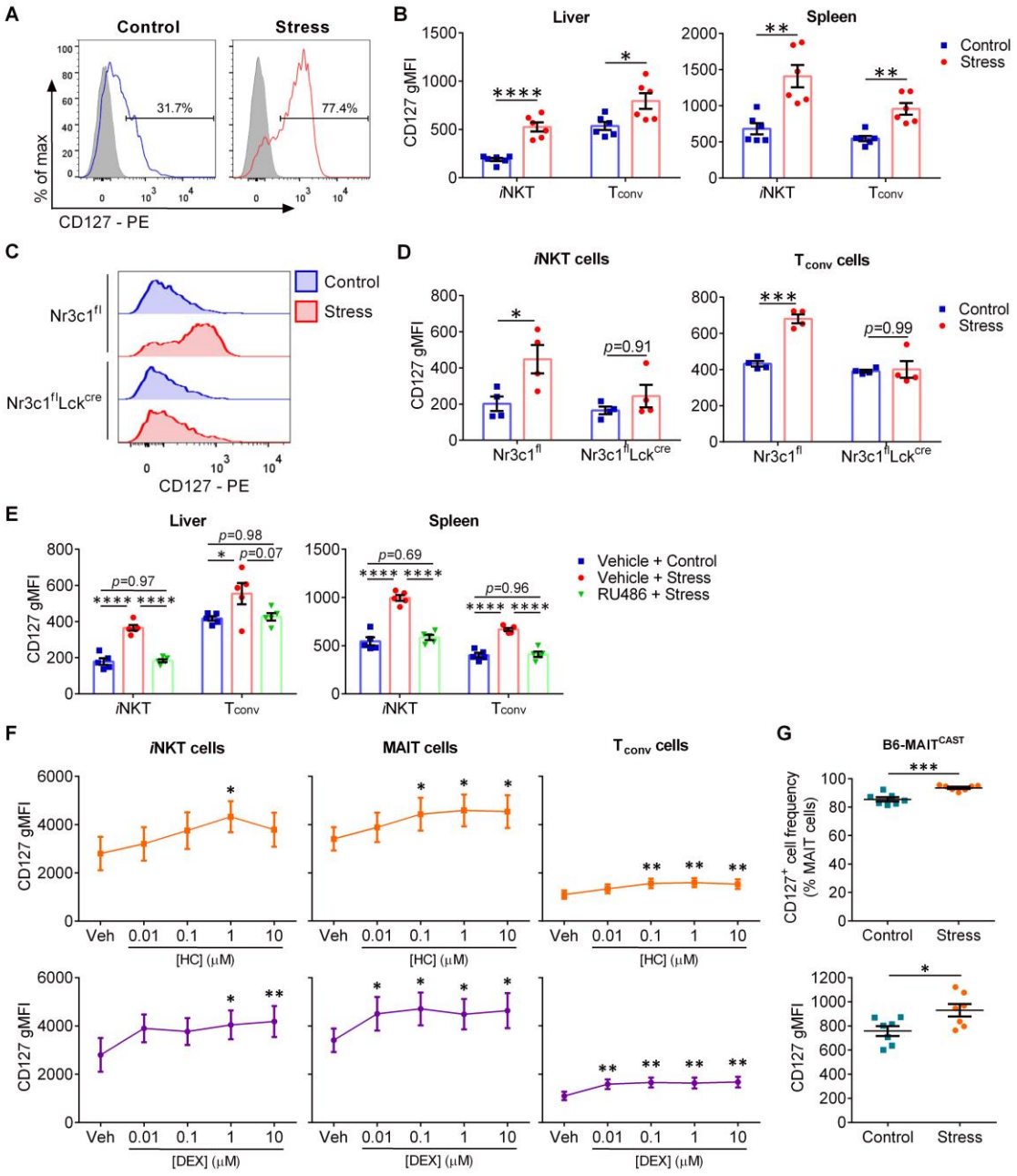

Figure 3.14: Mouse and human *i*NKT, MAIT and T_{conv} cells upregulate CD127 in response to GR signaling.

B6 mice were subjected to 12 h of restraint stress (or not). Shortly afterwards, HMNCs and splenocytes were prepared and stained to detect CD127 expression on the surface of *i*NKT and T_{conv} cells. Open and filled histograms correspond to staining with an anti-CD127 mAb and a rat IgG2aκ isotype control, respectively, after gating on hepatic *i*NKT cells **(A)**. Cumulative data depicting the gMFI of CD127 staining in indicated

populations are also shown **(B)**. $Nr3c1^{fl}$ and $Nr3c1^{fl}Lck^{cre}$ mice were physically restrained (or not) and assessed for CD127 expression in their *i*NKT and T_{conv} cell compartments **(C-D)**. Blue and red histograms correspond to CD127 expression on hepatic *i*NKT cells from control and stressed mice, respectively **(C)**, and cumulative data for indicated cell populations are summarized using bar graphs **(D)**. Separate cohorts of WT B6 mice received RU486 (or Veh) i.p. 1 h before they were physically restrained (or not). The gMFI of CD127 staining is shown **(E)**. Human PBMCs were cultured for 24 h in the presence of hydrocortisone (HC), dexamethasone (DEX) or vehicle before they were stained with either an anti-CD127 mAb or a mouse IgG1κ isotype control. The gMFI of CD127 staining in *i*NKT, MAIT and T_{conv} cells was assessed by flow cytometry (n=5) **(F)**. B6-$MAIT^{CAST}$ mice were physically restrained or left undisturbed for 12 h before they were sacrificed for their liver. HMNCs were stained with a mAb to CD127 or a rat IgG2aκ isotype control. After gating on $TCR\beta^{+}B220^{-}$MR1 $tetramer^{+}$ MAIT cells, the percentages of $CD127^{+}$ cells and the gMFI of CD127 staining were determined **(G)**. Each symbol in (**B**, **D-E** and **G**) represents an individual mouse. Error bars represent SEM. *, **, *** and **** denote differences with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, using unpaired Student's *t*-tests (**B**, **D** and **G**) or one-way ANOVA (**E-F**).

We then investigated whether the resistance of MAIT cells to apoptosis could be recapitulated in a mouse model of psychological stress. *In vivo* studies on MAIT cells can be challenging due to their low frequencies in standard mouse strains. We avoided this limitation by taking advantage of B6-MAIT^{CAST} mice that contain ~20 times more MAIT cells than do WT B6 mice (52). Subjecting these mice to prolonged restraint increased, rather than decreased, hepatic MAIT cell frequencies among αβ T lymphocytes (Figure 3.13C), which was accompanied by enhanced CD127 expression (Figure 3.14G). Furthermore, stress failed to reduce absolute MAIT cell numbers (Figure 3.13D).

To assess the impact of stress on MAIT cell functions, we first demonstrated that a single i.p. injection of 5-OP-RU gives rise to early serum IL-4 and IFN-γ spikes in B6-MAIT^{CAST} mice but not in their MR1$^{-/-}$ B6-MAIT^{CAST} (MR1$^{-/-}$) MAIT-deficient counterparts (Figure 3.13E). This indicated a requirement for MAIT cells in these responses and provided us with a reliable readout to work with. We found B6-MAIT^{CAST} mice that had undergone restraint stress before they received 5-OP-RU to have significantly lower blood levels of IL-4 and IFN-γ in comparison with control animals (Figure 3.13F). Likewise, as quickly as 30 minutes post-5-OP-RU administration, IL-4 and IFN-γ became detectable inside MAIT cells from control mice, but not in stressed animals (Figure 3.13G). Therefore, stress lessens MAIT cell capacities to potentiate T_H1- and T_H2-type responses *in vivo*, which mirrors the observed *i*NKT cell phenotype. The resistance of *i*T cells to stress/glucocorticoid-induced death and their functional impairments are robust and unique to these innate-like T cells.

3.4 Discussion

Although known to link the innate and adaptive arms of immunity (23, 27), *i*T cells have been ignored in the context of stress responses. Revealing how stress affects *i*T cells is important from a basic biological standpoint and also in light of their therapeutic potentials.

We have now demonstrated that stress compromises the ability of *i*NKT cells to trigger T_H1- and T_H2-type responses and to promote antimetastatic immune surveillance. Mechanistically, this hyporesponsive state is dependent upon direct GR signaling in *i*NKT cells, which remain uniquely and remarkably recalcitrant to glucocorticoid-inflicted apoptosis.

Many studies to date have suggested that mediators of stress promote T_H2-type immunity (14, 17, 18). Tamada et al. argued that glucocorticoids selectively retain IL-4-producing NKT cells in the T cell repertoire (81). Here, we demonstrate that psychological stress limits *i*NKT cell-mediated T_H2-type responses, albeit not exclusively. In fact, stress generates a mixed inflammatory signature and also skews *i*NKT cell responses in favor of 'select' anti-inflammatory cytokines, namely IL-10 and IL-27, but not TGF-β. To what extent these immunosuppressive cytokines inhibit T_H1 and/or T_H2 immunity following *i*NKT cell stimulation remains to be determined. We also found that stressed mice exposed to αGC generate greater levels of T_H17-associated cytokines, including IL-23 and, to a lesser degree, IL-17A. Although the exact cellular source(s) of these cytokines will need to be identified, our results point to an adaptation mechanism that may preserve fast-acting T_H17-type responses to a wide range of bacterial and fungal pathogens in the face of a stressful event.

The anticancer function of *i*NKT cells depends, in large part, on their ability to induce DC maturation and to transactivate additional effector cells (32). These functions require the expression of CD40L and IFN-γ, both of which were reduced in *i*NKT cells during stress. Serum concentrations of multiple chemokines (*e.g.*, IP-10, MCP-1 and RANTES) were also diminished in stressed mice receiving αGC, suggesting a weakened *i*NKT cell

capacity in mobilizing other immune cell types towards inflammatory sites and infectious foci.

We found *i*NKT cells from stressed mice to launch weak cytokine responses upon *i*TCR triggering, but to behave normally upon stimulation with PMA and ionomycin. Splenic *i*NKT cells from these animals had diminished *i*TCR, CD28 and ICOS levels on their surface. Further, we found reduced levels of phospho-SLP76 (pY128) and a trend towards lower phospho-CD3ζ (pY142) and phospho-ZAP70 (pY319)/phospho-Syk (pY352) levels, but not phospho-Lck (pY505), in hepatic *i*NKT cells (data not shown). These findings are consistent with a previous report that *in vitro* exposure to DEX lowers phospho-CD3ζ and phospho-ZAP70 levels, but not the kinase activity of Lck, in mouse T cells (82). Deciphering the biochemical bases of the above changes and their apparent tissue-selective patterns warrants further investigation.

The plasticity of *i*NKT cells was evident by virtue of their constitutive mRNA expression of T-bet, AP-1, NF-κB, and NFAT family transcription factors (83). These levels were either reduced or remained stable during stress, with the sole exception of the AP-1 subunit c-Fos. Intriguingly, the transcript levels of c-Jun, c-Fos's binding partner, were not impacted by stress. How the stoichiometric changes of Fos-Jun interactions may affect the activity of AP-1 during stress will also be a subject of future studies.

The SNS is known to control the activity of the HPA axis (84), and we found *i*NKT cells to amply express adrenergic receptors. Adrenergic agonists reportedly suppress *in vivo* NK and T_{conv} cell responses (85-87). Therefore, our finding that *i*NKT cell dysfunctions in prolonged stress stems, exclusively, from GR engagement, and not from post-ganglionic sympathetic neurotransmission, is curious. This is however in agreement with our recent report that OHDA-induced sympathectomy has no effect on glucocorticoid levels during prolonged stress (56). We propose that SNS's dominant role is likely to manifest during a fight-or-flight response to an acute stressor, which may not jeopardize *i*NKT cell functions. In fact, we have found that a brief period (15 minutes) of physical restraint, which results in acute stress (54), fails to alter αGC-elicited cytokine production (data not shown). Consistent with the above theory, plasma NE and epinephrine levels

reach their peak as early as 5-20 minutes after restraint in rats while CS levels peak later, around 60 minutes following physical restraint (88, 89).

In a mouse model of cerebral stroke, Wong et al. demonstrated that NE released by post-ganglionic sympathetic neurons induces IL-10 production by hepatic *i*NKT cells, which results in immunosuppression and secondary bacterial infection (90). However, whether or not SNS mediators, including NE, interfere with *i*TCR-mediated *i*NKT cell activation was not assessed. Nissen and coworkers reported that sustained adrenergic receptor stimulation has only minimal influence on the efficacy of αGC-based cancer immunotherapy (91), and our current work indicates that SNS mediators do not appreciably impact *i*NKT cell responses to glycolipid Ags. Therefore, in the absence or presence of concomitant *i*TCR stimulation, adrenergic receptor signaling may have different outcomes.

*i*NKT cell impairments in stressed animals were accompanied by increased intracellular levels of GILZ, a transcriptional target of the GR (92). GILZ exerts broad anti-inflammatory activities mediated, partially, by protein-protein interactions that inhibit NF-κB and AP-1 (93). It has been speculated that GILZ binds directly to DNA to repress the transcription of several genes that control T cell functions (94). GILZ was recently shown to abolish the efficacy of immunostimulatory therapies against murine tumors, and greater GILZ expression within tumor microenvironments correlated with poorer prognosis in human cancers (5). It is conceivable that GILZ contributes to suppressed *i*NKT cell responses in stressed individuals. As such, selective inhibitors of GILZ may be beneficial.

Consistent with the literature (16, 70), exposure to glucocorticoids contracted the size of several populations, including T_{conv}, B and NK cells. On the contrary, however, *i*NKT and MAIT cells were resistant to glucocorticoid-induced apoptosis. Our results thus unveil a glucocorticoid-induced signaling pathway within *i*T cells that operates in a fundamentally distinctive manner. We noted that *i*NKT cells express relatively high levels of Bcl-2, which becomes even more pronounced during physical restraint. This is reminiscent of a higher Bcl-2 content in NKT cells compared with "$NK1.1^-$ T cells" as

well as Bcl-2 upregulation upon radiotherapy or *in vitro* exposure to DEX as previously described (81, 95). Therefore, Bcl-2 expression may be crucial for *i*NKT cell survival during exposure to multiple apoptogenic stimuli, including but not limited to endogenous glucocorticoids.

GR transcriptionally controls *Cd127* in DEX-treated T cells *in vitro* (96). Here, we found GR to drive CD127 expression in *i*NKT and T_{conv} cells during stress. We also observed CD127 upregulation by hepatic mouse MAIT cells during physical confinement and also among human *i*NKT, MAIT and T_{conv} cells exposed to HC or DEX. CD127 may allow T cells to receive survival signals counteracting the pro-apoptotic properties of endogenous glucocorticoids. Of note, we detected no changes in serum IL-7 levels immediately or shortly after restraint stress (data not shown). Nevertheless, we cannot discount a role for locally produced IL-7 in conventional and unconventional T cell homeostasis and survival in the course of a stress response. IL-7 enhances the effector functions of both *i*NKT and MAIT cells (97, 98). Therefore, its administration may be useful in bolstering *i*T cell functions in stressed subjects.

Unlike T_{conv} cells that are restricted by polymorphic MHC molecules, *i*NKT and MAIT cells recognize cognate Ags complexed with monomorphic molecules, namely CD1d and MR1, respectively (28, 99). Therefore, CD1d and MR1 ligands should work across genetically distinct individuals, which makes *i*NKT and MAIT cells attractive targets in immunotherapeutic interventions for microbial infections and/or malignancies (21, 23). A growing body of evidence implicates psychological stress as an obstacle to cancer immunotherapy (5, 100, 101), and our findings provide a previously unappreciated mechanism underlying stress-induced immunosuppression with wide-ranging repercussions for antitumor immunity. In addition, stress curbed or drastically altered systemic inflammatory responses to αGC and 5-OP-RU, both of which are derived from microbes. It is thus likely that stress also impedes *i*T cell responses to pathogens in natural or therapeutic settings, which will be a subject of future investigations.

3.5 References

1. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol.* 2005;5(3):243-251.

2. Partecke LI, et al. Chronic stress increases experimental pancreatic cancer growth, reduces survival and can be antagonised by beta-adrenergic receptor blockade. *Pancreatology.* 2016;16(3):423-433.

3. Kamiya A, et al. Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat Neurosci.* 2019;22(8):1289-1305.

4. Saul AN, et al. Chronic stress and susceptibility to skin cancer. *J Natl Cancer Inst.* 2005;97(23):1760-1767.

5. Yang H, et al. Stress-glucocorticoid-TSC22D3 axis compromises therapy-induced antitumor immunity. *Nat Med.* 2019;25(9):1428-1441.

6. Kokolus KM, et al. Baseline tumor growth and immune control in laboratory mice are significantly influenced by subthermoneutral housing temperature. *Proc Natl Acad Sci USA.* 2013;110(50):20176-20181.

7. Batty GD, Russ TC, Stamatakis E, Kivimäki M. Psychological distress in relation to site specific cancer mortality: pooling of unpublished data from 16 prospective cohort studies. *Bmj.* 2017;356(j108.

8. Chida Y, Hamer M, Wardle J, Steptoe A. Do stress-related psychosocial factors contribute to cancer incidence and survival? *Nat Clin Pract Oncol.* 2008;5(8):466-475.

9. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev.* 2000;52(4):595-638.

10. Straub RH, et al. Neuropeptide Y cotransmission with norepinephrine in the sympathetic nerve-macrophage interplay. *J Neurochem.* 2000;75(6):2464-2471.

11. Prod'homme T, Weber MS, Steinman L, Zamvil SS. A neuropeptide in immune-mediated inflammation, Y? *Trends Immunol.* 2006;27(4):164-167.

12. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol.* 2017;17(4):233-247.

13. Pruett SB. Stress and the immune system. *Pathophysiology.* 2003;9(3):133-153.

14. Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Ann N Y Acad Sci.* 2006;1069(62-76.

15. Haeryfar SM, Berczi I. The thymus and the acute phase response. *Cell Mol Biol (Noisy-le-grand).* 2001;47(1):145-156.

16. Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci.* 2006;63(1):60-72.

17. Hu D, et al. Essential role of IL-10/STAT3 in chronic stress-induced immune suppression. *Brain Behav Immun.* 2014;36(118-127.

18. Hou N, et al. A novel chronic stress-induced shift in the Th1 to Th2 response promotes colon cancer growth. *Biochem Biophys Res Commun.* 2013;439(4):471-476.

19. Buttari B, et al. Neuropeptide Y induces potent migration of human immature dendritic cells and promotes a Th2 polarization. *FASEB J.* 2014;28(7):3038-3049.

20. Taves MD, Ashwell JD. Glucocorticoids in T cell development, differentiation and function. *Nat Rev Immunol.* 2020.

21. Haeryfar SM, Mallevaey T. Editorial: CD1- and MR1-Restricted T Cells in Antimicrobial Immunity. *Front Immunol.* 2015;6(611.

22. Lisbonne M, et al. In vivo activation of invariant V alpha 14 natural killer T cells by alpha-galactosylceramide sequentially induces Fas-dependent and -independent cytotoxicity. *Eur J Immunol.* 2004;34(5):1381-1388.

23. Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *J Leukoc Biol.* 2018;104(3):473-486.

24. Zhang Y, et al. alpha-GalCer and iNKT Cell-Based Cancer Immunotherapy: Realizing the Therapeutic Potentials. *Front Immunol.* 2019;10(1126.

25. Haeryfar SMM, Shaler CR, Rudak PT. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunol Immunother.* 2018;67(12):1885-1896.

26. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med.* 1994;180(3):1097-1106.

27. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol.* 2008;20(3):358-368.

28. Kawano T, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997;278(5343):1626-1629.

29. Smyth MJ, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* 2002;99(4):1259-1266.

30. Choi J, Rudak PT, Lesage S, Haeryfar SMM. Glycolipid Stimulation of Invariant NKT Cells Expands a Unique Tissue-Resident Population of Precursors to Mature NK Cells Endowed with Oncolytic and Antimetastatic Properties. *J Immunol.* 2019;203(7):1808-1819.

31. Nakagawa R, et al. Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by alpha-galactosylceramide. *J Immunol.* 2004;172(11):6550-6557.

32. Fujii SI, Shimizu K. Immune Networks and Therapeutic Targeting of iNKT Cells in Cancer. *Trends Immunol.* 2019;40(11):984-997.

33. Lepore M, et al. Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. *Nat Commun.* 2014;5(3866.

34. Rahimpour A, et al. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med.* 2015;212(7):1095-1108.

35. Dusseaux M, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood.* 2011;117(4):1250-1259.

36. Tang XZ, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol.* 2013;190(7):3142-3152.

37. Franciszkiewicz K, et al. MHC class I-related molecule, MR1, and mucosal-associated invariant T cells. *Immunol Rev.* 2016;272(1):120-138.

38. Corbett AJ, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* 2014;509(7500):361-365.

39. Kjer-Nielsen L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* 2012;491(7426):717-723.

40. Keller AN, et al. Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. *Nat Immunol.* 2017;18(4):402-411.

41. Kurioka A, et al. Shared and Distinct Phenotypes and Functions of Human CD161++ Valpha7.2+ T Cell Subsets. *Front Immunol.* 2017;8(1031.

42. Kelly J, et al. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol Cell Biol.* 2019;97(8):689-699.

43. Salio M, et al. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J Immunol.* 2017;199(8):2631-2638.

44. Constantinides MG, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science.* 2019;366(6464).

45. Salou M, Lantz O. A TCR-Dependent Tissue Repair Potential of MAIT Cells. *Trends Immunol.* 2019;40(11):975-977.

46. Lamichhane R, et al. TCR- or Cytokine-Activated CD8(+) Mucosal-Associated Invariant T Cells Are Rapid Polyfunctional Effectors That Can Coordinate Immune Responses. *Cell Rep.* 2019;28(12):3061-3076.e3065.

47. Leng T, et al. TCR and Inflammatory Signals Tune Human MAIT Cells to Exert Specific Tissue Repair and Effector Functions. *Cell Rep.* 2019;28(12):3077-3091.e3075.

48. Hinks TSC, et al. Activation and In Vivo Evolution of the MAIT Cell Transcriptome in Mice and Humans Reveals Tissue Repair Functionality. *Cell Rep.* 2019;28(12):3249-3262.e3245.

49. Yao T, Shooshtari P, Haeryfar SMM. Leveraging Public Single-Cell and Bulk Transcriptomic Datasets to Delineate MAIT Cell Roles and Phenotypic Characteristics in Human Malignancies. *Front Immunol.* 2020;11(1691.

50. Tyznik AJ, Tupin E, Nagarajan NA, Her MJ, Benedict CA, Kronenberg M. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. *J Immunol.* 2008;181(7):4452-4456.

51. van Wilgenburg B, et al. MAIT cells are activated during human viral infections. *Nat Commun.* 2016;7(11653.

52. Cui Y, et al. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J Clin Invest.* 2015;125(11):4171-4185.

53. Buynitsky T, Mostofsky DI. Restraint stress in biobehavioral research: Recent developments. *Neurosci Biobehav Rev.* 2009;33(7):1089-1098.

54. Dhabhar FS, Malarkey WB, Neri E, McEwen BS. Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. *Psychoneuroendocrinology.* 2012;37(9):1345-1368.

55. Franco AJ, et al. Sensitization of the Hypothalamic-Pituitary-Adrenal Axis in a Male Rat Chronic Stress Model. *Endocrinology.* 2016;157(6):2346-2355.

56. Rudak PT, et al. Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes. *Brain Behav Immun.* 2019;80(793-804.

57. Crowe NY, et al. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J Immunol.* 2003;171(8):4020-4027.

58. Hayakawa Y, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol.* 2001;31(6):1720-1727.

59. Chatila T, Silverman L, Miller R, Geha R. Mechanisms of T cell activation by the calcium ionophore ionomycin. *J Immunol.* 1989;143(4):1283-1289.

60. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* 2000;164(12):6166-6173.

61. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock.* 2004;22(5):460-466.

62. Flint MS, Tinkle SS. C57BL/6 mice are resistant to acute restraint modulation of cutaneous hypersensitivity. *Toxicol Sci.* 2001;62(2):250-256.

63. Gourdy P, et al. Relevance of sexual dimorphism to regulatory T cells: estradiol promotes IFN-gamma production by invariant natural killer T cells. *Blood.* 2005;105(6):2415-2420.

64. Bernin H, Fehling H, Marggraff C, Tannich E, Lotter H. The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. *Med Microbiol Immunol.* 2016;205(4):321-332.

65. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol.* 2002;3(9):867-874.

66. Fujii S, et al. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci USA.* 2006;103(30):11252-11257.

67. Blumenfeld HJ, Tohn R, Haeryfar SM, Liu Y, Savage PB, Delovitch TL. Structure-guided design of an invariant natural killer T cell agonist for optimum protection from type 1 diabetes in non-obese diabetic mice. *Clin Exp Immunol.* 2011;166(1):121-133.

68. Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med.* 2003;198(11):1631-1641.

69. Velazquez P, Cameron TO, Kinjo Y, Nagarajan N, Kronenberg M, Dustin ML. Cutting edge: activation by innate cytokines or microbial antigens can cause arrest of natural killer T cell patrolling of liver sinusoids. *J Immunol.* 2008;180(4):2024-2028.

70. Tseng RJ, Padgett DA, Dhabhar FS, Engler H, Sheridan JF. Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. *Brain Behav Immun.* 2005;19(2):153-164.

71. Yin D, Tuthill D, Mufson RA, Shi Y. Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J Exp Med.* 2000;191(8):1423-1428.

72. D'Andrea A, et al. Neonatal invariant Valpha24+ NKT lymphocytes are activated memory cells. *Eur J Immunol.* 2000;30(6):1544-1550.

73. Park SH, Benlagha K, Lee D, Balish E, Bendelac A. Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. *Eur J Immunol.* 2000;30(2):620-625.

74. Schober A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res.* 2004;318(1):215-224.

75. Cao L, Filipov NM, Lawrence DA. Sympathetic nervous system plays a major role in acute cold/restraint stress inhibition of host resistance to Listeria monocytogenes. *J Neuroimmunol.* 2002;125(1-2):94-102.

76. Bereshchenko O, Migliorati G, Bruscoli S, Riccardi C. Glucocorticoid-Induced Leucine Zipper: A Novel Anti-inflammatory Molecule. *Front Pharmacol.* 2019;10(308.

77. Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP, Spencer RL. Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience.* 2006;138(4):1067-1081.

78. van den Heuvel MJ, Garg N, Van Kaer L, Haeryfar SM. NKT cell costimulation: experimental progress and therapeutic promise. *Trends Mol Med.* 2011;17(2):65-77.

79. Salou M, et al. A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J Exp Med.* 2019;216(1):133-151.

80. Legoux F, Salou M, Lantz O. Unconventional or Preset αβ T Cells: Evolutionarily Conserved Tissue-Resident T Cells Recognizing Nonpeptidic Ligands. *Annu Rev Cell Dev Biol.* 2017;33(511-535.

81. Tamada K, Harada M, Abe K, Li T, Nomoto K. IL-4-producing NK1.1+ T cells are resistant to glucocorticoid-induced apoptosis: implications for the Th1/Th2 balance. *J Immunol.* 1998;161(3):1239-1247.

82. Van Laethem F, et al. Glucocorticoids attenuate T cell receptor signaling. *J Exp Med.* 2001;193(7):803-814.

83. Das R, Sant'Angelo DB, Nichols KE. Transcriptional control of invariant NKT cell development. *Immunol Rev.* 2010;238(1):195-215.

84. Engeland WC, Arnhold MM. Neural circuitry in the regulation of adrenal corticosterone rhythmicity. *Endocrine.* 2005;28(3):325-332.

85. Bucsek MJ, et al. beta-Adrenergic Signaling in Mice Housed at Standard Temperatures Suppresses an Effector Phenotype in CD8(+) T Cells and Undermines Checkpoint Inhibitor Therapy. *Cancer Res.* 2017;77(20):5639-5651.

86. Diaz-Salazar C, et al. Cell-intrinsic adrenergic signaling controls the adaptive NK cell response to viral infection. *J Exp Med.* 2020;217(4).

87. Wieduwild E, et al. beta2-adrenergic signals downregulate the innate immune response and reduce host resistance to viral infection. *J Exp Med.* 2020;217(4).

88. Kvetnansky R, Sun CL, Lake CR, Thoa N, Torda T, Kopin IJ. Effect of handling and forced immobilization on rat plasma levels of epinephrine, norepinephrine, and dopamine-beta-hydroxylase. *Endocrinology.* 1978;103(5):1868-1874.

89. Kvetnanský R, Fukuhara K, Pacák K, Cizza G, Goldstein DS, Kopin IJ. Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats. *Endocrinology.* 1993;133(3):1411-1419.

90. Wong CH, Jenne CN, Lee WY, Léger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science.* 2011;334(6052):101-105.

91. Nissen MD, Sloan EK, Mattarollo SR. beta-Adrenergic Signaling Impairs Antitumor CD8(+) T-cell Responses to B-cell Lymphoma Immunotherapy. *Cancer Immunol Res.* 2018;6(1):98-109.

92. Monczor F, Chatzopoulou A, Zappia CD, Houtman R, Meijer OC, Fitzsimons CP. A Model of Glucocorticoid Receptor Interaction With Coregulators Predicts Transcriptional Regulation of Target Genes. *Front Pharmacol.* 2019;10(214.

93. Ronchetti S, Migliorati G, Riccardi C. GILZ as a Mediator of the Anti-Inflammatory Effects of Glucocorticoids. *Front Endocrinol (Lausanne).* 2015;6(170.

94. Yosef N, et al. Dynamic regulatory network controlling TH17 cell differentiation. *Nature.* 2013;496(7446):461-468.

95. Yao Z, Liu Y, Jones J, Strober S. Differences in Bcl-2 expression by T-cell subsets alter their balance after in vivo irradiation to favor CD4+Bcl-2hi NKT cells. *Eur J Immunol.* 2009;39(3):763-775.

96. Lee HC, Shibata H, Ogawa S, Maki K, Ikuta K. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *J Immunol.* 2005;174(12):7800-7806.

97. de Lalla C, et al. Innate-like effector differentiation of human invariant NKT cells driven by IL-7. *J Immunol.* 2008;180(7):4415-4424.

98. Leeansyah E, et al. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. *PLoS Pathog.* 2015;11(8):e1005072.

99. Treiner E, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* 2003;422(6928):164-169.

100. Sommershof A, Scheuermann L, Koerner J, Groettrup M. Chronic stress suppresses anti-tumor TCD8+ responses and tumor regression following cancer immunotherapy in a mouse model of melanoma. *Brain Behav Immun.* 2017;65(140-149.

101. Levi B, et al. Continuous stress disrupts immunostimulatory effects of IL-12. *Brain Behav Immun.* 2011;25(4):727-735.

4 Chapter 4: Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes

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4.1 Introduction

Prolonged or chronic physiological stress impedes several important aspects of protective immunity (1). Experimental and clinical observations made in human subjects indicate that extended bouts of stress coincide with poorer outcomes related to infectious diseases (2, 3), antimicrobial vaccines (4, 5), and wound healing (6). Other studies have suggested a link between stress and the incidence or progression of hematological, respiratory system, and human papilloma virus-induced cervical malignancies among others (7-10). The reported connections between stress and impaired immunity have been further substantiated in animal-based models of infectious diseases (11, 12) and cancer (13, 14). However, mechanistic insight into the underlying mediators and potential molecular targets for intervention remains scarce.

Physiological stress culminates in the activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis, which orchestrate “fight-or-flight” adaptation to perceived threats (15). Both systems exert broad immunological effects. Lymphoid organs are densely innervated by catecholaminergic and peptidergic post-ganglionic nerve fibers, and also respond to circulating catecholamines produced by the adrenal medulla (16). The activation of the HPA axis results in adrenocortical release of glucocorticoid hormones into the circulation (15). Glucocorticoids display potent anti-inflammatory properties. Once activated by glucocorticoids, the glucocorticoid receptor (GR) undergoes nuclear translocation from the cytosol and acts as a ligand-activated transcription factor (17). In the nucleus, the GR binds to glucocorticoid response elements (GREs) characterized by two palindromic or semi-palindromic half-sites separated by three nucleotides (*e.g.*, 5’TGTACANNNTGTTCT3’) (18). Subsequent homodimerization allows the GR to enhance anti-inflammatory gene transcription or to repress pro-inflammatory gene transcription, among other anti-inflammatory actions of the GR.

Findings from *in vitro* models of T cell stimulation suggest that glucocorticoids promote the expression of the co-inhibitory molecules programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (19, 20). Importantly, blocking

these “checkpoint” receptors has shown significant promise in the treatment of patients with certain solid and hematological cancers (21). Recently, several additional co-inhibitory receptors have emerged as targets for “next-generation” immune checkpoint blockade (22). These include T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), a member of the CD28 family that interacts with CD155 and CD112 expressed by tumor cells and antigen (Ag)-presenting cells (APCs) among other cell types (23). The inherently inhibitory signal-transducing activities of TIGIT, through its immunoreceptor tyrosine-based inhibitory motif (ITIM) and/or immunoglobulin tail tyrosine (ITT)-like motif (24-27), regulate immune responses. Furthermore, TIGIT can outcompete the costimulatory receptor CD226 for binding to shared ligands (28) and also prevents the activity of CD226 via protein-protein interactions (29). Finally, TIGIT has been demonstrated to activate regulatory T cells (30, 31), and to induce IL-10-producing regulatory dendritic cells (DCs) (32). A role for TIGIT in restricting antiviral and antitumor immunity has been described (29, 33). However, whether altered expression and functions of TIGIT may explain at least certain aspects of stress-induced immunosuppression has been essentially unexplored.

Invariant natural killer T (*i*NKT) and mucosa-associated invariant T (MAIT) cells are innate-like T lymphocytes with remarkable immunomodulatory properties. *i*NKT cells recognize glycolipid antigens, typified by α-galactosylceramide (αGC), presented in the context of the MHC class I-like molecule CD1d (34). Once stimulated, they swiftly generate a burst of T helper-type 1 (T_H1), T_H2, and/or T_H17-type cytokines. MAIT cells are restricted by MHC-related protein 1 (MR1) that presents bacterial and yeast-derived vitamin B metabolites, such as 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (35), among other antigens. Activated MAIT cells release T_H1- and/or T_H17-type cytokines, and exhibit cytotoxic effector functions (36). Both *i*NKT and MAIT cells can also respond to inflammatory cytokine signals, such as those emanating from IL-12 and IL-18 receptors, even in the absence of cognate ligands (37, 38). They are thought to promote antimicrobial and antitumor immunity directly and/or through transactivation of other effector cell types such as NK cells and conventional αβ T cells (T_{conv}) cells (39-43).

Stress is known to modify T_{conv} cell responses (44, 45). However, to what extent and how *i*NKT cell and MAIT cell effector and regulatory functions can be influenced by stress is unclear. This is a particularly important question given the rapidity with which these unconventional T cells participate in immune responses. In this investigation, we have addressed this question in two mouse models of sustained physiological stress, namely prolonged psychological stress caused by physical restraint and chronic stress due to exposure to variable psychological and/or physical stressors. Here, we demonstrate a swift and selective rise in the expression of TIGIT on *i*NKT and MAIT cells during stress. Our mechanistic studies implicate GR signaling, in an invariant T cell-autonomous fashion, in TIGIT upregulation. The potential immunological consequences of these findings will be discussed.

4.2 Materials and Methods

4.2.1 Ethics statement

Mouse experiments were conducted in compliance with Animal Use Protocols #2010-241 and #2018-130 approved by the Animal Care Committee of Animal Care and Veterinary Services at Western University and following guidelines set by the Canadian Council on Animal Care.

4.2.2 Mice

Wild-type (WT) C57BL/6 (B6) mice between 8 and 16 weeks of age were purchased from Charles River Canada Inc. (Saint-Constant, QC, Canada). B6.$GR^{fl/fl}Lck^{Cre/wt}$ mice ($GR^{fl}Lck^{Cre}$ for short) were generated by mating a B6.$GR^{fl/fl}$ mouse with *loxP* sites flanking exon 3 of *Nr3c1* (46) (Jackson Laboratory, Bar Harbor, ME) to a B6.$Lck^{Cre/Cre}$ mouse, also from Jackson Laboratory. In the latter strain, Cre recombinase is expressed under the control of the proximal *Lck* promoter during thymic education of T cells (46, 47). This was followed by subsequent backcrossing of the heterozygous offspring with $GR^{fl/fl}$ mice. Polymerase chain reaction (PCR)-based genotyping of DNA extracted from ear tissue was conducted throughout the breeding process.

B6.$MAIT^{CAST}$ mice, which harbor MAIT cells in greater abundance compared with WT B6 mice (48), were generously provided by Dr. Olivier Lantz (Institut Curie, Paris, France) and bred in-house. All mice were housed in an institutional barrier facility with daily light/dark cycles. Closely age- and sex-matched mice were used in our experiments.

4.2.3 Physical restraint model of prolonged stress

Mice were subjected to physical restraint by being held horizontally in 50-mL conical tubes with ventilating holes for a single 12-hour period (49). In this procedure, mice are not compressed, do not experience pain, and can move slightly forward and backward. Prolonged restraint is a well-established model for inducing psychological stress in rodents, and is known to elevate circulating levels of catecholamines and gluocorticoids (50). Cage-mates of restrained mice remained in home cages without food and water and

served as non-stressed controls. Immediately after the restraint period, mice were sacrificed by cervical dislocation.

4.2.4 Chronic variable stress (CVS)

Mice were exposed to two distinct stressors, psychological and/or physical, each day over a 21-day period as outlined in Table 4.1. Daytime stressors included horizontal cage shaking at 80 rpm for 1 hour, restraint for 1 hour, cage placement at 4°C for 1 hour, and forced swimming in 30°C water for 15 minutes. Overnight stressors were exposure to light, cage tilting at 45°, wet bedding (using 200 mL of water poured onto cage bedding), food deprivation, and water deprivation. Using this model, mice do not habituate to stressful environments and have been described to exhibit hyperactive stress responses (51). Non-stressed control mice remained undisturbed in their home cages throughout the CVS period. Stressed and control mice were sacrificed by cervical dislocation at the conclusion of the experiments.

Table 4.1: Outline of the chronic variable stress (CVS) protocol used in the study highlighted in Chapter 4.

Days	Stressor (light cycle)	Overnight stressor (dark cycle)
1, 8, 15	Cage shake (1 hr)	Light on
2, 9, 16	Restraint (1 hr)	Cage tilt (45°)
3, 10, 17	4°C (1 hr)	Wet bedding
4, 11, 18	Cage shake (1 hr)	Food deprivation
5, 12, 19	Forced swim at 30°C (15 min)	Light on
6, 13, 20	Restraint (1 hr)	Cage tilt (45°)
7, 14, 21	4°C (1 hr)	Water deprivation

4.2.5 Chemical reagents and treatment protocols

To inhibit the action of endogenous glucocorticoids *in vivo*, mice were injected intraperitoneally (*i.p.*) with 25 mg/kg of the GR antagonist RU486 (Sigma-Aldrich), or with vehicle [dimethyl sulfoxide in phosphate-buffered saline (PBS)] 1 hour prior to restraint. To induce chemical sympathectomy, 6-hydroxidopamine (6-OHDA) (Sigma-Aldrich) was administered to mice at 200 mg/kg *i.p.* six days prior to restraint. Control mice received a vehicle containing 10^{-7} M ascorbic acid in PBS. Successful sympathectomy through 6-OHDA treatment was confirmed by Western blotting to detect tyrosine hydroxylase levels in the mouse spleen (52). To administer exogenous corticosterone (CS) orally, the powder from Sigma-Aldrich was dissolved in absolute ethanol, diluted 1:100 in standard drinking water to a final concentration of 25 μg/mL, and provided to mice for 21 days. Control mice received 1% ethanol in their drinking water. Freshly prepared solutions were supplied on a weekly basis. This regimen has been previously described to yield circulating CS levels similar to those found in mice experiencing chronic stress (53). For *in vitro* experiments, CS was dissolved at 1 mg/mL in ethanol, diluted in RPMI 1640 medium, and stored at -20°C until use.

4.2.6 Measurement of serum corticosterone levels

Serum was immediately collected from the whole blood and stored at -20°C until use. CS concentrations were measured using the DetectX Corticosterone Enzyme Immunoassay kit (Arbor Assays, Ann Arbor, Michigan, USA) according to manufacturer's instructions.

4.2.7 Cell lines and primary cell cultures

N38.3C3, a $CD4^+$ *i*NKT cell hybridoma that was initially created by fusing thymic *i*NKT cells with $\alpha^-\beta^-$ BW5147 thymoma cells (54), was provided by Dr. Kyoko Hayakawa (Fox Chase Cancer Center, Philadelphia, PA). DN32.D3, a $CD4^-CD8^-$ *i*NKT cell hybridoma (55), was obtained from Dr. Albert Bendelac (University of Chicago, Chicago, IL). Both lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM GlutaMAX-I, and 0.1 mM MEM nonessential amino acids at 37°C in a humidified incubator containing 6% CO_2. 17E6, a MAIT cell hybridoma that was generated through fusion of lymph node MAIT cells with BW5147 thymoma cells

(56), was provided by Dr. Olivier Lantz (Institut Curie). This hybridoma was maintained at 37°C in RPMI 1640 medium containing 10% FBS, 2 mM GlutaMAX-I, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES, which will hereafter be referred to as complete medium.

To isolate splenocytes, spleens were aseptically removed and mechanically homogenized in a Wheaton Dounce tissue grinder. To eliminate erythrocytes, cell pellets were exposed to ACK lysis buffer for 3 minutes at room temperature. To prepare non-parenchymal liver mononuclear cells (MNCs), livers were homogenized, and parenchymal cells were removed by density gradient centrifugation at 700 × *g* in a solution containing 33.75% Percoll PLUS (GE Healthcare). Cell pellets were then treated with ACK lysis buffer.

*i*NKT/MAIT hybridoma cells or bulk liver MNCs were seeded at 1×10^5 cells per well of a 96-well U-bottom polystyrene microplate and exposed to indicated concentrations of CS, or vehicle, in complete medium for 12 hours.

4.2.8 Cell staining for cytofluorimetric analyses

Single cell preparations were incubated on ice with 5 μg/mL of an anti-mouse CD16/CD32 mAb (clone 2.4G2) to prevent non-specific Fc receptor-mediated binding of mAbs. Cell surface staining with mAbs and/or tetramer reagents was performed for 30 minutes at 4°C in PBS containing 2% FBS. Fluorochrome-conjugated mAbs against TCRβ (clone H57-597), B220 (RA3-6B2), NK1.1 (PK136), 2B4 (eBio244F4), B and T lymphocyte attenuator (BTLA) (6F7), CD96 (6A6), CD160 (eBioCNX46-3), CTLA-4 (UC10-4B9), lymphocyte activation gene 3 (LAG-3) (eBioC9B7W), leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) (113), PD-1 (RMP1-30), TIGIT (GIGD7), T cell immunoglobulin and mucin-3 (TIM-3) (8B.2C12), and V-domain immunoglobulin suppressor of T cell activation (VISTA) (MIH64) were purchased from Thermo Scientific. Fluorochrome-conjugated mAbs against glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein (GITR) (DTA-1) were purchased from BioLegend (San Diego, CA).

CD1d and MR1 tetramers were supplied by the National Institutes of Health Tetramer Core Facility (Atlanta, GA), and employed to identify *i*NKT and MAIT cells, respectively (57-60). Primary *i*NKT cells were defined as $TCR\beta^+$PBS-57-loaded mouse CD1d $tetramer^+$ cells, and empty CD1d tetramers were used in parallel as a staining control. MAIT cells were identified as $B220^-TCR\beta^+$5-OP-RU-loaded mouse MR1 $tetramer^+$ cells. We also stained for $TCR\beta^+tetramer^-$ T_{conv} cells and $TCR\beta^-NK1.1^+$ NK cells in indicated experiments. To assess the expression of various co-inhibitory molecules, proper gates were first set based on negative staining with appropriate isotype controls. We also used Fixable Viability Dye eFluor 780 (Thermo Scientific), as per manufacturer's instructions, to exclude dead cells. Stained cells were interrogated using a BD FACSCanto II flow cytometer, and data analysis was conducted using FlowJo software Version 10 (Tree Star, Ashland, OR).

4.2.9 Quantitative PCR

Fresh hepatic *i*NKT cells, ~100% pure, from ≥ 5 stressed or control mice were isolated using a BD FACSAria III Cell Sorter. RNA from each population was extracted using the Invitrogen PureLink RNA Mini Kit with on-column DNase treatment (Thermo Scientific), and cDNA was generated using the Invitrogen SuperScript VILO cDNA Synthesis Kit (Thermo Scientific). cDNA from transcripts of interest were amplified and detected using a StepOne Plus Real-Time PCR instrument (Applied Biosystems) and TaqMan-based fluorescent probe/primer sets (Thermo Scientific) targeting mRNA for BTLA (Mm00616981_m1), CTLA-4 (Mm00486849_m1), GITR (Mm00437136_m1), killer cell lectin-like receptor subfamily G member 1 (KLRG1) (Mm00516879_m1), LAG-3 (Mm00493071_m1), PD-1 (Mm00435532_m1), TIGIT (Mm03807522_m1), and TIM-3 (Mm00454540_m1). Cycle threshold (Ct) values were normalized to those of the housekeeping genes TBP (Mm00446973_m1) and β-actin (Mm00607939_s1) to generate ΔCt values. Differences in mRNA levels of *i*NKT cells from stressed and control mice were then calculated using the $2^{-(\Delta\Delta Ct)}$ method.

4.2.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism Version 6.0 software (La Jolla, CA) and Student's *t*-tests or one- or two-way ANOVA as appropriate. *, **, *** and **** denote statistical differences with $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

4.3 Results

4.3.1 Prolonged psychological stress raises the expression of TIGIT in *i*NKT cells

Despite numerous studies reporting that physiological stress can be detrimental to immunological competence (2, 11, 14), much remains to be learned regarding molecular targets that can be potentially exploited to overcome stress-induced immunosuppression. Furthermore, whether stress impairs unconventional innate-like T cells, such as *i*NKT cells, is not understood. To explore whether and how stress may change the expression of co-inhibitory receptors by *i*NKT cells, we determined mRNA transcript levels of several such receptors in hepatic *i*NKT cells isolated from mice that had been subjected to physical restraint and in control animals. Liver was chosen for our initial screening studies since it accommodates many *i*NKT cells in mice (61). We found upregulated expression of TIGIT, by ~8-fold, at the mRNA level in stressed animals in comparison with controls (Figure 4.1). This effect appeared to be selective for TIGIT since there were no detectable increases in the transcript levels of six other immune checkpoint molecules, namely BTLA (denoted by its gene name, *Btla*), CTLA-4 (*Ctla4*), GITR (*Tnfrsf18*), KLRG1 (*Klrg1*), LAG-3 (*Lag3*), PD-1 (*Pdcd1*) and TIM-3 (*Havcr2*). We next asked whether TIGIT expression by *i*NKT cells was also similarly augmented at the protein level. Indeed, our cytofluorimetric analyses revealed that both hepatic (Figure 4.2A-B) and splenic (Figure 4.2C) *i*NKT cells from stressed mice display significantly elevated levels of TIGIT on their surface. This was reflected by increases in both the frequencies of $TIGIT^+$ *i*NKT cells and the expression levels of TIGIT on a per-cell basis as judged by the mean fluorescence intensity (MFI) of staining for this molecule (Figure 4.2B-C). Therefore, prolonged psychological stress distinctively upregulates the expression of the immune checkpoint receptor TIGIT in *i*NKT cells.

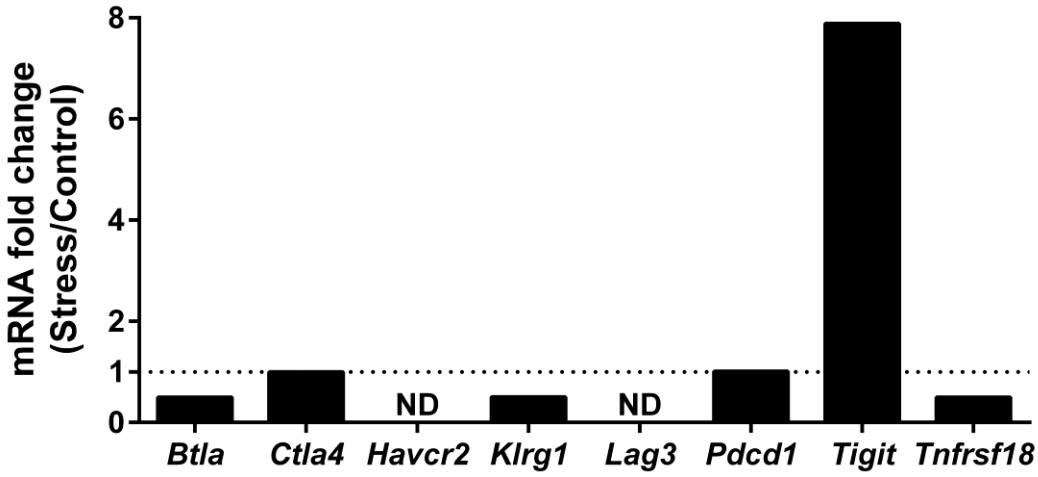

Figure 4.1: Hepatic *i*NKT cells from stressed mice contain elevated TIGIT mRNA levels.

Male B6 mice (n=5) were subjected to physical restraint for 12 hours. Cage-mate control mice (n=10) remained in home cages without food and water for 12 hours. Mice were euthanized, and non-parenchymal hepatic MNCs were isolated and pooled. TCRβ^+CD1d tetramer$^+$ *i*NKT cells were sorted followed by mRNA isolation and conversion to cDNA. Taqman-based primer/probe sets were used to amplify indicated gene products. After 40 cycles of PCR, ΔCt values were determined using TATA-binding protein and β-actin as reference genes. Differences in mRNA levels were then calculated using the $2^{-(\Delta\Delta Ct)}$ method. The dotted line represents a scenario in which transcript levels are identical between samples from stressed and control animals.

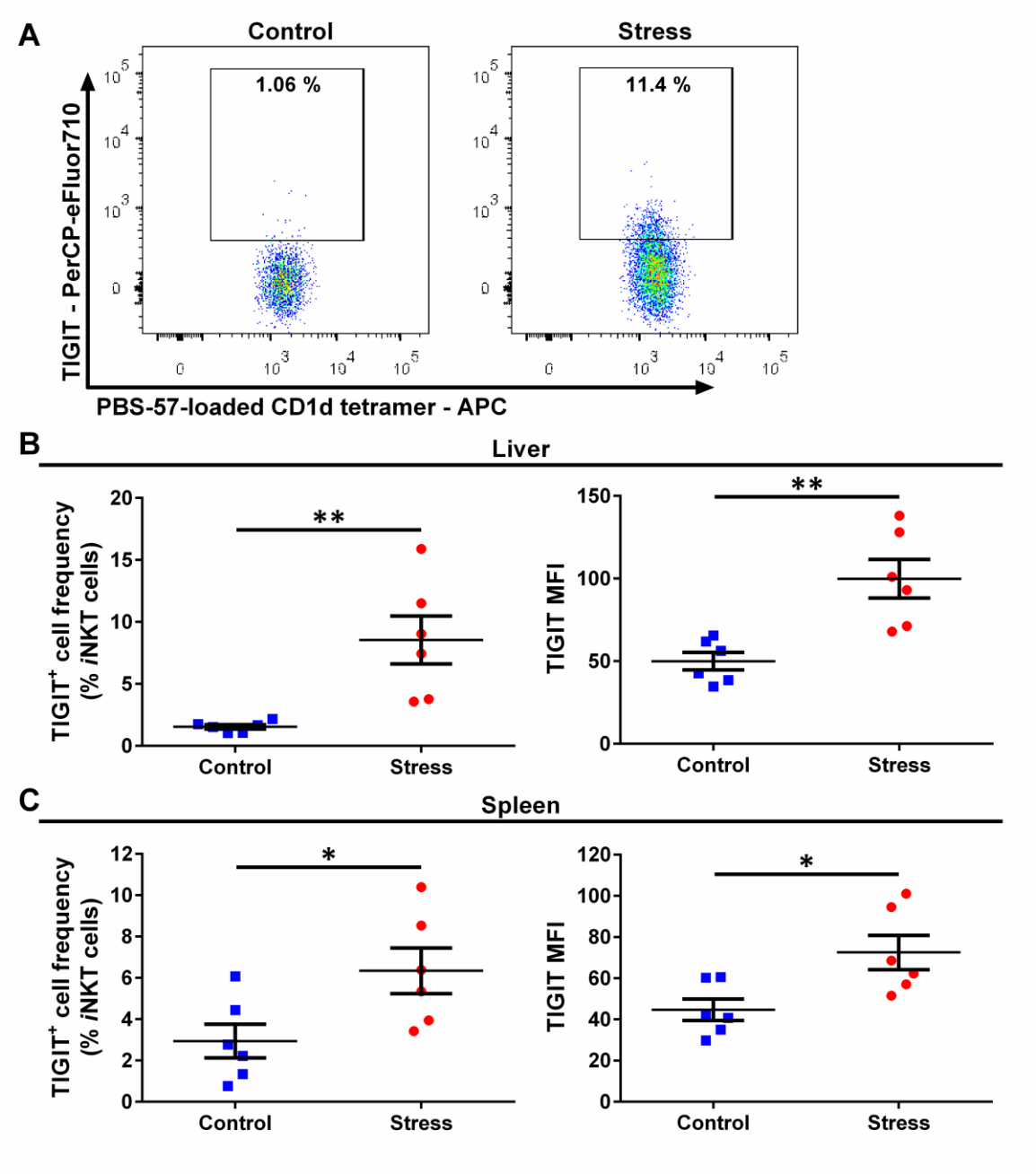

Figure 4.2: Physical restraint results in elevated TIGIT expression on the surface of hepatic and splenic *i*NKT cells.

Following 12 hours of physical confinement, liver MNCs from stressed and control B6 mice were stained with an anti-mouse TIGIT mAb or with a rat IgG2a isotype control. Representative cytofluorimetric plots illustrate TIGIT expression by hepatic CD1d tetramer$^+$ *i*NKT cells after initial gating on TCRβ$^+$ events **(A)**. The percentages of TIGIT$^+$ cells (left panels) and the geometric mean fluorescence intensity (MFI) of TIGIT

staining (right panels) are depicted for both hepatic **(B)** and splenic **(C)** *i*NKT cells. Each symbol represents an individual mouse. Mean ± SEM values are shown. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively, using unpaired Student's *t*-tests.

In a more comprehensive analysis, we examined the expression of several co-inhibitory receptors on *i*NKT cells by flow cytometry (Figure 4.3). These included cell surface molecules whose expression at the mRNA level was evaluated in our initial experiment (Figure 4.1) in addition to several other checkpoint receptors. We observed no stress-induced increases in the frequencies of *i*NKT cells that stained positively for BTLA, CD96, CD160, CTLA-4, GITR, LAG-3, LAIR-1, PD-1, TIM-3 or VISTA (Figure 4.3). We found a relatively modest but statistically significant increase (~2-fold) in the percentage of $2B4^+$ cells among hepatic, but not splenic, *i*NKT cells (Figure 4.3), which was not nearly as pronounced as the observed changes in TIGIT expression (Figure 4.2B-C). These results reinforce our conclusion that psychological stress selectively provokes the upregulation TIGIT on *i*NKT cells as opposed to globally impacting the expression of various co-inhibitory receptors.

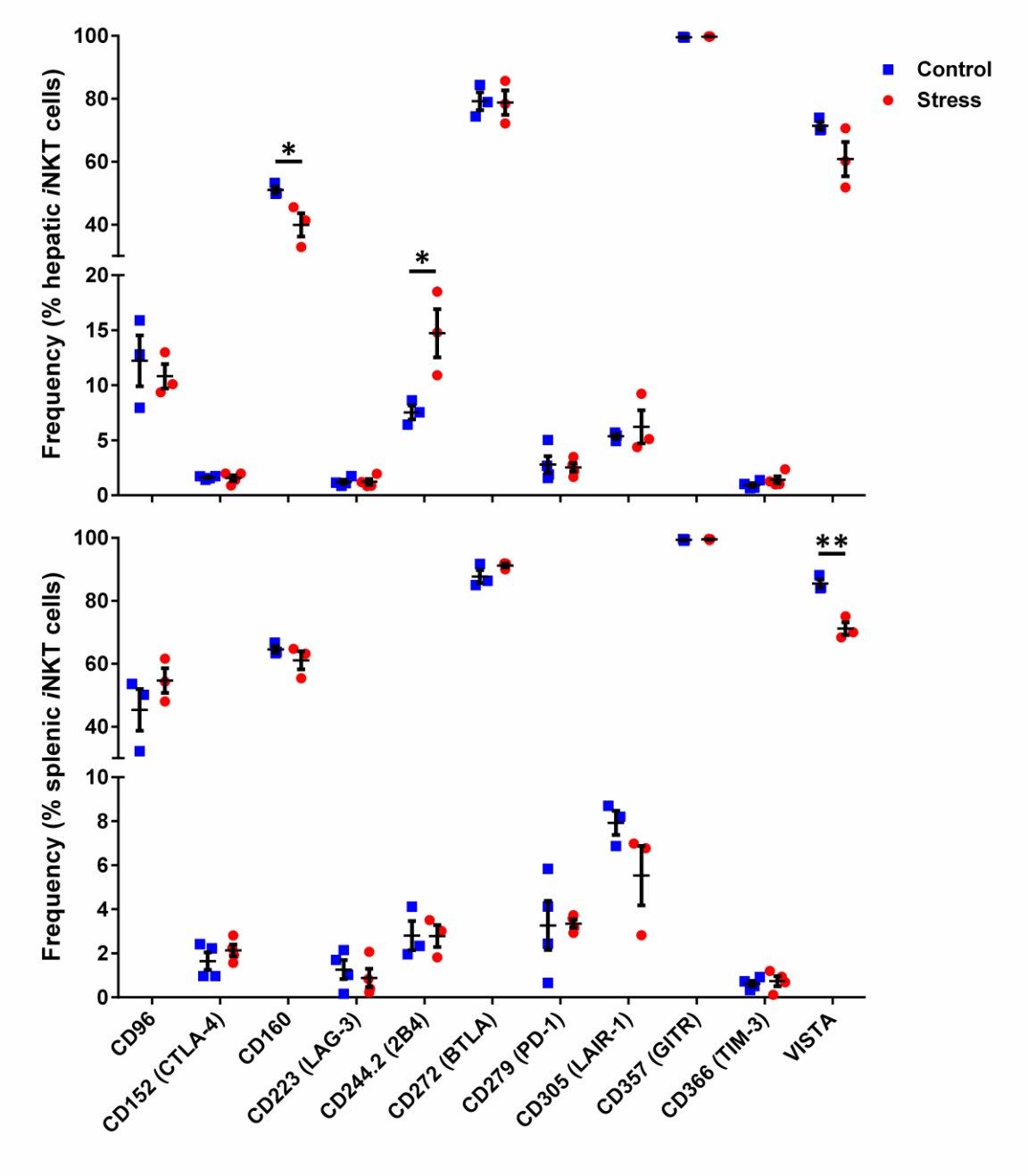

Figure 4.3: Prolonged physical confinement does not globally increase the expression of immune checkpoint molecules by *i*NKT cells.

Following 12 hours of restraint stress, hepatic MNCs from stressed and control B6 mice (n=3-4) were stained with mouse CD1d tetramer and anti-mouse mAbs against TCRβ, 2B4, BTLA, CD96, CD160, CTLA-4, GITR, LAG-3, LAIR-1, PD-1, TIM-3, and VISTA, or with respective isotype control reagents. The percentages of hepatic (upper panel) and splenic (lower panel) *i*NKT cells staining positively for each coinhibitory

molecule are summarized. Each symbol represents an individual mouse, and mean ± SEM values are shown. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively, using unpaired Student's *t*-tests.

4.3.2 Endogenous glucocorticoids are responsible for stress-induced TIGIT upregulation in *i*NKT cells

Stress responses are marked by the activation of both the SNS and the HPA axis (50). Therefore, we sought to ascertain whether the observed TIGIT upregulation in *i*NKT cells was caused by the activation of one or both of these powerful physiological systems. To this end, we injected mice with 6-OHDA, which causes long-term destruction of post-ganglionic sympathetic nerve termini (62), or with RU486, a strong GR antagonist (11). Mice were then subjected to physical restraint before they were sacrificed for their liver in which the frequency of $TIGIT^+$ *i*NKT cells was determined. Pre-treatment with 6-OHDA had no impact on TIGIT expression (Figure 4.4A). In sharp contrast, systemic administration of RU486 prevented TIGIT upregulation on *i*NKT cells (Figure 4.4B). To rule out the remote possibility that 6-OHDA administration may have fortuitously altered the activity of the HPA axis, we quantified the serum levels of corticosterone (CS), the main glucocorticoid hormone in mice, in 6-OHDA-treated animals. The results illustrated in Figure 4.5 confirm that circulating CS concentrations in stressed mice were comparable between OHDA- and vehicle-treated mice, strongly suggesting that endogenous glucocorticoids are solely responsible for TIGIT upregulation in the *i*NKT cell compartment.

Given the ubiquitous nature of GR expression, it was of interest to explore whether the observed phenomenon was *i*NKT cell-intrinsic or -extrinsic. We employed a Cre/*lox*-based gene targeting system in which the GR is specifically deleted in all T cell subsets to address the above question. In sharp contrast with *i*NKT cells from stressed Cre-negative GR^{fl} mice that were used as positive controls, *i*NKT cells from restrained $GR^{fl}Lck^{Cre}$ mice failed to upregulate TIGIT (Figure 4.4C-D). Taken together, these findings indicate that restraint stress results in upregulation of TIGIT on *i*NKT cells via direct GR signaling in a predominantly, if not entirely, *i*NKT cell-autonomous fashion. In addition, signals emanating from sympathetic nerve endings do not contribute to TIGIT upregulation by *i*NKT cells.

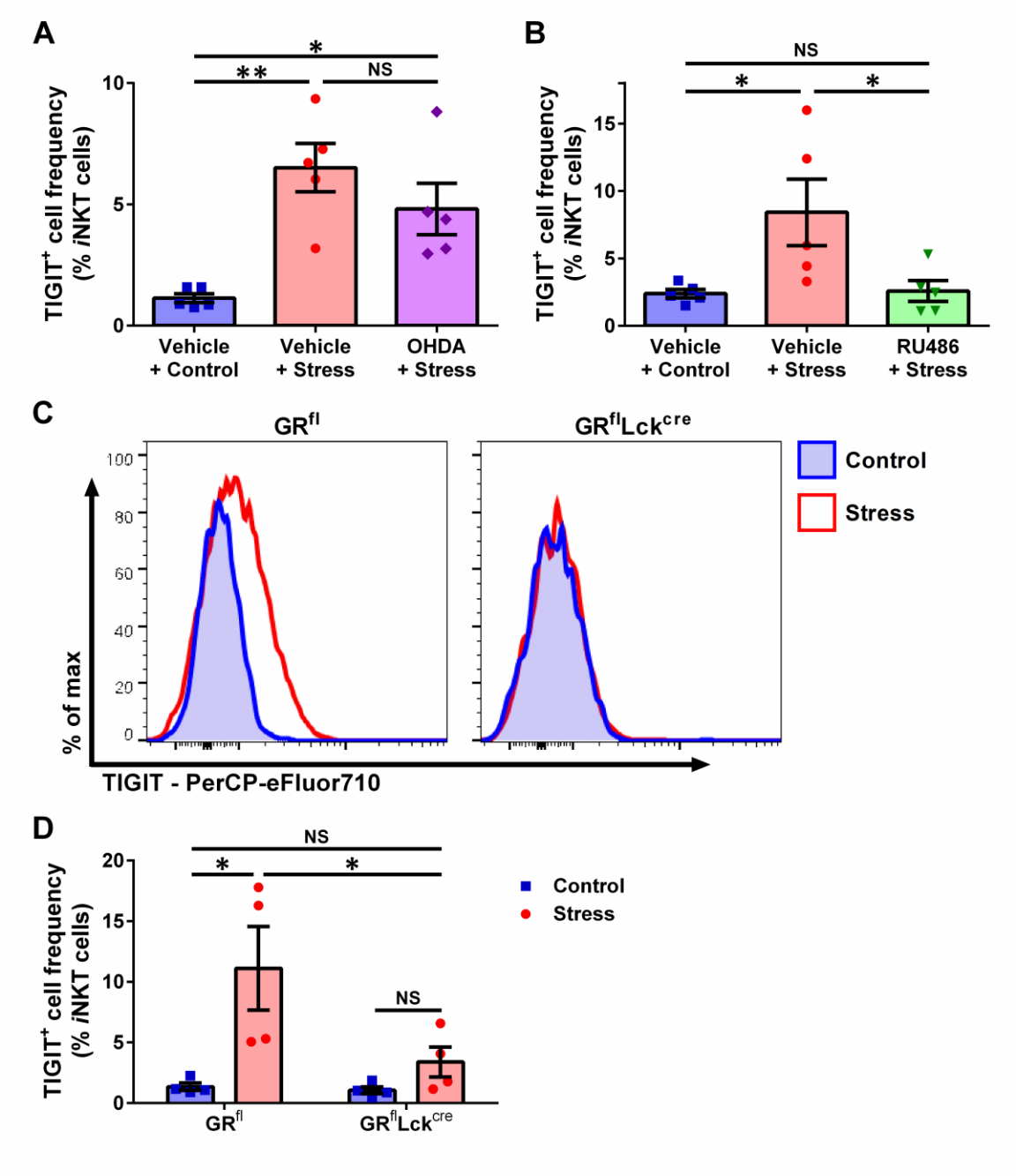

Figure 4.4: Stress-induced TIGIT upregulation on *i*NKT cells is mediated by endogenous GC signaling, but not by SNS mediators, and occurs in an *i*NKT cell-intrinsic manner.

Male B6 mice received a single *i.p.* injection of 6-OHDA to induce chemical sympathectomy, or vehicle, six days before they were subjected to 12 hours of restraint stress **(A)**. Separate cohorts of mice received the GR antagonist RU486, or vehicle, *i.p.* one hour before they were physically restrained **(B)**. Mice were sacrificed for their liver,

and the frequencies of TIGIT$^+$ *i*NKT cells were determined by flow cytometry among liver MNCs. Cohorts of GR^{fl} and GR^{fl}Lck^{Cre} mice were or were not subjected to 12 hours of physical restraint. Liver MNCs were subsequently isolated and stained with anti-TCRβ, CD1d tetramer and anti-TIGIT for cytofluorimetric analysis of TIGIT expression. Open and filled histograms correspond to TIGIT expression in stressed and control animals, respectively, after gating on TCRβ$^+$CD1d tetramer$^+$ cells **(C)**. Summary data are also depicted **(D)**. Each symbol represents an individual mouse, and error bars represent SEM. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively, by one-way ANOVA **(A-B)** or two-way ANOVA **(D)**. NS = not significant

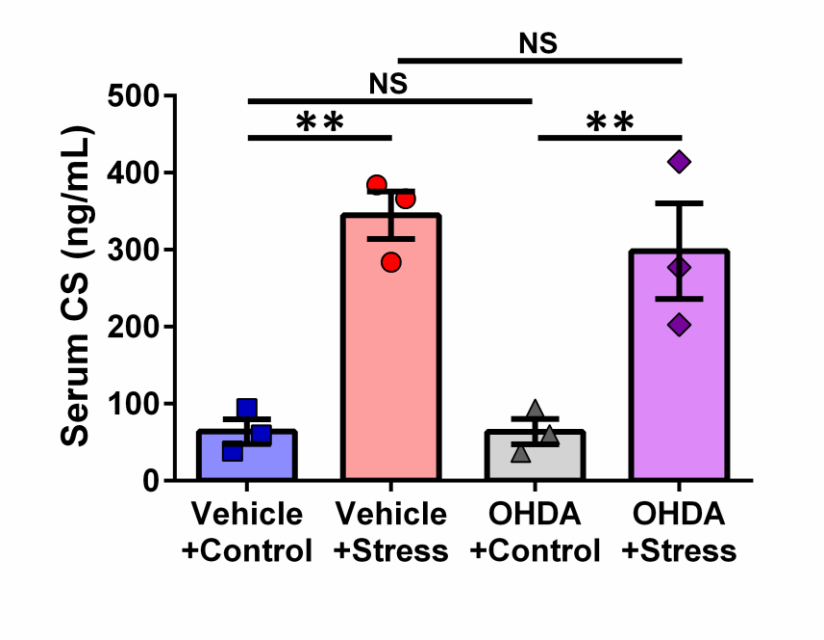

Figure 4.5: Chemical sympathectomy does not alter circulating CS levels in stressed and control mice.

Male B6 mice (n=3/group) were given an *i.p.* injection of 6-OHDA or vehicle six days before they were subjected to 12 hours of restraint stress. Parallel cohorts of mice receiving 6-OHDA or vehicle (n=3/group) were left undisturbed. Immediately after restraint, mice were sacrificed, and serum CS concentrations were quantitated by ELISA. Error bars represent SEM. ** denotes a statistical difference with $p \leq 0.01$ by one-way ANOVA. NS = not significant

4.3.3 Treatment with exogenous CS augments the expression of TIGIT by *i*NKT cells

To more directly link glucocorticoids to TIGIT expression, we first treated mice for 21 days with a standard dose of CS that was added to their drinking water (53, 63, 64). Consistent with the previous literature, this regimen raised the circulating levels of CS (Figure 4.6A, left panel). As predicted, *i*NKT cells from CS-treated mice exhibited significantly increased levels of TIGIT on their surface (Figure 4.6A, right panel). Therefore, the effect of physical restraint on TIGIT expression could be phenocopied by oral CS administration.

Next, we exposed bulk non-parenchymal hepatic MNCs, which are known to contain a large *i*NKT cell fraction (61), to several concentrations of CS in an *in vitro* setting. The dose range of CS was chosen based on the available literature and also on our own quantitation of this glucocorticoid in the serum of restrained mice, thus mimicking our *in vivo* model. To be exact, the average serum CS level in control mice (n=3) was 63.87 (±16.11) ng/mL, which approximates 0.18 (±0.05) μM. In contrast, physical restraint led to a >5-fold rise in CS levels (344.83 ± 30.93 ng/mL or 1.00 ± 0.09 μM; n=3) (Figure 4.6.5). We found *in vitro* exposure of hepatic MNCs to increasing doses of CS to gradually enhance the surface expression of TIGIT, but not LAG-3, on *i*NKT cells, which reached its plateau at the 0.1-μM dose (Figure 4.6B).

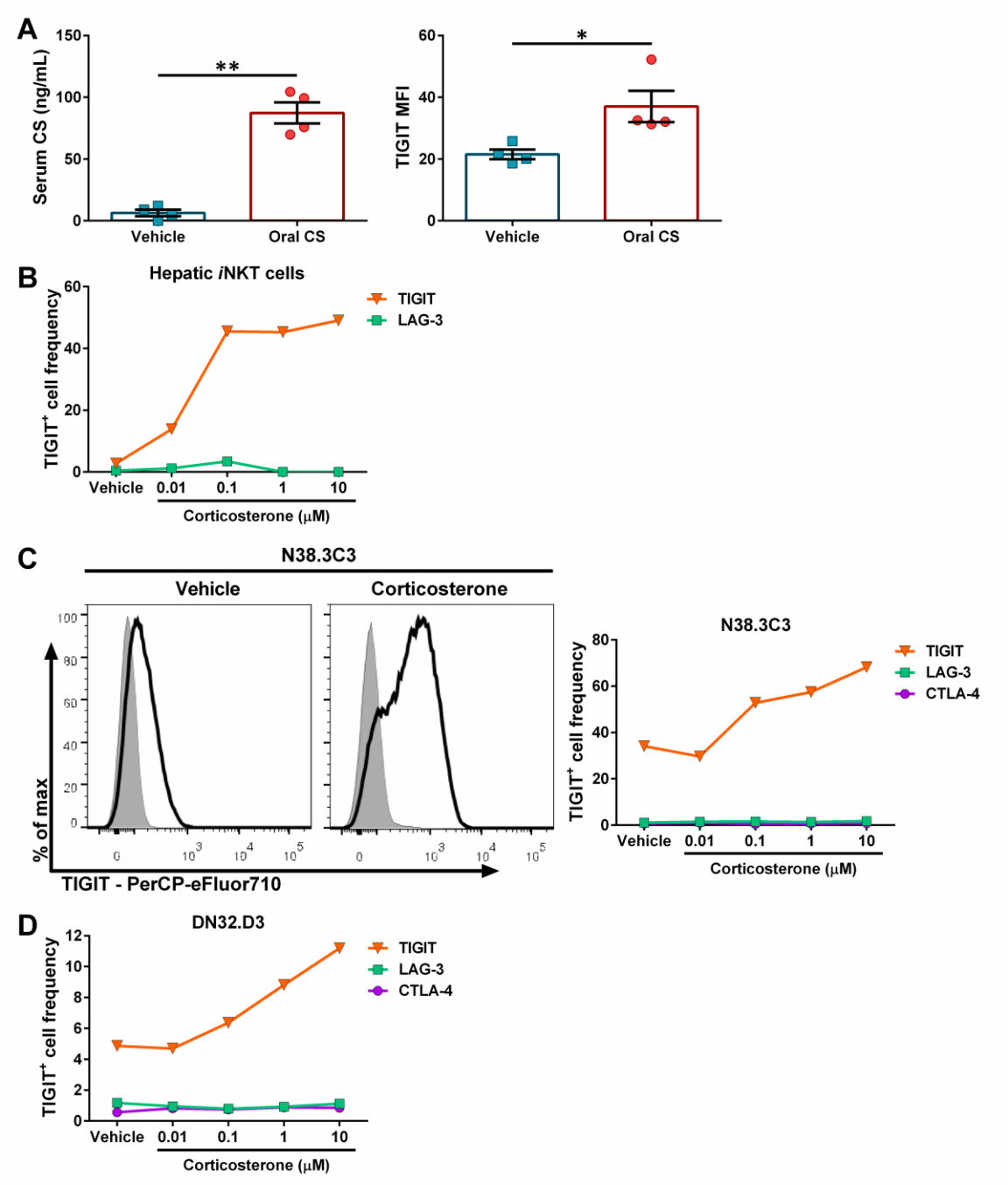

Figure 4.6: *In vivo* or *in vitro* treatment with corticosterone (CS) elevates the surface expression of TIGIT on *i*NKT cells.

Male B6 mice received drinking water containing 25 μg/mL of CS or 1% ethanol for 21 days. They were then euthanized followed immediately by isolation of serum and non-parenchymal liver MNCs. Serum CS concentrations were measured by ELISA **(A, left panel)**. In parallel, the geometric MFI of TIGIT staining among hepatic TCRβ$^+$CD1d

tetramer$^+$ cells was determined by flow cytometry **(A, right panel)**. One hundred thousand primary hepatic MNCs from naïve B6 mice were treated for 12 hours with indicated doses of CS, or with vehicle, before they were stained with an anti-TCRβ mAb and CD1d tetramer along with an anti-TIGIT mAb or an anti-LAG-3 mAb (or appropriate isotype controls). The frequencies of TIGIT$^+$ and LAG-3$^+$ events among TCRβ$^+$CD1d tetramer$^+$ cells were then determined by flow cytometry **(B)**. N38.3C3 **(C)** and DN32.D3 hybridoma *i*NKT cells **(D)** were similarly cultured in the presence or absence of CS followed by staining for TIGIT, LAG-3 and CTLA-4. Open and filled representative histograms **(C)** correspond to the staining of N38.3C3 cells with an anti-mouse TIGIT mAb or a rat IgG2a isotype control, respectively, upon treatment with 1 μM CS (right panel) or vehicle (left panel). The frequencies of hybridoma cells expressing TIGIT, LAG-3 or CTLA-4 are demonstrated **(C-D)**. Each symbol in **A** represents an individual mouse, and error bars represent SEM. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively (by unpaired Student's *t*-tests). Data in **B-D** are representative of 2 independent experiments yielding similar results.

T_{conv} cells are present among bulk hepatic MNCs used in the above experiments. Furthermore, our *in vivo* Cre/*lox* system does not rule out the possibility, however remote, that TIGIT upregulation by *i*NKT cells may be secondary to GR signaling in T_{conv} cells. The rapid kinetics of TIGIT upregulation in our model is somewhat reassuring. Nevertheless, to definitively address the direct effect of glucocorticoids on *i*NKT cells in the complete absence of T_{conv} cells, we used simple but informative culture systems in which two separate mouse *i*NKT hybridomas were exposed to physiologically relevant concentrations of CS. We found that a far greater percentage of either N38.3C3 (Figure 4.6C) or DN32.D3 *i*NKT cells (Figure 4.6D) expressed TIGIT after 12 hours of CS treatment in a dose-dependent manner, thus recapitulating our other *in vitro* and *in vivo* findings. Importantly, neither LAG-3 nor CTLA-4 was inducible by CS.

Collectively, the above results support the notion that glucocorticoids are direct regulators of TIGIT expression by *i*NKT cells.

4.3.4 Chronic variable stress (CVS) similarly augments TIGIT expression by *i*NKT cells

In the next series of experiments, we employed a chronic variable stress (CVS) model (65) to address whether long-term exposure to unpredictable psychological and/or physical stressors (Table 4.1) affects TIGIT expression in the *i*NKT cell compartment. This model is particularly useful because it prevents habituation. This is in contrast with several other models in which study subjects are repeatedly exposed to an identical stressor, resulting in desensitization and gradually in diminished stress responses over time (66).

Consistent with our findings in the prolonged restraint stress model, *i*NKT cells from animals enduring CVS increased their TIGIT expression appreciably (Figure 4.7). This was manifest for both $TIGIT^+$ cell frequencies and the MFI of staining for TIGIT. Therefore, TIGIT upregulation by *i*NKT cells can be viewed as a consequence of sustained physiological stress across multiple mouse models.

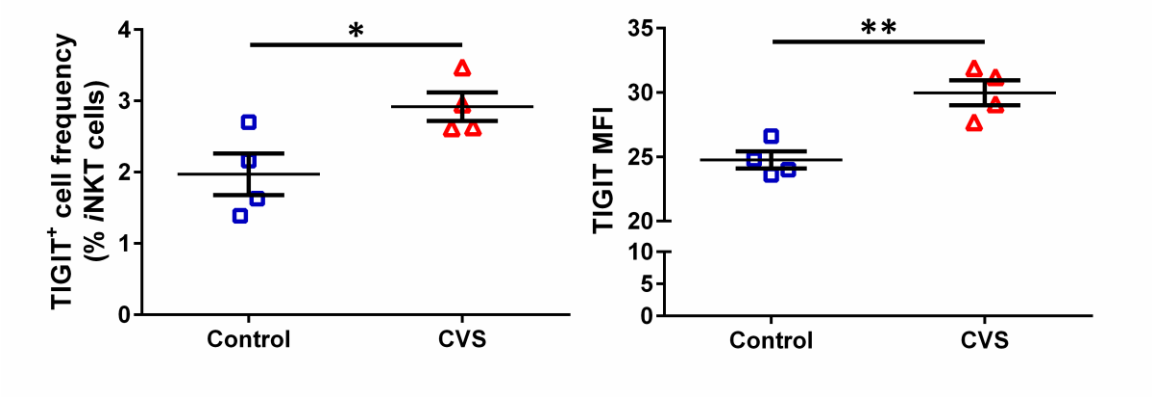

Figure 4.7: Long-term exposure to unpredictable heterotypic stressors results in enhanced TIGIT expression by *i*NKT cells.

B6 mice (n=4/group) were subjected to 21 days of chronic variable stress (CVS) according to a protocol outlined in Table 1. Control mice were left undisturbed in their home cages. Upon completion of the CVS procedure, animals were sacrificed, and liver MNCs were stained with CD1d tetramer and anti-mouse mAbs to TCRβ and TIGIT (or a rat IgG2a control). The percentages of $TIGIT^+$ cells (left panel) and the geometric MFI of TIGIT staining (right panel) after gating on $TCR\beta^+CD1d$ $tetramer^+$ cells are depicted. Error bars represent SEM. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively, using unpaired Student's *t*-tests.

4.3.5 Physical restraint and CS treatment raise the surface expression of TIGIT on MAIT cells

*i*NKT cells are prominent, but certainly not the only, innate-like invariant T lymphocytes. MAIT cells, another subset of innate-like T cells that home to mucosal layers and that are abundant in humans, have become a subject of intense investigations in recent years. However, whether they respond to stress is unknown. Therefore, we asked whether the glucocorticoid-GR-TIGIT regulatory axis also operates in MAIT cells. MAIT cells are scarce in conventional strains of laboratory mice. Therefore, we took advantage of a unique strain, called B6.MAIT^{CAST} mice, in which MAIT cells are readily detectable (48). Subjecting these animals to physical restraint increased both the frequency of TIGIT^{+} cells and the MFI of TIGIT expression in the hepatic MAIT cell compartment of these animals (Figure 4.8A). MAIT cells from stressed OHDA- and vehicle-treated B6.MAIT^{CAST} mice displayed comparable TIGIT levels, indicating that their heightened TIGIT expression was not dependent on the activity of post-ganglionic sympathetic nerve termini (Figure 4.8B). In stark contrast, GR antagonism via systemic administration of RU486 prevented stress-induced TIGIT upregulation on MAIT cells (Figure 4.8C). Consistent with this observation, exposing B6.MAIT^{CAST} non-parenchymal liver MNCs to CS dose-dependently enhanced the frequency of TIGIT^{+} MAIT cells (Figure 4.8D). Similarly, escalating doses of CS dramatically increased the proportion of TIGIT^{+} cells among 17E6 MAIT hybridoma cells (Figure 4.8E). In contrast, LAG-3 was not detectable in primary or hybridoma MAIT cells (Figure 4.8D-E). Therefore, MAIT cells closely mirror *i*NKT cells in their responsiveness to stress and GR signaling resulting in TIGIT upregulation.

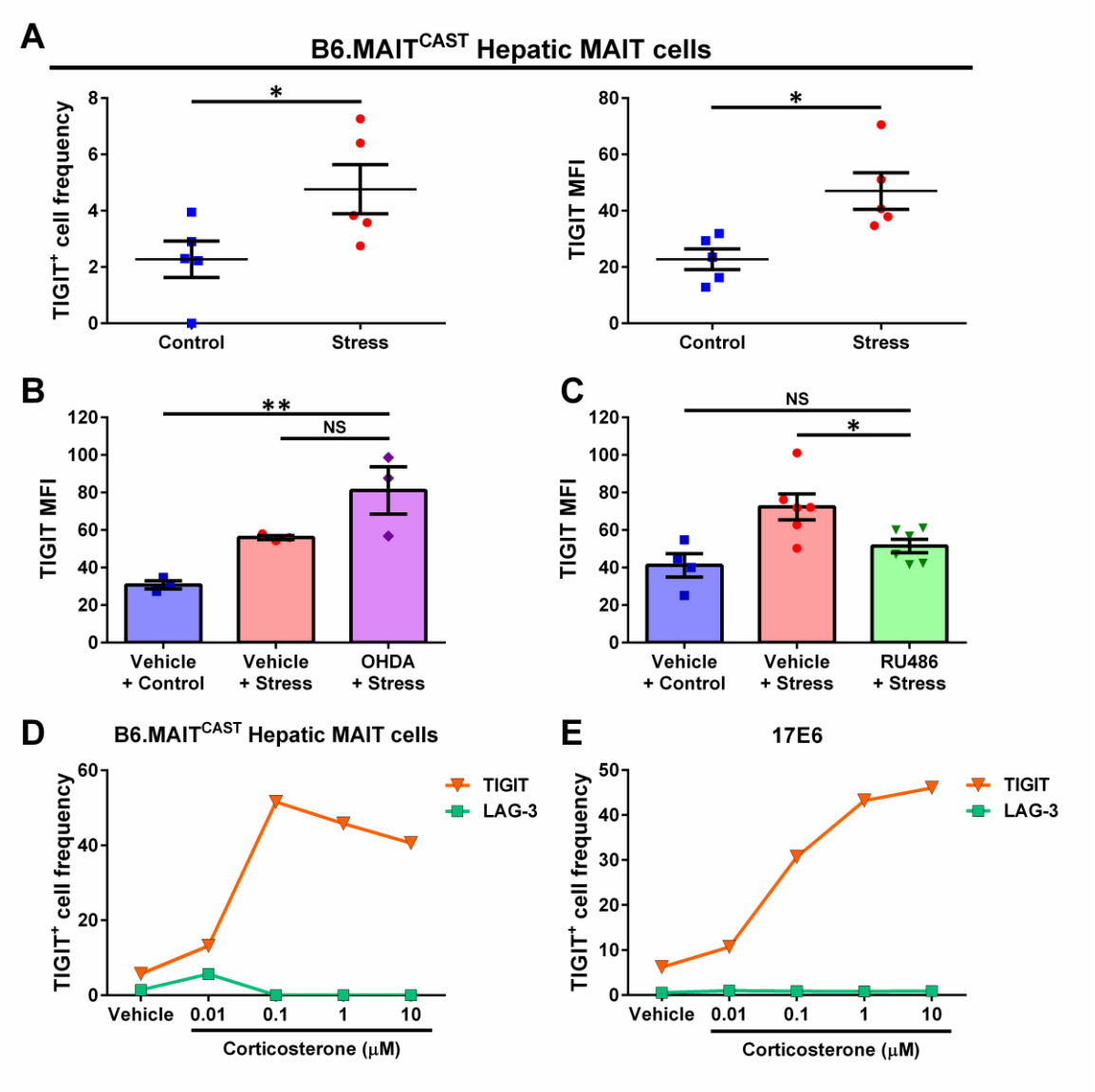

Figure 4.8: Endogenous GR signaling during restraint stress and *in vitro* treatment with CS similarly upregulate TIGIT on MAIT cells.

Male B6.MAIT^{CAST} mice were physically confined for 12 hours or left unstressed before they were sacrificed for their liver. Hepatic MNCs were harvested and stained with anti-TCRβ, -B220 and -TIGIT mAbs (or with a rat IgG2a isotype control) along with 5-OPRU-loaded mouse MR1 tetramers. The frequencies of TIGIT^{+} events among B220^{-} TCRβ^{+}MR1 tetramer^{+} MAIT cells (left panel) and the geometric MFI of TIGIT expression (right panel) were then determined by flow cytometry **(A)**. Separate cohorts of B6.MAIT^{CAST} mice were treated with 6-OHDA or vehicle six days before being subjected to restraint stress **(B)** or with RU486, or vehicle, one hour before physical

restraint **(C)**. The geometric MFI of TIGIT staining among hepatic *i*NKT cells were subsequently determined. One hundred thousand pooled liver MNCs from naïve male B6.MAIT^{CAST} mice **(D)** or 17E6 mouse hybridoma MAIT cells **(E)** were cultured for 12 hours with increasing doses of CS or vehicle, followed by staining with anti-TIGIT and -LAG-3 mAbs (or isotype controls). TIGIT^{+} and LAG-3^{+} MAIT cell frequencies were determined by flow cytometry. Each symbol in **A-C** represents an individual mouse, and error bars represent SEM. * and ** denote statistical differences with $p \leq 0.05$ and $p \leq 0.01$, respectively, by Student's *t*-tests **(A)** or one-way ANOVA **(B-C)**. Data depicted in **D-E** are representative of two independent experiments yielding similar results. NS = not significant

4.3.6 Stress-induced GR signaling elevates the expression of TIGIT on T_{conv} and NK cells

In parallel with invariant T cells, we assessed the impact of stress on TIGIT expression by conventional lymphocytes, including T_{conv} and NK cells. We found both prolonged restraint stress (Figure 4.9) and CVS (Figure 4.10) to significantly increase TIGIT levels on the surface of T_{conv} and NK cells. Furthermore, stress-induced TIGIT upregulation in T_{conv} and NK cell compartments could be reversed by the GR antagonist RU486, but not by chemical sympathectomy (Figure 4.11A-B). Physical restraint did not prompt TIGIT upregulation on T_{conv} cells of $GR^{fl}Lck^{Cre}$ mice (Figure 4.11C). By contrast, NK cells, which maintain their GR expression in the $GR^{fl}Lck^{Cre}$ model, retained their elevated TIGIT expression in stressed $GR^{fl}Lck^{Cre}$ mice (Figure 4.11C). Finally, CS supplementation of primary hepatic MNC cultures increased the proportion of T_{conv} and NK cells that stained positively for TIGIT (Figure 4.12). Therefore, glucocorticoids promote the expression of TIGIT not only on *i*NKT and MAIT cells, but also on effector cell types that can be transactivated by invariant T cells under normal conditions. TIGIT has been previously described as an inducible activation marker expressed by anti-CD3/CD28-stimulated T cells and by tumor-infiltrating NK cells (33, 67). To our knowledge, hormone-based regulation of TIGIT expression has never been described before. Our findings introduce a novel mode of cross-talk between the neuroendocrine and the immune system that may influence the overall immunological status in the face of perceived threats to our survival and homeostasis.

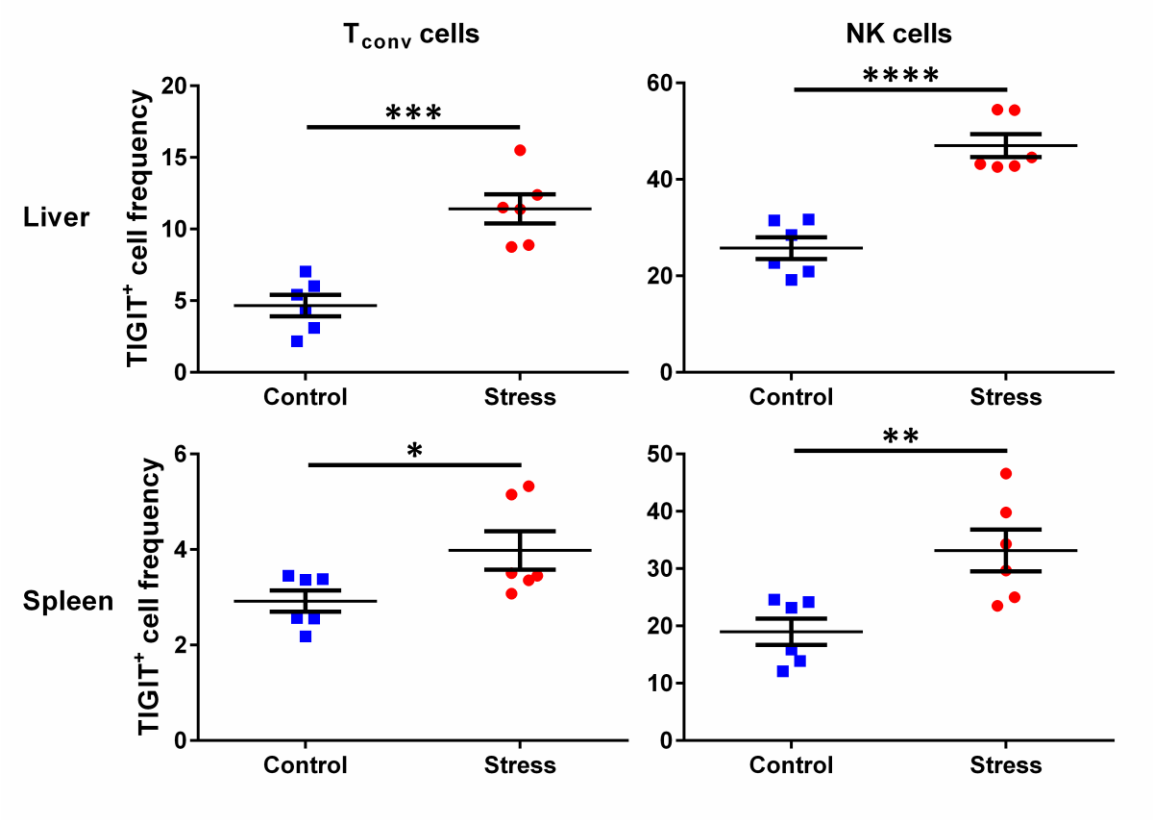

Figure 4.9: Restraint stress upregulates TIGIT on hepatic and splenic T_{conv} and NK cells.

B6 mice were subjected to physical restraint for12 hours or were left unstressed in their home cages without food and water. Liver MNCs and splenocytes from stressed and control mice were stained with CD1d tetramer and mAbs to TCRβ, NK1.1 and TIGIT (or a rat IgG2a isotype control). The frequencies of $TIGIT^+$ cells among hepatic (upper panels) and splenic (lower panels) $TCR\beta^+CD1d$ $tetramer^-$ T_{conv} cells (left panels) and $NK1.1^+TCR\beta^-$ NK cells (right panels) were determined by flow cytometry. Each symbol represents an individual mouse. Mean ± SEM values are shown, and *, **, *** and **** denote statistical differences with $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively, using unpaired Student's *t*-tests.

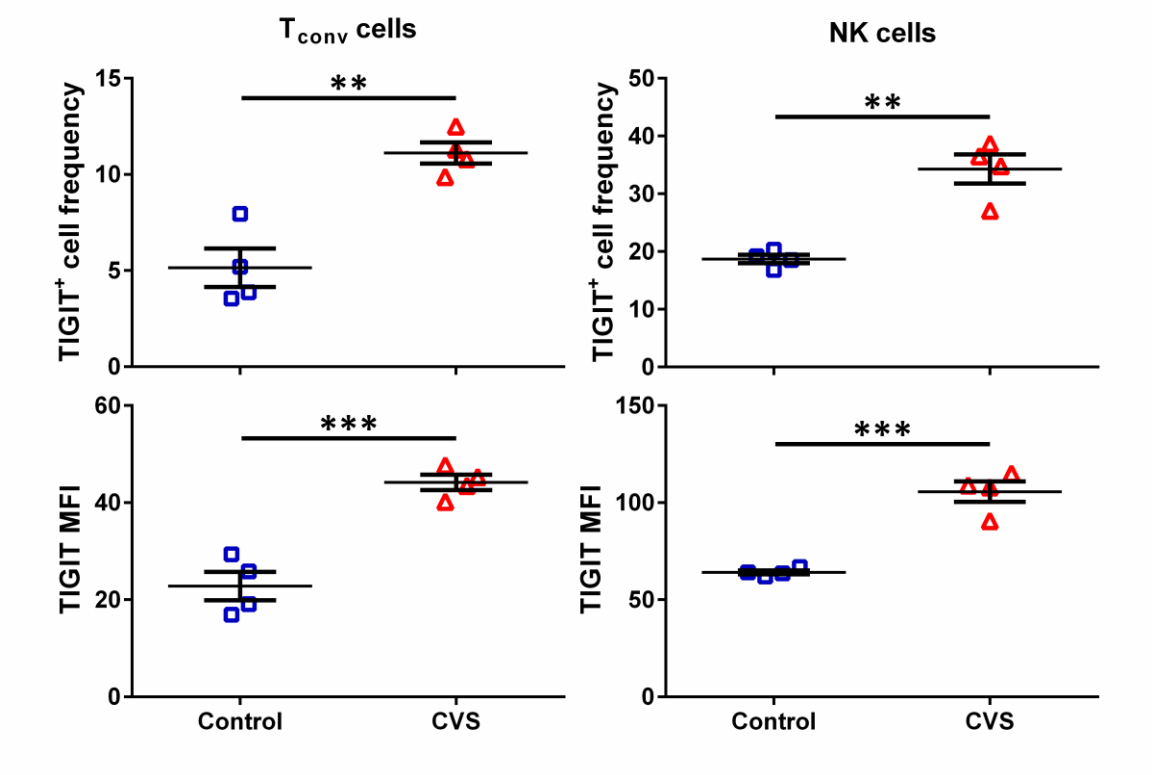

Figure 4.10: Increased TIGIT levels are detectable on the surface of T_{conv} and NK cells following CVS.

B6 mice (n=4) were subjected to 21 days of CVS according to the protocol outlined in Table 1, while control mice (n=4) remained unmanipulated. Mice were subsequently sacrificed, and hepatic MNCs were stained with mouse CD1d tetramers and mAbs to TCRβ, NK1.1, and TIGIT. The percentages of $TIGIT^+$ cells (upper panels) and the geometric MFI of TIGIT staining (lower panels) are shown for $TCR\beta^+CD1d$ $tetramer^-$ T_{conv} cells (left panels) and $TCR\beta^-NK1.1^+$ NK cells (right panels). Mean ± SEM values are shown. ** and *** denote statistical differences with $p \leq 0.01$ and $p \leq 0.001$, respectively, using unpaired Student's *t*-tests.

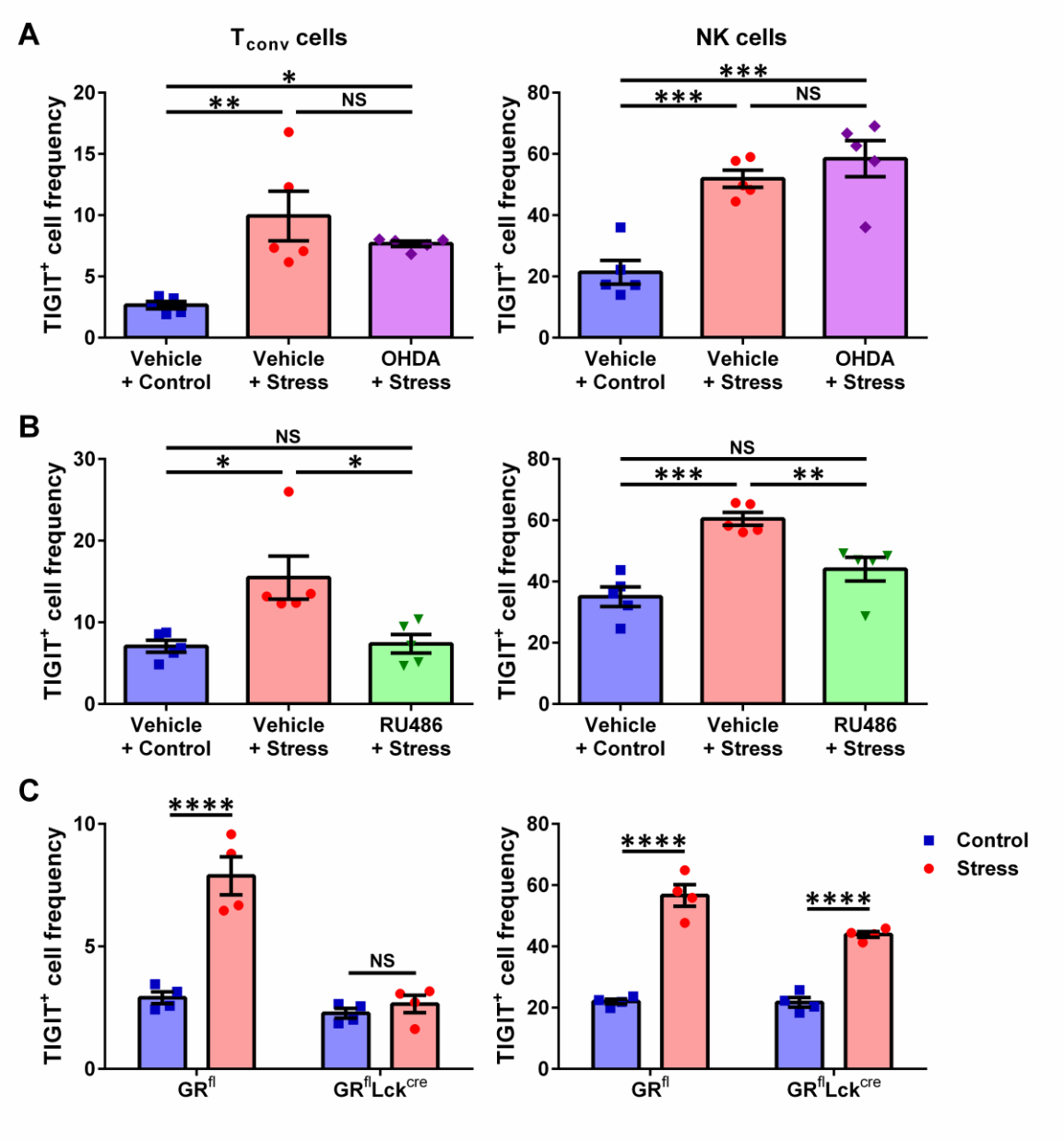

Figure 4.11: Stress-induced GR signaling, but not SNS priming, results in TIGIT upregulation on T_{conv} and NK cells.

Male B6 mice were injected with 6-OHDA or vehicle six days before they were subjected to 12 hours of restraint stress **(A)**. Separate cohorts of B6 mice were given RU486, or vehicle, one hour before they were physically restrained **(B)**. GR^{fl} and $GR^{fl}Lck^{Cre}$ mice were either physically confined or left unstressed **(C)**. The frequencies of $TIGIT^+$ T_{conv} ($TCR\beta^+CD1d$ $tetramer^-$) cells and NK ($NK1.1^+TCR\beta^-$) cells were determined by flow cytometry among hepatic MNCs. Each symbol represents an individual mouse, and error

bars represent SEM. *, **, *** and **** denote statistical differences with $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively, by one-way ANOVA **(A-B)** or two-way ANOVA **(C)**. NS = not significant

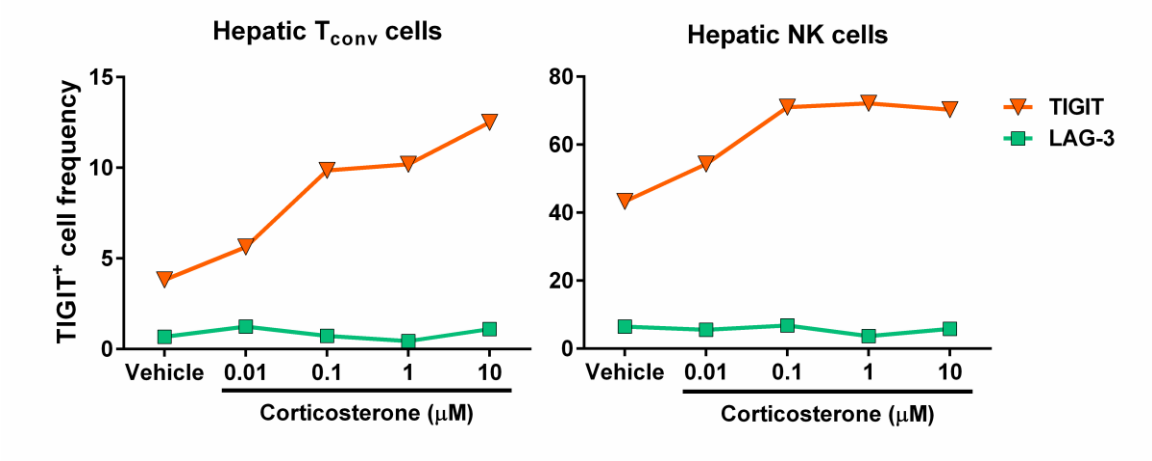

Figure 4.12: *In vitro* exposure to exogenous CS raises the proportion of $TIGIT^+$ cells among hepatic T_{conv} and NK cells.

One hundred thousand B6 liver MNCs were cultured with escalating doses of CS for 12 hours, and then stained with anti-TIGIT and -LAG-3 mAbs (or isotype controls). $TIGIT^+$ cell frequencies were determined among $TCR\beta^+CD1d$ tetramer$^-$ T_{conv} cells and $NK1.1^+TCR\beta^-$ NK cells. Data are representative of 2 independent experiments.

4.4 Discussion

The cellular and molecular mechanisms by which stressful experiences hamper immunological functions, especially those elicited by innate-like T cells, are far from clearly understood. In this work, we found augmented expression of TIGIT in *i*NKT and MAIT cells following exposure to physiological stress, which was linked to GR signaling in these cell types.

*i*NKT cells exhibit antimicrobial and antitumor properties *in vivo* (68). Once stimulated, they rapidly secrete IFN-γ, transactivate downstream effector cells such as NK cells, and promote cell-mediated cytotoxicity (69). Encouraging pre-clinical studies sparked clinical trials of the *i*NKT cell superagonist αGC in cancer and viral diseases, which yielded promising but varying results (34). Nonetheless, αGC and its derivatives continue to receive attention as prospective drug candidates in the clinic (70). For instance, DCs loaded with αGC and peptides derived from the tumor Ag NY-ESO-1 were recently found to boost tumor-specific $CD4^+$ and $CD8^+$ T cell responses in melanoma patients (71).

MAIT cells are found in the circulation, in the mucosal tissues of the gut, lungs and female genital tract, and in the liver, where they sense and respond to MR1-bound microbial vitamin B metabolites such as 5-OP-RU (36). 5-OP-RU-stimulated MAIT cells upregulate CD40L and potentiate the maturation of APCs, ultimately resulting in transactivation of NK cells to produce IFN-γ (42). We and others have reported the presence of MAIT cells in primary and metastatic tumors (72-74) and suggested that MAIT cells may be targeted in anticancer immunotherapeutic regimens (36, 75). Stress is known to compromise antimicrobial and anticancer responses, and the findings presented herein may introduce a novel mechanism underlying such effects. Our ongoing studies are addressing how stress alters invariant T cell responses to cognate ligands (*e.g.*, αGC and 5-OP-RU) and nonspecific stimuli (*e.g.*, inflammatory cytokines).

Of note, the effect of CS on $PD\text{-}1^+$ cell frequencies among *i*NKT hybridoma cells could not be examined since 100% of these cells expressed PD-1 at the baseline (data not shown). However, we noticed considerably increased MFI of PD-1 staining upon 12

hours of CS treatment in N38.3C3 cultures (MFIs of 10,740 and 4,121 in cultures containing 1 μM CS and vehicle, respectively). This was not evident in DN32.D3 cell cultures (data not shown). While stress selectively upregulated TIGIT in primary hepatic *i*NKT cells (Figure 4.1), it is possible that stress mediators, including glucocorticoids, may induce the expression of additional co-inhibitory receptors in a context-, tissue- and system-specific manner. Indeed, we found physical confinement to modestly raise the expression levels of 2B4 on *i*NKT cells in the liver (Figure 4.3). Future studies will be required to elucidate whether 2B4 plays a role in stress-induced immunomodulation.

Our work constitutes the first report that TIGIT can be expressed by invariant T cells. Future investigations should analyze the expression pattern of TIGIT and other checkpoint receptors on innate-like and conventional lymphocytes present in lymphoid tissues and mucosal sites other than the spleen and the liver. We are currently exploring the *in vivo* functional repercussions of stress-induced TIGIT upregulation. We anticipate far-reaching consequences in host defense.

The notably robust upregulation of TIGIT in the liver may be related to the inherently tolerogenic properties of this vital organ. Future studies are warranted to extend our outlook on how stress regulates liver functions in the context of antimicrobial and antitumor immune surveillance, via TIGIT and/or other co-inhibitory receptors. This is particularly important in light of the fact that invariant T cells, especially MAIT cells, can comprise up to 50% of all T lymphocytes residing in the human liver (73, 76).

Signaling through GR is known to positively or negatively influence the transcription of numerous immunologically relevant genes, including those encoding glucocorticoid-induced leucine zipper (GILZ) (77), IL-7Rα (CD127) (18) and Fas ligand (CD178) (78) just to name a few. Whether there exist GRE(s) that may dictate the transcription of TIGIT in immunocytes remains an open question at this point.

Immune checkpoint inhibitors targeting PD-1/PD-L1 or CTLA-4 have achieved unprecedented success as immunotherapies for several late-stage malignancies. However, a poor response rate in many cancer patients constitutes a major hurdle for their widespread use, prompting investigations into combination-based therapeutic modalities.

We propose that the activation of the HPA axis upon exposure to physical, chemical and psychological stressors should not be overlooked as it may render human subjects unresponsive to adjuvant treatments. Our findings suggest that glucocorticoids make crucial effector cells prone to co-inhibitory signaling through TIGIT. Therefore, it is plausible to envisage scenarios in which anti-TIGIT agents are beneficial in restoring immunological competence. Of note, in pre-clinical models of colorectal carcinoma and glioblastoma, combination therapies with PD-1- and TIGIT-blocking reagents were found to be more efficacious than anti-PD-1 or anti-TIGIT alone (29, 79).

Glucocorticoids continue to be prescribed for cancer patients as analgesics and antiemetics, or even as anti-inflammatories for adverse reactions encountered during immunotherapy (80). This presents a logical contradiction since pharmacological usage of glucocorticoids may negate certain aspects of protective antitumor immunity. In this work, we have found that oral CS administration over a three-week period results in noticeable increases in TIGIT expression by mouse *i*NKT and T_{conv} cells (Figure 4.6A and data not shown). Although we did not analyze NK cells in these experiments, we expect TIGIT expression by NK cells to follow a similar trend. Overall, these findings, once validated in clinical settings, may suggest that glucocorticoid therapy can be counter-productive in patients with malignancies. They may also pave the way for future strategies in mitigating glucocorticoid-induced immunosuppression while retaining the advantageous properties of glucocorticoids.

Although our findings have been predominantly discussed in the context of protective immunity, glucocorticoid-mediated regulation of TIGIT may aid in resolution of inflammation when immune responses turn pathogenic. TIGIT is reportedly important for limiting T cell-mediated inflammation in experimental autoimmune encephalomyelitis (79) and in collagen-induced arthritis (23). Psychological stress is associated with increased circulating levels of TNF-α and/or IL-6. This has been demonstrated in chronically stressed caregivers of patients with dementia (81) and in rodent models of restraint stress (82, 83). Under these and similar circumstances, elevated TIGIT expression may thus serve to counter harmful inflammatory responses.

To summarize, we have identified a novel signaling pathway that links stress-induced activation of the HPA axis and glucocorticoid release to the expression of the co-inhibitory receptor TIGIT. This pathway is operational in multiple critical subsets of immune effector cells, including *i*NKT, MAIT, T_{conv} and NK cells, and is therefore likely to broadly influence immune responses in health and disease.

4.5 References

1. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol.* 2005;5(3):243-251.

2. Cohen S, Tyrrell DA, Smith AP. Psychological stress and susceptibility to the common cold. *N Engl J Med.* 1991;325(9):606-612.

3. Pereira DB, et al. Stress as a predictor of symptomatic genital herpes virus recurrence in women with human immunodeficiency virus. *J Psychosom Res.* 2003;54(3):237-244.

4. Kiecolt-Glaser JK, Glaser R, Gravenstein S, Malarkey WB, Sheridan J. Chronic stress alters the immune response to influenza virus vaccine in older adults. *Proc Natl Acad Sci U S A.* 1996;93(7):3043-3047.

5. Morag M, Morag A, Reichenberg A, Lerer B, Yirmiya R. Psychological variables as predictors of rubella antibody titers and fatigue--a prospective, double blind study. *J Psychiatr Res.* 1999;33(5):389-395.

6. Kiecolt-Glaser JK, Marucha PT, Malarkey WB, Mercado AM, Glaser R. Slowing of wound healing by psychological stress. *Lancet.* 1995;346(8984):1194-1196.

7. Byrnes DM, et al. Stressful events, pessimism, natural killer cell cytotoxicity, and cytotoxic/suppressor T cells in HIV+ black women at risk for cervical cancer. *Psychosom Med.* 1998;60(6):714-722.

8. Levav I, Kohn R, Iscovich J, Abramson JH, Tsai WY, Vigdorovich D. Cancer incidence and survival following bereavement. *Am J Public Health.* 2000;90(10):1601-1607.

9. Lutgendorf SK, et al. Social support, psychological distress, and natural killer cell activity in ovarian cancer. *J Clin Oncol.* 2005;23(28):7105-7113.

10. Spiegel D, Bloom JR, Kraemer HC, Gottheil E. Effect of psychosocial treatment on survival of patients with metastatic breast cancer. *Lancet.* 1989;2(8668):888-891.

11. Elftman MD, Hunzeker JT, Mellinger JC, Bonneau RH, Norbury CC, Truckenmiller ME. Stress-induced glucocorticoids at the earliest stages of herpes simplex virus-1 infection suppress subsequent antiviral immunity, implicating impaired dendritic cell function. *J Immunol.* 2010;184(4):1867-1875.

12. Hunzeker J, Padgett DA, Sheridan PA, Dhabhar FS, Sheridan JF. Modulation of natural killer cell activity by restraint stress during an influenza A/PR8 infection in mice. *Brain Behav Immun.* 2004;18(6):526-535.

13. Saul AN, et al. Chronic stress and susceptibility to skin cancer. *J Natl Cancer Inst.* 2005;97(23):1760-1767.

14. Sommershof A, Scheuermann L, Koerner J, Groettrup M. Chronic stress suppresses anti-tumor TCD8+ responses and tumor regression following cancer immunotherapy in a mouse model of melanoma. *Brain Behav Immun.* 2017;65(140-149.

15. Pruett SB. Stress and the immune system. *Pathophysiology.* 2003;9(3):133-153.

16. Madden KS. Catecholamines, sympathetic innervation, and immunity. *Brain Behav Immun.* 2003;17 Suppl 1(S5-10.

17. Scheschowitsch K, Leite JA, Assreuy J. New Insights in Glucocorticoid Receptor Signaling-More Than Just a Ligand-Binding Receptor. *Front Endocrinol (Lausanne).* 2017;8(16.

18. Lee HC, Shibata H, Ogawa S, Maki K, Ikuta K. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *J Immunol.* 2005;174(12):7800-7806.

19. Xia M, Gasser J, Feige U. Dexamethasone enhances CTLA-4 expression during T cell activation. *Cell Mol Life Sci.* 1999;55(12):1649-1656.

20. Xing K, Gu B, Zhang P, Wu X. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC Immunol.* 2015;16(39.

21. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell.* 2015;27(4):450-461.

22. Marin-Acevedo JA, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. *J Hematol Oncol.* 2018;11(1):39.

23. Levin SD, et al. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur J Immunol.* 2011;41(4):902-915.

24. Li M, et al. T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-gamma production of natural killer cells via beta-arrestin 2-mediated negative signaling. *J Biol Chem.* 2014;289(25):17647-17657.

25. Liu S, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ.* 2013;20(3):456-464.

26. Stanietsky N, et al. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol.* 2013;43(8):2138-2150.

27. Stanietsky N, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A.* 2009;106(42):17858-17863.

28. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. *J Immunol.* 2012;188(8):3869-3875.

29. Johnston RJ, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell.* 2014;26(6):923-937.

30. Joller N, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity.* 2014;40(4):569-581.

31. Kurtulus S, et al. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest.* 2015;125(11):4053-4062.

32. Yu X, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol.* 2009;10(1):48-57.

33. Zhang Q, et al. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat Immunol.* 2018.

34. van den Heuvel MJ, Garg N, Van Kaer L, Haeryfar SM. NKT cell costimulation: experimental progress and therapeutic promise. *Trends Mol Med.* 2011;17(2):65-77.

35. Kjer-Nielsen L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* 2012;491(7426):717-723.

36. Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *J Leukoc Biol.* 2018;104(3):473-486.

37. Ussher JE, et al. CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol.* 2014;44(1):195-203.

38. Velazquez P, Cameron TO, Kinjo Y, Nagarajan N, Kronenberg M, Dustin ML. Cutting edge: activation by innate cytokines or microbial antigens can cause arrest

of natural killer T cell patrolling of liver sinusoids. *J Immunol.* 2008;180(4):2024-2028.

39. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med.* 2003;198(2):267-279.

40. Hayakawa Y, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol.* 2001;31(6):1720-1727.

41. Hermans IF, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol.* 2003;171(10):5140-5147.

42. Salio M, et al. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J Immunol.* 2017;199(8):2631-2638.

43. Smyth MJ, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* 2002;99(4):1259-1266.

44. Kiank C, Holtfreter B, Starke A, Mundt A, Wilke C, Schutt C. Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. *Brain Behav Immun.* 2006;20(4):359-368.

45. Steelman AJ, et al. Restraint stress modulates virus specific adaptive immunity during acute Theiler's virus infection. *Brain Behav Immun.* 2009;23(6):830-843.

46. Mittelstadt PR, Monteiro JP, Ashwell JD. Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. *J Clin Invest.* 2012;122(7):2384-2394.

47. Hennet T, Hagen FK, Tabak LA, Marth JD. T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl-transferase gene by site-directed recombination. *Proc Natl Acad Sci U S A.* 1995;92(26):12070-12074.

48. Cui Y, et al. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J Clin Invest.* 2015;125(11):4171-4185.

49. Hu D, et al. Essential role of IL-10/STAT3 in chronic stress-induced immune suppression. *Brain Behav Immun.* 2014;36(118-127.

50. Buynitsky T, Mostofsky DI. Restraint stress in biobehavioral research: Recent developments. *Neurosci Biobehav Rev.* 2009;33(7):1089-1098.

51. Franco AJ, et al. Sensitization of the Hypothalamic-Pituitary-Adrenal Axis in a Male Rat Chronic Stress Model. *Endocrinology.* 2016;157(6):2346-2355.

52. Grebe KM, Hickman HD, Irvine KR, Takeda K, Bennink JR, Yewdell JW. Sympathetic nervous system control of anti-influenza CD8+ T cell responses. *Proc Natl Acad Sci U S A.* 2009;106(13):5300-5305.

53. Gourley SL, Swanson AM, Koleske AJ. Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience. *J Neurosci.* 2013;33(7):3107-3112.

54. Gui M, Li J, Wen LJ, Hardy RR, Hayakawa K. TCR beta chain influences but does not solely control autoreactivity of V alpha 14J281T cells. *J Immunol.* 2001;167(11):6239-6246.

55. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med.* 1994;180(3):1097-1106.

56. Tilloy F, et al. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med.* 1999;189(12):1907-1921.

57. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med.* 2000;191(11):1895-1903.

58. Matsuda JL, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med.* 2000;192(5):741-754.

59. Reantragoon R, et al. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med.* 2013;210(11):2305-2320.

60. Corbett AJ, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* 2014;509(7500):361-365.

61. Bandyopadhyay K, Marrero I, Kumar V. NKT cell subsets as key participants in liver physiology and pathology. *Cell Mol Immunol.* 2016;13(3):337-346.

62. Kostrzewa RM, Jacobowitz DM. Pharmacological actions of 6-hydroxydopamine. *Pharmacol Rev.* 1974;26(3):199-288.

63. Kinlein SA, Shahanoor Z, Romeo RD, Karatsoreos IN. Chronic Corticosterone Treatment During Adolescence Has Significant Effects on Metabolism and Skeletal Development in Male C57BL6/N Mice. *Endocrinology.* 2017;158(7):2239-2254.

64. Cassano AE, White JR, Penraat KA, Wilson CD, Rasmussen S, Karatsoreos IN. Anatomic, hematologic, and biochemical features of C57BL/6NCrl mice maintained on chronic oral corticosterone. *Comp Med.* 2012;62(5):348-360.

65. Salter EW, Sunstrum JK, Matovic S, Inoue W. Chronic stress dampens excitatory synaptic gain in the paraventricular nucleus of the hypothalamus. *J Physiol.* 2018;596(17):4157-4172.

66. Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP, Spencer RL. Habituation to repeated restraint stress is associated with lack of stress-induced c-fos

expression in primary sensory processing areas of the rat brain. *Neuroscience.* 2006;138(4):1067-1081.

67. Joller N, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol.* 2011;186(3):1338-1342.

68. Haeryfar SM, Mallevaey T. Editorial: CD1- and MR1-Restricted T Cells in Antimicrobial Immunity. *Front Immunol.* 2015;6(611.

69. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol.* 2010;185(5):2721-2729.

70. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T Cell Targets for Cancer Immunotherapy. *Immunity.* 2018;48(3):453-473.

71. Gasser O, et al. A phase I vaccination study with dendritic cells loaded with NY-ESO-1 and alpha-galactosylceramide: induction of polyfunctional T cells in high-risk melanoma patients. *Cancer Immunol Immunother.* 2018;67(2):285-298.

72. Peterfalvi A, et al. Invariant Valpha7.2-Jalpha33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells. *Int Immunol.* 2008;20(12):1517-1525.

73. Shaler CR, et al. Mucosa-associated invariant T cells infiltrate hepatic metastases in patients with colorectal carcinoma but are rendered dysfunctional within and adjacent to tumor microenvironment. *Cancer Immunol Immunother.* 2017;66(12):1563-1575.

74. Sundstrom P, et al. Human Mucosa-Associated Invariant T Cells Accumulate in Colon Adenocarcinomas but Produce Reduced Amounts of IFN-gamma. *J Immunol.* 2015;195(7):3472-3481.

75. Haeryfar SMM, Shaler CR, Rudak PT. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunol Immunother.* 2018.

76. Dusseaux M, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood.* 2011;117(4):1250-1259.

77. Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq C, Yamamoto KR. Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. *Proc Natl Acad Sci U S A.* 2004;101(44):15603-15608.

78. Baumann S, et al. Glucocorticoids inhibit activation-induced cell death (AICD) via direct DNA-dependent repression of the CD95 ligand gene by a glucocorticoid receptor dimer. *Blood.* 2005;106(2):617-625.

79. Dixon KO, et al. Functional Anti-TIGIT Antibodies Regulate Development of Autoimmunity and Antitumor Immunity. *J Immunol.* 2018;200(8):3000-3007.

80. Garant A, Guilbault C, Ekmekjian T, Greenwald Z, Murgoi P, Vuong T. Concomitant use of corticosteroids and immune checkpoint inhibitors in patients with hematologic or solid neoplasms: A systematic review. *Crit Rev Oncol Hematol.* 2017;120(86-92.

81. Kiecolt-Glaser JK, Preacher KJ, MacCallum RC, Atkinson C, Malarkey WB, Glaser R. Chronic stress and age-related increases in the proinflammatory cytokine IL-6. *Proc Natl Acad Sci U S A.* 2003;100(15):9090-9095.

82. Nukina H, Sudo N, Aiba Y, Oyama N, Koga Y, Kubo C. Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. *J Neuroimmunol.* 2001;115(1-2):46-52.

83. Shimizu T, et al. Resistance of extrathymic T cells to stress and the role of endogenous glucocorticoids in stress associated immunosuppression. *Scand J Immunol.* 2000;51(3):285-292.

5 Chapter 5: Discussion

Parts of Chapter 5 have been adapted from a published research article:

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 physically spares but functionally impairs innate-like invariant T cells. *Cell Reports*. 2021; 35(2): 108979. DOI: 10.1016/j.celrep.2021.108979. Please refer to Appendix B to view the relevant copyright policy enabling the reuse of this work.

5.1 General discussion

Invariant T or *i*T cells, a general term used to describe two functionally similar yet distinct subsets of innate-like T lymphocytes, namely CD1d-restricted *i*NKT cells and MR1-restricted MAIT cells (1-3), serve pivotal roles in launching innate and adaptive immune responses at the early stages of Ag exposure (4). Accordingly, *i*T cells have been reported to: i) exit development as pre-primed, effector memory-like cells (5, 6); ii) readily exert their effector functions upon stimulation by cognate Ags and/or inflammatory cues (7); iii) release an array of immunomodulatory cytokines at sizeable quantities (8, 9); iv) elicit direct cytotoxicity of CD1d- or MR1-expressing target cells (10, 11); and v) facilitate the transactivation of several innate and adaptive effector cells (12-14). Of importance, *i*T cells are attractive targets for immunotherapy given their notable prevalence at particular tissue locations in humans (15-17) and the monomorphic nature of CD1d and MR1, allowing *i*T cell ligands to take effect in genetically distinct individuals (4, 18-20). Despite their diverse effector functions and potential therapeutic applicability, it was curious that the impacts of psychological stress on *i*T cell biology had essentially gone unexplored since the discovery of *i*NKT and MAIT cells' respective *i*TCRs nearly three decades ago (21, 22). Thus, the unified objective of this thesis was to define how mediators of psychological stress affect the persistence and function of *i*T cells and to identify pathways that may be exploited to mitigate the detrimental impacts of stress on the protective immune responses they mediate. The studies presented throughout this thesis were the first to determine how psychological stress influences *i*T cell dynamics using the appropriate tetramers and agonists with which to specifically analyze *i*T cell populations (23, 24). Moreover, to my knowledge, they collectively represent the most comprehensive study to date describing how the survival, phenotype, and function of *i*T cells are regulated by mediators of stress in general.

I demonstrated in Chapter 3 that, under psychological stress, *i*T cells lose their ability to generate robust levels of T_H1- and T_H2-type cytokines, exhibit an atypical inflammatory signature, and fail to orchestrate oncolytic and antimetastatic host responses. These effects are surprisingly independent of post-ganglionic sympathetic neurotransmitters but are mediated by *i*T cell-intrinsic glucocorticoid receptor signaling. At the same time,

mouse and human *i*T cells are uniquely resistant to stress- and glucocorticoid-induced apoptosis. This finding reveals the intriguing possibility that the responsiveness of *i*T cells, which remain physically intact during stress, may be restorable should the correct interventional approach be identified.

In Chapter 4, I reported that long-term stress upregulates the immune checkpoint molecule TIGIT on *i*NKT cells in a cell-autonomous, GR-dependent fashion (25). Since stress-induced suppression of cytokine responses towards αGC also resulted from *i*NKT cell-intrinsic GR signaling, I asked whether this functional inhibition operated through a TIGIT-dependent pathway. My preliminary results indicate that systemic blockade of TIGIT moderately but significantly restores serum IFN-γ levels in stressed animals mounting responses to αGC (Figure 5.1). Therefore, TIGIT appears to be partially responsible for *i*NKT cells' functional impairments during stress. As such, its blockade may be a viable option in reversing stress-induced immunosuppression. Of note, although TIGIT may mediate cellular exhaustion (26, 27), the impact of its engagement appears to be transient since *i*NKT cells from stressed mice do not exhibit signs of long-term dysfunction (Figure 3.10). I also demonstrated in Chapter 4 that, similar to *i*NKT cells, stress-induced GR signaling upregulates TIGIT on MAIT cells. Future analyses will be required to determine whether TIGIT blockade in stressed mice improves cytokine responses to 5-OP-RU.

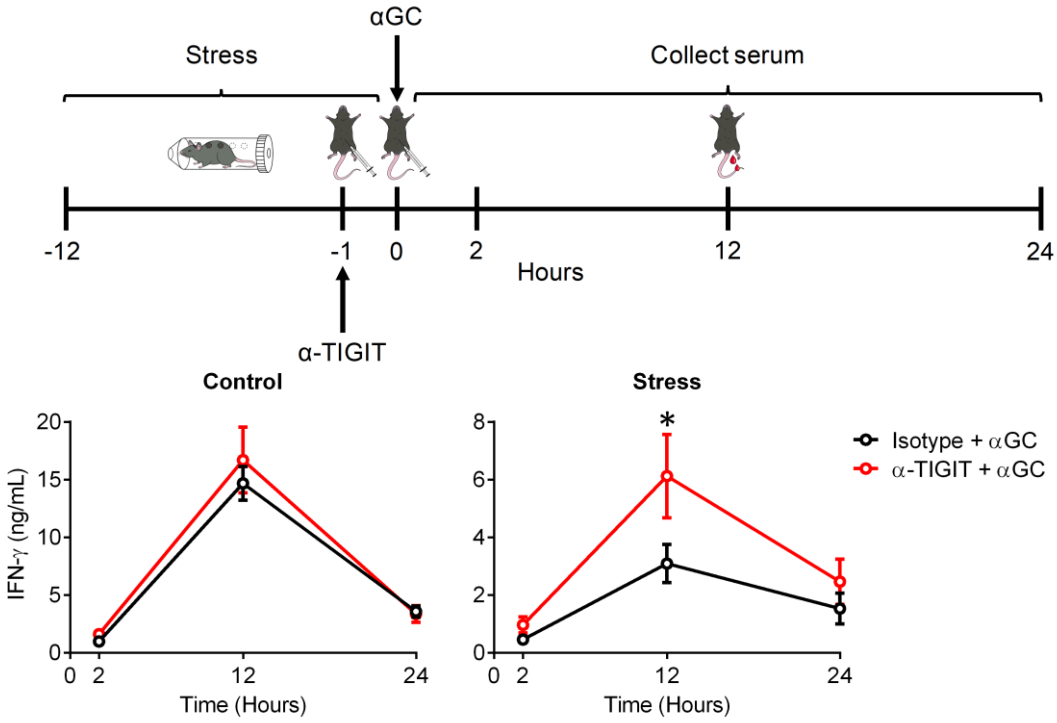

Figure 5.1: Stress-induced *i*NKT cell impairments are partially mediated by TIGIT. B6 mice were restrained or left undisturbed before they received a 200-μg i.p. dose of an anti-mouse TIGIT mAb (or a mouse IgG1κ isotype control) followed by αGC administration as schematically illustrated. Mice were bled at indicated time points, and serum IFN-γ levels were quantified (n=8-9/group). Error bars represent SEM. * denotes differences with $p<0.05$ using two-way ANOVA with Sidak's post-hoc analysis.

Although the primary focus of Chapter 4 was to analyze changes in the expression of co-inhibitory receptors on *i*T cells pre- and post-stress, the discovery that GR signaling induced greater expression of TIGIT was not restricted solely to *i*T cells. Stress was also associated with a marked increase in TIGIT expression on hepatic and splenic T_{conv} and NK cells in a GR-dependent manner (Figures 4.9-4.12). Since TIGIT was upregulated on multiple effector cell types during stress, I questioned whether *in vivo* TIGIT blockade could bolster overall antitumor immune responses in stressed mice. However, an antagonistic mAb towards TIGIT failed to affect the growth of B16 melanoma or EL4 lymphoma metastases after restraint (Figure 5.2). Nevertheless, given its ability to enhance T_H1-type cytokine responses towards αGC in stressed mice (Figure 5.1), *in vivo* blockade of TIGIT may be capable of augmenting antitumor immunity mediated by stimulated *i*NKT cells under stressful conditions. In addition, should a MAIT ligand be shown to elicit potent antitumor efficacy in the future, understanding whether psychological stress disrupts these effects and, if so, whether anti-TIGIT mAbs prevent this from occurring will be important to deduce.

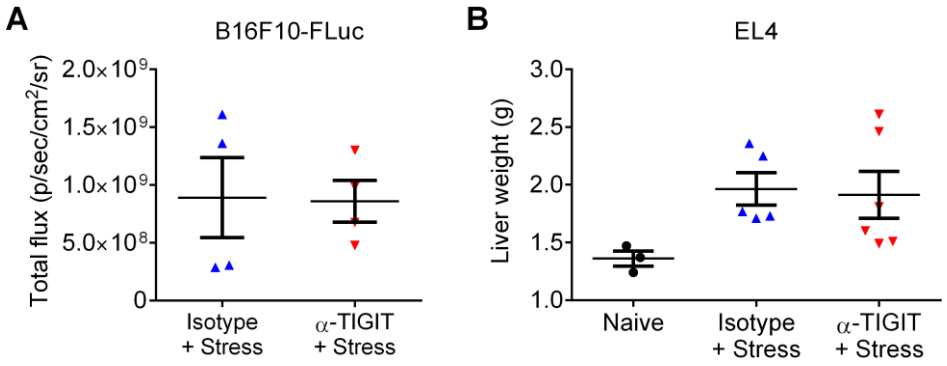

Figure 5.2: *In vivo* blockade of TIGIT in stressed mice fails to broadly enhance antitumor immune responses.

(A-B) Two hours before inoculating B16F10-Fluc or EL4 cells i.v. into stressed mice, as well as every other day thereafter, 200 μg anti-mouse TIGIT (clone: 1B4) or mIgG1κ isotype control (clone: MOPC-21) mAbs were injected i.p. Fourteen days later, metastatic tumor burden was measured by whole body bioluminescence imaging of B16F10-FLuc melanoma **(A)** or by liver weight for EL4 lymphoma **(B)**. Each symbol represents an individual mouse. Error bars represent SEM.

One of the major questions introduced in Chapter 3 was to determine whether the inhibition of *i*T cell function during stress was merely a consequence of their apoptosis. In doing so, we discovered that both *i*NKT and MAIT cells are refractory to stress-induced apoptosis, resulting in the significant expansion of their frequencies as other subsets of lymphocytes succumb to cell death. Vast reductions in the cellular abundance of lymphocytes during restraint stress have been reported (28, 29). Multiple groups have proposed that restraint stress induces lymphocyte apoptosis via the release of endogenous opioids (30, 31). By injecting the pan opioid receptor antagonists naloxone and naltrexone into stressed mice, Yin et al. demonstrated that opioids mediate splenocyte death by triggering their upregulation of the death receptor Fas/CD95 (30). As a confirmatory measure, they validated that stress failed to decrease splenocyte counts in loss-of-function Fas mutant mice. Furthermore, splenocyte numbers continued to be diminished in adrenalectomized mice, ruling out the possibility that the HPA axis mediates this effect (30). Later, Wang et al. reproduced these findings and used μ-opioid receptor knockout mice to establish that opioids deplete the number of B cells and T cells in the spleen via the μ-opioid receptor (31). In contrast, Tseng et al. reported that restraint stress-induced decreases in splenocyte counts are prevented by pretreatment with the GR antagonist RU486, whereas naltrexone bears little to no effect (32). In this thesis, I demonstrated that stress-induced apoptosis fails to occur in T cells lacking the GR and in NK and B cells from RU486-treated mice (Figures 3.5 and 3.6, respectively). Clearly, the relationship between psychological stress, GR and opioid receptor signaling, and lymphocyte apoptosis is currently difficult to interpret, and may be complicated by the ability of opioids to stimulate glucocorticoid production (31, 33, 34). Nevertheless, the data in Chapter 3 contribute to a body of evidence suggesting that glucocorticoids are the chief mediators of psychological stress-induced apoptosis in various lymphocyte subsets.

Recently, the abundance and function of *i*NKT cells were examined in a 72-hour model of sleep deprivation inducing a physical stress response (35). Mice exposed to sleep deprivation harbored greater levels of serum CS which, as expected (36), was accompanied by a loss in overall and DP thymocyte counts. Sleep deprivation also led to a loss in the number of $CD1d^+$ DP thymocytes from which *i*NKT cells are positively selected (refer to Chapter 1.2), mirroring the effects that I witnessed in the CVS model

(Figure 5.3). Flow cytometric analyses of *i*NKT cells in the periphery revealed that sleep deprivation did not alter their frequencies or absolute numbers in the spleen and liver. This contrasted with a previous study in which sleep deprivation was found to decrease the total number of $CD3^+NKp46^+$ NKT cells in the spleen (37). In comparison with the data shown in this thesis, *i*NKT cell frequencies and absolute numbers increase and remain constant, respectively, in the spleen and liver during prolonged restraint stress (Figure 3.5). Next, phenotyping of *i*NKT cell populations indicated that their expression of the activation marker CD69 remains unchanged during sleep deprivation (35); likewise, it is unchanged during prolonged restraint (Figure 3.9). Interestingly, IFN-γ and IL-4 responses to αGC, both in the serum and in *i*NKT cells intracellularly, were completely unobstructed in sleep deprived mice. Moreover, *i*NKT cell-mediated responses against B16 melanoma metastases were unaffected by sleep deprivation (35). These data differ heavily from my own, as I demonstrated here that stress drastically impairs the ability of *i*NKT cells to elicit IFN-γ and IL-4 responses and to protect against metastases of B16 melanoma (see Chapter 3). As a potential source of error between systems, the antitumor properties of *i*NKT cells in the sleep deprivation model were tested upon injection of αGC-pulsed B16 cells (35), whereas mice subjected to restraint stress received αGC i.p. prior to being inoculated with unpulsed B16 cells (Figure 3.12). This caveat notwithstanding, the similarities and dissimilarities in the effects of different stress models on *i*NKT cell responses highlight the context dependent nature by which stress likely regulates their phenotypic and functional profiles, as has been described for other subsets of T cells (38).

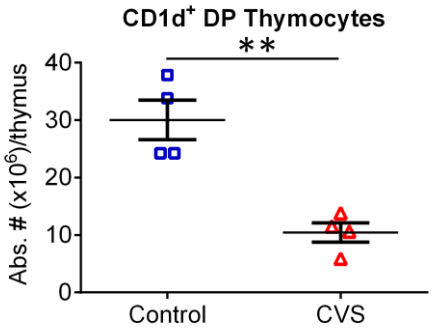

Figure 5.3: CVS reduces the cellular abundance of CD1d-expressing $CD4^+CD8^+$ DP thymocytes.

B6 mice were exposed to CVS or control conditions. On day 21, after the final stressor was applied, mice were sacrificed and thymocytes were stained with a mAb against mouse CD1d or a rat IgG2bκ isotype control before being analyzed by flow cytometry. The absolute number of $CD1d^+$ $CD4^+CD8^+$ DP thymocytes was then calculated. Each symbol denotes an individual mouse and error bars represent SEM. ** denotes a difference with $p<0.01$ using an unpaired Student's *t*-test.

5.2 Outstanding questions

Collectively, the data presented throughout this thesis provide an in-depth characterization of the mechanisms by which sustained psychological stress can impact *i*T cell biology. However, due to the complexities of the physiological stress response and the diverse effector functions elicited by *i*T cells at various tissue sites, defining all of the intricate pathways by which stress may regulate *i*T cell behavior would not be feasible in a limited time frame. Naturally, several unresolved questions and opportunities to expand on this work remain.

A major conclusion of this thesis is that, contrary to the prevailing dogma which states that stress promotes a T_H2-type bias (39, 40), *i*T cell responses do not become skewed in this way. Rather, the ability of *i*T cells to initiate T_H2-type responses are surprisingly hampered during psychological stress. Hepatic and splenic *i*NKT cells activated by αGC display a limited capacity to produce T_H2-type cytokines including IL-4, IL-5, and IL-13 in stressed mice (Figures 3.1, 3.2, and 3.4). Likewise, hepatic MAIT cells stimulated with 5-OP-RU generate weaker levels of IL-4 in stressed mice (Figure 3.13). While these data might suggest that *i*T cells do not participate in creating a T_H2-type bias during stress, the possibility that *i*T cells facilitate such a bias in the context of T_{conv} cell activation cannot necessarily be ruled out. T_{conv} cell activation in mice lacking *i*T cells through germline deficiencies in Jα18 or MR1 genes (41, 42), the administration of *i*T cell depleting antibodies (43), or CRISPR/Cas9 approaches (44, 45) would be helpful in determining the net contribution, if any, of *i*T cells in supporting T_H2 cell differentiation during stress.

Figures 3.1 and 3.4 highlight the abnormal systemic inflammatory response triggered by αGC-stimulated *i*NKT cells in stressed mice. This was characterized in part by stark increases in the production of the anti-inflammatory cytokines IL-10 and IL-27 as well as the T_H17-type cytokines IL-17A and IL-23. In addition, several other cytokines and chemokines were found to be either upregulated or downregulated in stressed mice. However, the cellular sources of the majority of these cytokines as well as the implications of these differences on the ability of *i*NKT cells to mediate antitumor immunity remains to be determined.

While decreased T_H1- and T_H2-type cytokine production in stressed mice treated with αGC is mediated by glucocorticoids (Figure 3.8), we cannot discount the potential involvement of the ANS, which communicates bi-directionally with the HPA axis (46, 47), in imposing these and other effects. In Figure 3.8, we demonstrate using OHDA-treated animals that stress-induced suppression of *i*NKT cell responses is completely independent of signals derived from postganglionic neurons of the SNS. This was curious given that *i*NKT cells express many adrenergic receptors and are sensitive to regulation by NE *in vitro* (Figure 3.7). However, since OHDA only destroys postganglionic sympathetic neurons (48), we cannot rule out whether the SNS participates in imposing these stress-induced effects by controlling the secretion of catecholamines from the adrenal glands. Experiments in which adrenergic receptor antagonists are administered systemically before stress would be beneficial in determining the role of the sympathetic-adrenal-medullary system in governing the effects reported here. Moreover, since the SNS and PSNS are mutually antagonistic (49) and the immunomodulatory effects of acetylcholine are increasingly becoming documented (50, 51), reduced PSNS activity during stress or increased PSNS activity in the aftermath of stress may also influence the outcomes observed in our studies. Thus, the use of acetylcholine receptor inhibitors could be informative in revealing the contribution, if any, of the PSNS in these settings.

In addition to the questions described above, it remains unclear whether glucocorticoids mediate the increased production of anti-inflammatory and T_H17-type cytokines in stressed mice. While the existing literature provides evidence that the activated GR can stimulate the production of cytokines such as IL-10 and IL-17A (52, 53), the elevated levels of these cytokines may instead be due to increased or decreased release of mediators of the ANS. For instance, NE and epinephrine have both been described to promote the production of IL-10, IL-17A, and IL-23 (54-56). On a similar note, whether GR signaling also mediates the increases or decreases in the production of numerous other cytokines/chemokines examined in our multiplexing analyses (e.g., eotaxin, GM-CSF, IL-1α/β, IL-2, IP-10, etc.) remains undetermined.

Like *i*NKT cells, stress suppresses the production of IFN-γ and IL-4 by stimulated MAIT cells (Figure 3.13). Whether these outcomes are also GR-dependent has not yet been

validated. Thus, pretreating B6-MAIT^{CAST} mice with inhibitors of glucocorticoid signaling (e.g., metyrapone, RU486) prior to stress should be useful in future experiments. In addition, how stress alters the production of other cytokines after MAIT cell activation is currently unknown since our multiplex analyses were conducted only on serum samples from mice treated with αGC (or its corresponding vehicle). Of note, my supplemental analyses indicated that stress also diminishes MAIT cell-mediated production of TNF-α and, interestingly, IL-17A (data not shown). Therefore, how stress affects the secretion of certain cytokines after MAIT cell activation is not necessarily identical to that observed in the context of *i*NKT cell activation.

Assessing how stress influences other facets of *i*T cell function was beyond the scope of this thesis. For instance, *i*NKT and MAIT cells can recognize and destroy CD1d^{+} and MR1^{+} target cells, respectively, via direct cell-mediated cytotoxic activity (57-61). Additionally, although antitumor immune responses were examined in this thesis (Figure 3.12), the primary mission of *i*T cells is arguably to participate in antimicrobial host defense (4). Accordingly, both *i*NKT and MAIT cells have been demonstrated to elicit protective immunity against bacterial and viral infections *in vivo* (62-65). The impact of stress on the above and other *i*T cell functions should be a subject of future investigations.

Another key finding highlighted in Chapter 3 is that *i*T cells are unusually resistant to stress- and glucocorticoid-induced apoptosis (Figures 3.5 and 3.13). This was accompanied by increased expression of the anti-apoptotic protein Bcl-2 within the *i*NKT cell compartment. Whether MAIT cells also upregulate Bcl-2 during stress remains to be understood. Importantly, Bcl-2 has been described to allow other T cell subsets to resist glucocorticoid-induced apoptosis, namely T_H17 cells and likely T_H2 cells (66). Whether mouse and human *i*T cells similarly resist glucocorticoid-induced apoptosis via the upregulation of Bcl-2 has yet to be elucidated. *In vitro* culture systems in which glucocorticoids are introduced in the presence or absence of the Bcl-2-specific inhibitor venetoclax (67) may reveal whether *i*T cells' resistance glucocorticoid-induced apoptosis is dependent on Bcl-2.

Consistent with prior reports indicating that the GR exerts transcriptional control over CD127 in T cells (68-71), we found that GR signaling drives CD127 expression in human and mouse *i*NKT, MAIT, and T_{conv} cells (Figure 3.14). CD127 associates with CD132 (*aka*., the common-γ chain) to form the IL-7 receptor complex (70), which provides signals of survival to T cells in part by driving Bcl-2 transcription (71, 72). Although we did not identify any changes in serum IL-7 levels in stressed mice (data not shown), we cannot rule out whether local IL-7 concentrations increase without washing out into the circulation or whether T cells become more sensitive to baseline levels of IL-7. Thus, whether and to what extent elevated CD127 expression contributes to *i*T and/or T_{conv} cell survival during stress will be of interest in future studies. As such, *in vivo* blocking antibodies towards CD127, CD132, and IL-7 could be useful in determining how IL-7 receptor signaling influences the numerical abundance and Bcl-2 expression patterns of different T cell populations during stress.

Interestingly, the resistance of T_H17 cells to the effects of glucocorticoids have been suggested to be partly dependent on their expression of multi-drug resistance protein 1 (MDR1) (73, 74). Both *i*NKT and MAIT cells have also been reported to constitutively express relatively high levels of MDR1 (16, 75, 76). How the expression pattern of MDR1 changes in *i*T cells exposed to glucocorticoids and whether this at least partially mediates their resistance to glucocorticoid-induced cell death could be informative in future inquiries.

Throughout Chapter 4, I demonstrate that GR signaling mediates the upregulation of TIGIT on *i*T, T_{conv}, and NK cells in stressed mice. Given that the GR is a ligand-activated transcription factor, it is conceivable that it facilitates the transcription of *Tigit* via binding to a proximal GRE (68). As an initial approach, model cell culture systems can be treated with glucocorticoids in the presence or absence of the translation inhibitor cycloheximide before analyzing *Tigit* transcript levels (77). These experiments would determine whether the GR induces *Tigit* transcription directly or indirectly (i.e., through the transcription of an intermediate transcription factor). If GR-mediated transcription of *Tigit* is found to be direct, the associated GRE could then be identified using chromatin immunoprecipitation sequencing, reporter gene assays, and/or other methodologies.

Consistent with their broad anti-inflammatory actions, glucocorticoids have been demonstrated to enhance the immunosuppressive functions of T_{reg} cells (78, 79). Recently, Kim et al. reported that the therapeutic effects of glucocorticoids in autoimmune and allergic airway diseases are dependent on GR-mediated metabolic reprogramming in T_{reg} cells (78). T_{reg} cells also appear to be less sensitive to glucocorticoid-induced apoptosis than other subsets of $CD4^+$ T cells (80). In this thesis, I found that GR signaling upregulates TIGIT in T cells, a molecule which potentiates the suppressive functions of T_{reg} cells (81, 82). Thus, the possibility that T_{reg} cells contribute to stress-induced suppression of *i*T cell responses cannot be ruled out, particularly since the mechanisms of such effects were determined in part using mice in which the GR is lacking in all T cells. Mice treated with T_{reg}-depleting antibodies (83) and/or mice in which the GR is selectively deleted in T_{reg} cells (e.g., $Nr3c1^{fl/fl}Foxp3^{cre}$ mice) (78, 79) would be useful in determining whether glucocorticoids impair the functions of *i*T cells in stressed mice through T_{reg}-mediated immunosuppression. These models may also reveal whether T_{reg} cells are a source of immunoregulatory cytokines (e.g., IL-10) after the administration of αGC in stressed mice (Figure 3.1).

Given that, unlike T_{conv} cells, *i*T cells are insensitive to glucocorticoid-induced apoptosis and do not preferentially produce T_H2-type cytokines during stress, it appears that mediators of stress may evoke fundamentally distinct signaling pathways in *i*T and T_{conv} cells. However, throughout this thesis, *i*T cell populations were assessed only at select tissue locations. Our analyses of *i*NKT cells were largely limited to the liver and spleen, wherein NKT1 cells represent the most prominent *i*NKT cell subset by far (84). Moreover, when administered systemically, αGC predominantly activates hepatic and splenic *i*NKT cells (84). Thus, our findings are based heavily on how stress impacts the numerical abundance, phenotype, and functional capacity of NKT1 cells in particular. Importantly, *i*NKT cells also occupy the thymus, lungs, lymph nodes, and intestines, where NKT2 and/or NKT17 cells amass and exhibit unique characteristics compared to NKT1 cells (12, 84). For instance, while NKT2 cells express IL-17RB, a receptor for the T_H2 polarizing cytokine IL-25, NKT1 cells lack expression of this molecule (85). Of note, we found IL-25 to be elevated in stressed mice regardless of whether they received αGC or vehicle (Figure 3.1). It is therefore conceivable that NKT2 cells are more

sensitive to the T_H2-promoting effects of stress, which may have been missed in our investigation. Likewise, analyses of MAIT cells were exclusively conducted on those found in the liver. Mouse MAIT cells can also be detected at sufficient quantities in the spleen, lungs, and lamina propria (86). How our findings from *i*T cell populations in the spleen and/or liver compare to the outcomes exhibited by *i*T cells in other organs may reveal tissue-specific regulatory mechanisms governed by mediators of stress.

Lastly, a major finding of this thesis is that αGC-stimulated *i*NKT cells from stressed mice fail to elicit antimetastatic host defenses against B16 melanoma. This is preventable by delivering the GR antagonist RU486 systemically prior to stress (Figure 3.12). However, on which cell type(s) glucocorticoids are acting to impose these effects remains unclear. Previous studies have indicated that the antitumor properties of αGC depend on the ability of *i*NKT cells to transactive NK cells (87, 88), $CD8^+$ T cells (89, 90), γδ T cells (91), and pre-MNK cells (92), each of which can be sensitive to the actions of glucocorticoids. Moreover, GR signaling can enhance the immunosuppressive functions of regulatory cells such as T_{reg} cells (78) and MDSCs (93), both of which facilitate tumor growth (94). Adding another layer of complexity, glucocorticoids can exert their effects on non-immune cells, including on cancer cells directly, to promote tumor cell proliferation and the development of metastases (95-97), which may overwhelm the oncolytic capacity of effector cells activated by αGC. In future studies, in-depth analyses of the immune landscape in the TME as well as Cre/*lox*-based targeting approaches could be used to identify the precise cellular mechanisms by which glucocorticoids impair the antitumor functions of *i*NKT cells during stress.

5.3 Limitations

As expected, the accuracy and relevance of the conclusions drawn throughout this thesis are limited by the inherent imperfections and weaknesses of the models chosen. Here, the most frequent method used to induce psychological stress in mice was prolonged restraint stress, which was applied for a total of 12 hours. Although this has been described as a model of "chronic" stress in the past (98), it does not fit the accepted definition of chronic stress (i.e., a stress response lasting multiple days or weeks) (49). Therefore, caution should be taken when broadly relating the results from the prolonged restraint stress model to circumstances in which bona fide chronic stress responses are involved.

Although it is a highly common and established method for inducing psychological stress in rodents (99), a criticism of physical restraint is that it can result in different immunological outcomes than those resulting from naturalistic psychological stressors such as social disruption (38, 100). Alternative mouse models of psychological stress could be employed to compare their effects on *i*T cell biology to those of prolonged restraint stress. Potential examples include the presence of a predator such as a rat (101), the introduction of an aggressive cage-mate (102), social isolation (100), inescapable scream sounds (103), and other approaches (104).

An issue related to the validity of prolonged restraint stress as a model for inducing psychological stress is how to adequately control for the effects of nutrient deprivation, a physical stressor (104). In this thesis, non-stressed control mice were also deprived of access to food and water during the 12-hour period of restraint. However, it is clear that mice subjected to restraint will consume more metabolic resources over this period of time as they attempt to escape from physical confinement with urgency. Indeed, concerns regarding whether physical restraint represents a purely psychogenic stressor have been discussed previously (103). Nevertheless, as mentioned above, physical restraint continues to be an accepted practice for introducing a stressor that is considered to be primarily psychogenic in nature (105).

In Chapters 3 and 4, I leveraged the CVS model to verify my findings from prolonged restraint stress in an established paradigm of chronic stress. These experiments proved

highly useful as they enabled me to reproduce many of my findings in an independent physiological setting in which a protracted stress response is evident (106). Although these data were meant to reflect how *i*T cells are regulated during chronic psychological stress, the CVS model is comprised of a mix of psychological and physical stressors. While several of these stressors are indeed psychogenic, including restraint, cage shaking, forced swimming, cage tilting, and wet bedding, some physical stressors are also introduced in the CVS model, including a cold environment, keeping the lights on overnight, and food and water deprivation. The relative contributions of psychogenic and physical stressors to the observations obtained from the CVS-based experiments would thus be difficult to discern.

In Chapter 3, I showed that, like *i*T cells in mice, human *i*NKT and MAIT cells isolated from the liver and PB are abnormally refractory to glucocorticoid-induced cell death (Figure 3.13). These findings notwithstanding, the studies presented in this thesis are limited by a lack of understanding of how closely these experimental findings in mice translate to humans. Studying prolonged or chronic psychological stress in humans remains challenging (107). Accordingly, the available literature on the immunological consequences of psychological stress in humans is relatively scarce. However, analyzing *i*T cells from human cohorts exposed to high degrees of occupational stress, such as emergency room physicians (108) or caregivers of dementia patients (109, 110), may provide viable representations of how these cell populations are influenced by naturalistic psychological stressors. Alternatively, *i*T cell-related readouts of interest can be correlated with subjective psychological distress scores reported by study participants, such as those generated by the perceived stress scale, as described previously (111). Nevertheless, the mouse studies in this thesis provide a framework for identifying indicators of prolonged or chronic psychological stress that manifest in human *i*T cell populations. These include, but are not limited to, poorer capacity to produce T_H1- and T_H2-type cytokines, a greater propensity to produce T_H17-type and anti-inflammatory cytokines, higher frequencies among lymphocytes and/or T cells, and increased expression of Bcl-2, CD127, GILZ, and TIGIT relative to cohorts experiencing psychological stress to a lesser degree.

Sex-related differences in *i*T cell responses have previously been reported (112-114). For example, estrogen receptor signaling drives greater IFN-γ production in *i*NKT cells from female mice than male mice (112). Likewise, in Figure 3.3, I demonstrate that serum IFN-γ and IL-4 levels after αGC administration are higher in female mice than in males. In Chapter 3, both male and female mice were included in our initial serum cytokine analyses (Figures 3.1B-C, 3.3, and 3.13E-F) and immunophenotyping studies (Figures 3.5A-B and 3.13C-D). However, male mice were used for all *in vivo* mechanism-of-action experiments in Chapter 3 and most *in vivo* experiments in Chapter 4. The reasons for this were twofold: i) *i*NKT cell responses exhibit less variability in male mice (Figure 3.3), thereby minimizing the number of mice needed for mechanistic delineations; and ii) I frequently used RU486, which is both a GR and progesterone receptor inhibitor. Although the effects of stress on *i*NKT cells appeared to be comparable between male and female mice (Figure 3.3), the notion that similar or identical mechanisms operate in both males and females remains an assumption that has yet to be confirmed *a posteriori*. Validating that at least some of our mechanistic findings from male mice also apply to female mice will be important in follow-up work.

A caveat that should be considered when interpreting the data in this thesis is that the parameters used to define T_{conv} cell populations are imperfect. Throughout Chapters 3 and 4, mouse T_{conv} cells were defined as TCRβ^+PBS-57-loaded CD1d tetramer$^-$ cells. However, this gating strategy fails to exclude certain subsets of non-conventional T cells including *v*NKT cells and MAIT cells. Of note, the latter are highly infrequent in WT B6 mice (86) and thus their overall contribution to the readouts presented can be considered quite negligible. Nevertheless, this T_{conv} cell definition is advantageous for excluding γδ T cells, a fairly prevalent subset of innate-like T cells in mice (115). In Chapter 3, human T_{conv} cells were identified as CD3$^+$PBS-57-loaded CD1d tetramer$^-$5-OP-RU-loaded MR1 tetramer$^-$ events. However, this definition does not rule out γδ T cells and *v*NKT cells, both of which represent minor yet appreciable proportions of CD3$^+$ cells in humans (115, 116). Adding the appropriate staining reagents to allow for the exclusion of these non-conventional T cell subsets from mouse and human T_{conv} cell gates should be done in future efforts to validate the findings in this thesis.

Finally, I demonstrated in Figure 3.12 that GR signaling during stress diminishes the protective effects of αGC in the B16-F10 metastatic melanoma model. In this system, αGC was injected 6 hours prior to the inoculation of B16-F10 cells. Therefore, its relevance to spontaneous neoplasia or cancer immunotherapy may be questioned since target cancer cells were introduced when *i*NKT cells were already pre-activated. However, αGC administration after the injection of B16-F10 cells also protects against subsequent lung nodule formation to a similar extent (92). Thus, it is reasonable to assume that stress would have a comparable effect had mice received αGC after the injection of B16-F10 cells. On another note, a general limitation of the B16-F10 metastatic melanoma model is that it is induced by the intravenous injection of a cell line. This non-spontaneous tumor model bypasses the process of metastasis in which tumor cells intravasate into the bloodstream from a primary tumor (117). Assessing how stress influences the antimetastatic effects of αGC after the establishment of a primary tumor, for example after the intrasplenic injection of B16-F10 cells resulting in hepatic metastases (118, 119), may be an opportunity to expand on this work in the future.

5.4 Concluding remarks

Unfortunately, experiencing sustained psychological stress is commonplace in modern day society (107). Given the now well-recognized cross-talk that occurs between the nervous system and the immune system (120), it is unsurprising that psychological stress can impose a myriad of detrimental effects on the function of immune cells (121). As a consequence, exposure to sustained psychological stress is accepted as a potential determinant of increased susceptibility to diseases related to immunity, a concept which has prevailed for many decades (122). Although our understanding of the pathways by which stress affects immune cells has become more sophisticated in recent years, much remains to be learned about these highly complex and nuanced relationships.

Psychological stress has frequently been reported to be capable of impairing cell-mediated immune responses, rendering the host vulnerable to opportunistic infection and cancer establishment and/or progression (39, 40, 123). Recent decades have seen *i*NKT and MAIT cells rise to the forefront of immunological inquiry due to their powerful immunomodulatory capabilities and participation in tumor immune surveillance and antimicrobial defense (4, 9, 11). As a result, *i*NKT and MAIT cells are actively being scrutinized for their utility as targets for immunotherapy (124, 125). Yet, the impacts of long-term psychological stress on *i*T cell survival, phenotype, and function have historically remained elusive.

In this thesis, I demonstrated for the first time that the ability of *i*T cells to trigger T_H1- and T_H2-type cytokine responses and participate in antitumor immunity are abrogated during psychological stress. These effects are independent of post-ganglionic sympathetic neurotransmitters but are dependent on GR signaling despite the fact that *i*T cells are resistant to glucocorticoid-induced apoptosis. Moreover, in a GR-dependent manner, *i*T cells upregulate the immune checkpoint molecule TIGIT during stress. This receptor can be targeted by specific blocking antibodies to partially restore the impaired cytokine-producing capacity of *i*T cells. Taken together, I have uncovered a previously unappreciated mechanism of stress-induced immunosuppression involving innate-like *i*T cells with wide-ranging implications for innate immunity and cancer immunotherapy (Figure 5.4).

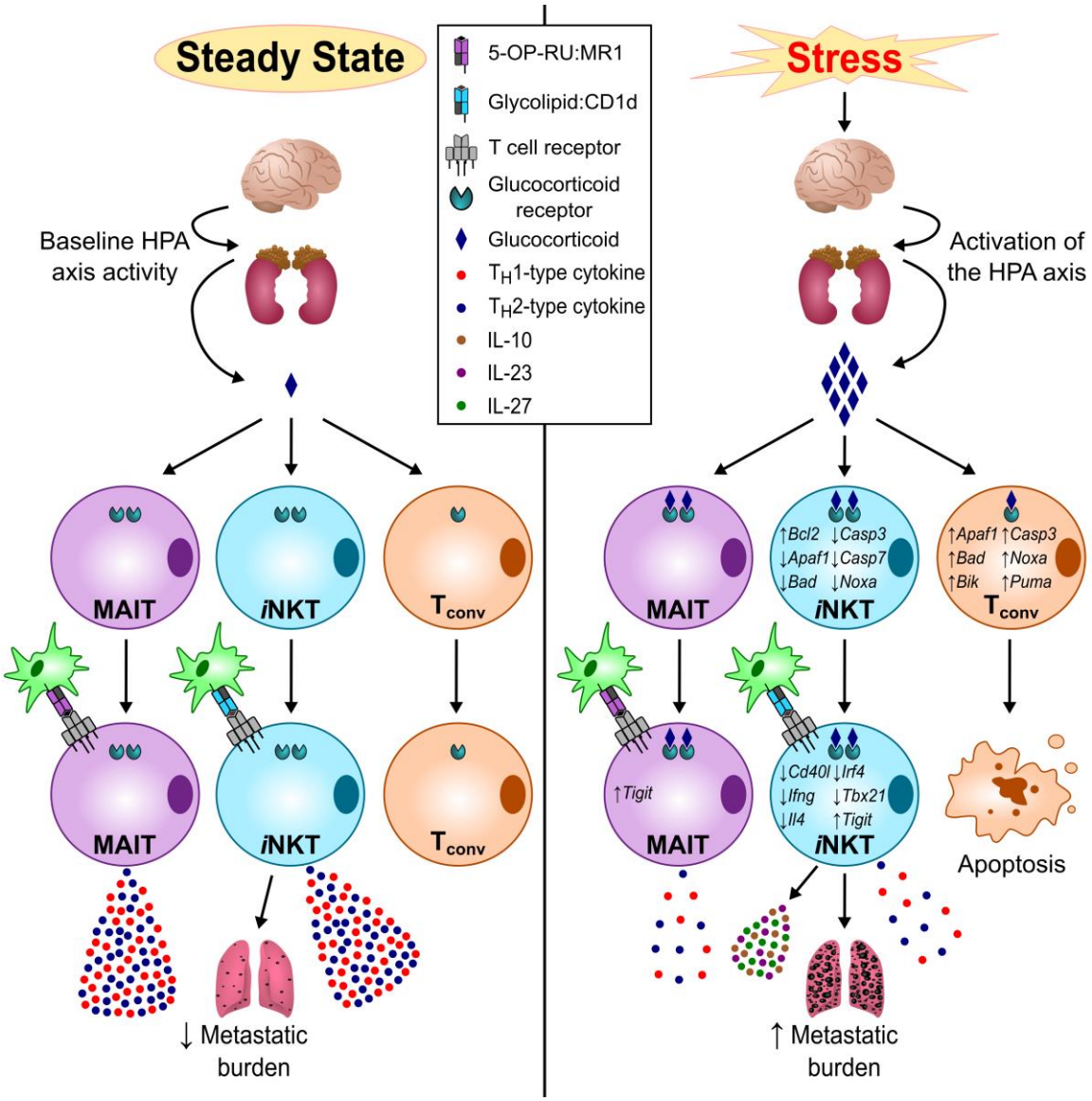

Figure 5.4: Graphical summary depicting the central findings described in this thesis.

5.5 References

1. Salou M, et al. A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J Exp Med.* 2019;216(1):133-151.

2. Legoux F, Salou M, Lantz O. Unconventional or Preset αβ T Cells: Evolutionarily Conserved Tissue-Resident T Cells Recognizing Nonpeptidic Ligands. *Annu Rev Cell Dev Biol.* 2017;33(511-535.

3. Xiao X, et al. Mucosal-Associated Invariant T Cells Expressing the TRAV1-TRAJ33 Chain Are Present in Pigs. *Front Immunol.* 2019;10(2070.

4. Haeryfar SM, Mallevaey T. Editorial: CD1- and MR1-Restricted T Cells in Antimicrobial Immunity. *Front Immunol.* 2015;6(611.

5. Godfrey DI, Berzins SP. Control points in NKT-cell development. *Nat Rev Immunol.* 2007;7(7):505-518.

6. Le Bourhis L, Mburu YK, Lantz O. MAIT cells, surveyors of a new class of antigen: development and functions. *Curr Opin Immunol.* 2013;25(2):174-180.

7. Chandra S, Kronenberg M. Activation and Function of iNKT and MAIT Cells. *Adv Immunol.* 2015;127(145-201.

8. Hinks TSC, Zhang XW. MAIT Cell Activation and Functions. *Front Immunol.* 2020;11(1014.

9. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol.* 2008;20(3):358-368.

10. Díaz-Basabe A, Strati F, Facciotti F. License to Kill: When iNKT Cells Are Granted the Use of Lethal Cytotoxicity. *Int J Mol Sci.* 2020;21(11).

11. Rudak PT, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. *J Leukoc Biol.* 2018;104(3):473-486.

12. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol.* 2013;13(2):101-117.

13. Salio M, et al. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J Immunol.* 2017;199(8):2631-2638.

14. Miyazaki Y, Miyake S, Chiba A, Lantz O, Yamamura T. Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis. *Int Immunol.* 2011;23(9):529-535.

15. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur J Immunol.* 2009;39(7):1893-1901.

16. Dusseaux M, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood.* 2011;117(4):1250-1259.

17. Hinks TS, Wallington JC, Williams AP, Djukanovic R, Staples KJ, Wilkinson TM. Steroid-induced Deficiency of Mucosal-associated Invariant T Cells in the Chronic Obstructive Pulmonary Disease Lung. Implications for Nontypeable Haemophilus influenzae Infection. *Am J Respir Crit Care Med.* 2016;194(10):1208-1218.

18. Parrot T, et al. Expansion of donor-unrestricted MAIT cells with enhanced cytolytic function suitable for TCR-redirection. *JCI Insight.* 2021.

19. Crowther MD, et al. Genome-wide CRISPR-Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1. *Nat Immunol.* 2020;21(2):178-185.

20. Kawano T, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997;278(5343):1626-1629.

21. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med.* 1993;178(1):1-16.

22. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med.* 1994;180(3):1097-1106.

23. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M. Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc.* 2008;3(1):70-78.

24. Xiao X, Cai J. Mucosal-Associated Invariant T Cells: New Insights into Antigen Recognition and Activation. *Front Immunol.* 2017;8(1540.

25. Rudak PT, et al. Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes. *Brain Behav Immun.* 2019;80(793-804.

26. Johnston RJ, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell.* 2014;26(6):923-937.

27. Zhang Q, et al. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat Immunol.* 2018;19(7):723-732.

28. Oya H, et al. The differential effect of stress on natural killer T (NKT) and NK cell function. *Clin Exp Immunol.* 2000;121(2):384-390.

29. Shi Y, Devadas S, Greeneltch KM, Yin D, Allan Mufson R, Zhou JN. Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain Behav Immun.* 2003;17 Suppl 1(S18-26.

30. Yin D, Tuthill D, Mufson RA, Shi Y. Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J Exp Med.* 2000;191(8):1423-1428.

31. Wang J, Charboneau R, Barke RA, Loh HH, Roy S. Mu-opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. *J Immunol.* 2002;169(7):3630-3636.

32. Tseng RJ, Padgett DA, Dhabhar FS, Engler H, Sheridan JF. Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. *Brain Behav Immun.* 2005;19(2):153-164.

33. Fuchs BA, Pruett SB. Morphine induces apoptosis in murine thymocytes in vivo but not in vitro: involvement of both opiate and glucocorticoid receptors. *J Pharmacol Exp Ther.* 1993;266(1):417-423.

34. Roy S, et al. Role of hypothalamic-pituitary axis in morphine-induced alteration in thymic cell distribution using mu-opioid receptor knockout mice. *J Neuroimmunol.* 2001;116(2):147-155.

35. Sousa MEP, et al. Invariant Natural Killer T cells resilience to paradoxical sleep deprivation-associated stress. *Brain Behav Immun.* 2020;90(208-215.

36. Tarcic N, Ovadia H, Weiss DW, Weidenfeld J. Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. *J Neuroimmunol.* 1998;82(1):40-46.

37. De Lorenzo BH, de Oliveira Marchioro L, Greco CR, Suchecki D. Sleep-deprivation reduces NK cell number and function mediated by β-adrenergic signalling. *Psychoneuroendocrinology.* 2015;57(134-143.

38. Pruett SB. Stress and the immune system. *Pathophysiology.* 2003;9(3):133-153.

39. Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Ann N Y Acad Sci.* 2006;1069(62-76.

40. Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol.* 2004;5(10):617-625.

41. Chandra S, et al. A new mouse strain for the analysis of invariant NKT cell function. *Nat Immunol.* 2015;16(8):799-800.

42. Cui Y, et al. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J Clin Invest.* 2015;125(11):4171-4185.

43. Szabo PA, et al. Invariant Natural Killer T Cells Are Pathogenic in the HLA-DR4-Transgenic Humanized Mouse Model of Toxic Shock Syndrome and Can Be Targeted to Reduce Morbidity. *J Infect Dis.* 2017;215(5):824-829.

44. Ren Y, et al. A Novel Mouse Model of iNKT Cell-deficiency Generated by CRISPR/Cas9 Reveals a Pathogenic Role of iNKT Cells in Metabolic Disease. *Sci Rep.* 2017;7(1):12765.

45. Koay HF, et al. Diverse MR1-restricted T cells in mice and humans. *Nat Commun.* 2019;10(1):2243.

46. Ziegler DR, Cass WA, Herman JP. Excitatory influence of the locus coeruleus in hypothalamic-pituitary-adrenocortical axis responses to stress. *J Neuroendocrinol.* 1999;11(5):361-369.

47. Agorastos A, et al. Vagal effects of endocrine HPA axis challenges on resting autonomic activity assessed by heart rate variability measures in healthy humans. *Psychoneuroendocrinology.* 2019;102(196-203.

48. Kostrzewa RM, Jacobowitz DM. Pharmacological actions of 6-hydroxydopamine. *Pharmacol Rev.* 1974;26(3):199-288.

49. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci.* 2009;10(6):397-409.

50. Cox MA, et al. Choline acetyltransferase-expressing T cells are required to control chronic viral infection. *Science.* 2019;363(6427):639-644.

51. Rosas-Ballina M, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* 2011;334(6052):98-101.

52. de Castro Kroner J, Knoke K, Kofler DM, Steiger J, Fabri M. Glucocorticoids promote intrinsic human T(H)17 differentiation. *J Allergy Clin Immunol.* 2018;142(5):1669-1673.e1611.

53. Peek EJ, et al. Interleukin-10-secreting "regulatory" T cells induced by glucocorticoids and beta2-agonists. *Am J Respir Cell Mol Biol.* 2005;33(1):105-111.

54. Takenaka MC, et al. Norepinephrine Controls Effector T Cell Differentiation through β2-Adrenergic Receptor-Mediated Inhibition of NF-κB and AP-1 in Dendritic Cells. *J Immunol.* 2016;196(2):637-644.

55. Wong CH, Jenne CN, Lee WY, Léger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science.* 2011;334(6052):101-105.

56. Kim BJ, Jones HP. Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-γ by CD4+ T cells. *Brain Behav Immun.* 2010;24(7):1126-1136.

57. Kuylenstierna C, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *Eur J Immunol.* 2011;41(7):1913-1923.

58. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol.* 2010;185(5):2721-2729.

59. Kurioka A, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* 2015;8(2):429-440.

60. Le Bourhis L, et al. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog.* 2013;9(10):e1003681.

61. Leeansyah E, et al. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. *PLoS Pathog.* 2015;11(8):e1005072.

62. Kawakami K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *Eur J Immunol.* 2003;33(12):3322-3330.

63. De Santo C, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest.* 2008;118(12):4036-4048.

64. Wang H, et al. MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun.* 2018;9(1):3350.

65. van Wilgenburg B, et al. MAIT cells contribute to protection against lethal influenza infection in vivo. *Nat Commun.* 2018;9(1):4706.

66. Banuelos J, et al. BCL-2 protects human and mouse Th17 cells from glucocorticoid-induced apoptosis. *Allergy.* 2016;71(5):640-650.

67. Anderson MA, et al. The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. *Blood.* 2016;127(25):3215-3224.

68. Lee HC, Shibata H, Ogawa S, Maki K, Ikuta K. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *J Immunol.* 2005;174(12):7800-7806.

69. Franchimont D, et al. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. *J Immunol.* 2002;168(5):2212-2218.

70. Kakal JA, Ghazawi FM, Faller EM, Sugden SM, Parmar P, MacPherson PA. Transcriptional regulation of the IL-7Rα gene by dexamethasone and IL-7 in primary human CD8 T cells. *Immunogenetics.* 2017;69(1):13-27.

71. Shimba A, et al. Glucocorticoids Drive Diurnal Oscillations in T Cell Distribution and Responses by Inducing Interleukin-7 Receptor and CXCR4. *Immunity.* 2018;48(2):286-298.e286.

72. Mackall CL, Fry TJ, Gress RE. Harnessing the biology of IL-7 for therapeutic application. *Nat Rev Immunol.* 2011;11(5):330-342.

73. Ramesh R, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med.* 2014;211(1):89-104.

74. Banuelos J, Cao Y, Shin SC, Lu NZ. Immunopathology alters Th17 cell glucocorticoid sensitivity. *Allergy.* 2017;72(3):331-341.

75. Chen ML, et al. Physiological expression and function of the MDR1 transporter in cytotoxic T lymphocytes. *J Exp Med.* 2020;217(5).

76. Fergusson JR, Ussher JE, Kurioka A, Klenerman P, Walker LJ. High MDR-1 expression by MAIT cells confers resistance to cytotoxic but not immunosuppressive MDR-1 substrates. *Clin Exp Immunol.* 2018;194(2):180-191.

77. U M, Shen L, Oshida T, Miyauchi J, Yamada M, Miyashita T. Identification of novel direct transcriptional targets of glucocorticoid receptor. *Leukemia.* 2004;18(11):1850-1856.

78. Kim D, et al. Anti-inflammatory Roles of Glucocorticoids Are Mediated by Foxp3(+) Regulatory T Cells via a miR-342-Dependent Mechanism. *Immunity.* 2020;53(3):581-596.e585.

79. Rocamora-Reverte L, et al. Glucocorticoid Receptor-Deficient Foxp3(+) Regulatory T Cells Fail to Control Experimental Inflammatory Bowel Disease. *Front Immunol.* 2019;10(472.

80. Prenek L, et al. Regulatory T cells are less sensitive to glucocorticoid hormone induced apoptosis than CD4(+) T cells. *Apoptosis.* 2020;25(9-10):715-729.

81. Joller N, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity.* 2014;40(4):569-581.

82. Kurtulus S, et al. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest.* 2015;125(11):4053-4062.

83. Haeryfar SM, DiPaolo RJ, Tscharke DC, Bennink JR, Yewdell JW. Regulatory T cells suppress CD8+ T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities. *J Immunol.* 2005;174(6):3344-3351.

84. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. *Immunity.* 2015;43(3):566-578.

85. Terashima A, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med.* 2008;205(12):2727-2733.

86. Kurioka A, Walker LJ, Klenerman P, Willberg CB. MAIT cells: new guardians of the liver. *Clin Transl Immunology*. 2016;5(8):e98.

87. Hayakawa Y, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol.* 2001;31(6):1720-1727.

88. Smyth MJ, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* 2002;99(4):1259-1266.

89. Nishimura T, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol.* 2000;12(7):987-994.

90. Nakagawa R, et al. Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by alpha-galactosylceramide. *J Immunol.* 2004;172(11):6550-6557.

91. Paget C, Chow MT, Duret H, Mattarollo SR, Smyth MJ. Role of γδ T cells in α-galactosylceramide-mediated immunity. *J Immunol.* 2012;188(8):3928-3939.

92. Choi J, Rudak PT, Lesage S, Haeryfar SMM. Glycolipid Stimulation of Invariant NKT Cells Expands a Unique Tissue-Resident Population of Precursors to Mature NK Cells Endowed with Oncolytic and Antimetastatic Properties. *J Immunol.* 2019;203(7):1808-1819.

93. Lu Y, et al. Glucocorticoid receptor promotes the function of myeloid-derived suppressor cells by suppressing HIF1α-dependent glycolysis. *Cell Mol Immunol.* 2018;15(6):618-629.

94. Hatziioannou A, Alissafi T, Verginis P. Myeloid-derived suppressor cells and T regulatory cells in tumors: unraveling the dark side of the force. *J Leukoc Biol.* 2017;102(2):407-421.

95. Gündisch S, Boeckeler E, Behrends U, Amtmann E, Ehrhardt H, Jeremias I. Glucocorticoids augment survival and proliferation of tumor cells. *Anticancer Res.* 2012;32(10):4251-4261.

96. Tian D, Tian M, Han G, Li JL. Increased glucocorticoid receptor activity and proliferation in metastatic colon cancer. *Sci Rep.* 2019;9(1):11257.

97. Obradović MMS, et al. Glucocorticoids promote breast cancer metastasis. *Nature.* 2019;567(7749):540-544.

98. Hu D, et al. Essential role of IL-10/STAT3 in chronic stress-induced immune suppression. *Brain Behav Immun.* 2014;36(118-127.

99. Glavin GB, Paré WP, Sandbak T, Bakke HK, Murison R. Restraint stress in biomedical research: an update. *Neurosci Biobehav Rev.* 1994;18(2):223-249.

100. Budiu RA, et al. Restraint and Social Isolation Stressors Differentially Regulate Adaptive Immunity and Tumor Angiogenesis in a Breast Cancer Mouse Model. *Cancer Clin Oncol.* 2017;6(1):12-24.

101. Lu ZW, Song C, Ravindran AV, Merali Z, Anisman H. Influence of a psychogenic and a neurogenic stressor on several indices of immune functioning in different strains of mice. *Brain Behav Immun.* 1998;12(1):7-22.

102. Sommershof A, Scheuermann L, Koerner J, Groettrup M. Chronic stress suppresses anti-tumor TCD8+ responses and tumor regression following cancer immunotherapy in a mouse model of melanoma. *Brain Behav Immun.* 2017;65(140-149.

103. Hou N, et al. A novel chronic stress-induced shift in the Th1 to Th2 response promotes colon cancer growth. *Biochem Biophys Res Commun.* 2013;439(4):471-476.

104. Patchev VK, Patchev AV. Experimental models of stress. *Dialogues Clin Neurosci.* 2006;8(4):417-432.

105. Servatius RJ, Salameh G, Coyle KM, Paré WP. In: Fink G ed. *Encyclopedia of Stress (Second Edition)*. New York: Academic Press; 2007:389-390.

106. Salter EW, Sunstrum JK, Matovic S, Inoue W. Chronic stress dampens excitatory synaptic gain in the paraventricular nucleus of the hypothalamus. *J Physiol.* 2018;596(17):4157-4172.

107. Sood P, Priyadarshini S, Aich P. Estimation of psychological stress in humans: a combination of theory and practice. *PLoS One.* 2013;8(5):e63044.

108. Estryn-Behar M, et al. Emergency physicians accumulate more stress factors than other physicians-results from the French SESMAT study. *Emerg Med J.* 2011;28(5):397-410.

109. Kiecolt-Glaser JK, Preacher KJ, MacCallum RC, Atkinson C, Malarkey WB, Glaser R. Chronic stress and age-related increases in the proinflammatory cytokine IL-6. *Proc Natl Acad Sci U S A.* 2003;100(15):9090-9095.

110. Damjanovic AK, et al. Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients. *J Immunol.* 2007;179(6):4249-4254.

111. Cohen S, Tyrrell DA, Smith AP. Psychological stress and susceptibility to the common cold. *N Engl J Med.* 1991;325(9):606-612.

112. Gourdy P, et al. Relevance of sexual dimorphism to regulatory T cells: estradiol promotes IFN-gamma production by invariant natural killer T cells. *Blood.* 2005;105(6):2415-2420.

113. Bernin H, Fehling H, Marggraff C, Tannich E, Lotter H. The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. *Med Microbiol Immunol.* 2016;205(4):321-332.

114. Yu C, et al. Mucosal Associated Invariant T (MAIT) Cell Responses Differ by Sex in COVID-19. *Med (N Y).* 2021.

115. Holderness J, Hedges JF, Ramstead A, Jutila MA. Comparative biology of γδ T cell function in humans, mice, and domestic animals. *Annu Rev Anim Biosci.* 2013;1(99-124.

116. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory type II natural killer T lymphocytes in health and disease. *Scand J Immunol.* 2012;76(3):246-255.

117. Miles FL, Pruitt FL, van Golen KL, Cooper CR. Stepping out of the flow: capillary extravasation in cancer metastasis. *Clin Exp Metastasis.* 2008;25(4):305-324.

118. Cullen R, Germanov E, Shimaoka T, Johnston B. Enhanced tumor metastasis in response to blockade of the chemokine receptor CXCR6 is overcome by NKT cell activation. *J Immunol.* 2009;183(9):5807-5815.

119. Kopper L, Van Hanh T, Lapis K. Experimental model for liver metastasis formation using Lewis lung tumor. *J Cancer Res Clin Oncol.* 1982;103(1):31-38.

120. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* 2006;6(4):318-328.

121. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol.* 2003;24(8):444-448.

122. Yaribeygi H, Panahi Y, Sahraei H, Johnston TP, Sahebkar A. The impact of stress on body function: A review. *Excli j.* 2017;16(1057-1072.

123. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol.* 2005;5(3):243-251.

124. Fujii SI, Shimizu K. Immune Networks and Therapeutic Targeting of iNKT Cells in Cancer. *Trends Immunol.* 2019;40(11):984-997.

125. Lukasik Z, Elewaut D, Venken K. MAIT Cells Come to the Rescue in Cancer Immunotherapy? *Cancers (Basel).* 2020;12(2).

Appendices

Appendix A: Copyright approval obtained from John Wiley and Sons

Appendix A continued

Type of use	Dissertation/Thesis
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Portion	Full article
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Title	The impacts of psychological stress on innate-like invariant T cell survival, phenotype, and function
Institution name	The University of Western Ontario
Expected presentation date	Apr 2021
Requestor Location	Mr. Patrick Rudak [redacted] [redacted] Canada Attn: Mr. Patrick Rudak
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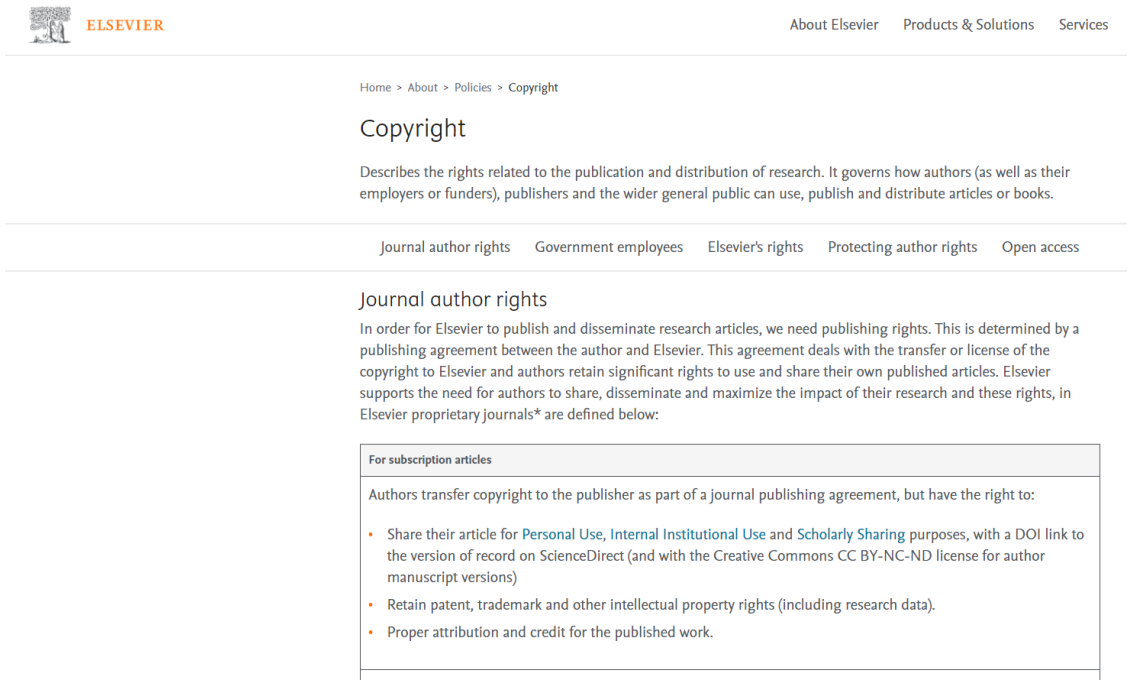

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Part II

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Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes

Author: Patrick T. Rudak,Rakshith Gangireddy,Joshua Choi,Amer M. Burhan,Kelly L. Summers,Dwayne N. Jackson,Wataru Inoue,S.M. Mansour Haeryfar

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Chronic stress physically spares but functionally impairs innate-like invariant T cells

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BACK CLOSE WINDOW

Curriculum Vitae

Patrick Rudak

Educational Background:

2015 – present **Doctor of Philosophy**, Microbiology and Immunology
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2010 – 2015 **Honours Bachelor of Science**, Biochemistry (Co-Op)
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Senior thesis project in Dr. Jonathan Bramson's laboratory (McMaster Immunology Research Centre)
Thesis title: "Rapamycin and salicylate modulate the metabolic and phenotypic characteristics of chimeric antigen receptor (CAR) T cells"

Publications:

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 physically spares but functionally impairs innate-like invariant T cells. *Cell Reports*. 2021; 35(2): 108979. DOI: 10.1016/j.celrep.2021.108979

2. **Rudak PT**, Yao T, Richardson CD, Haeryfar SMM. Measles Virus Infects and Programs MAIT Cells for Apoptosis. *The Journal of Infectious Diseases*. 2021; 223(4): 667-672. DOI: 10.1093/infdis/jiaa407

3. Choi J, **Rudak PT**, Lesage S, Haeryfar SMM. Glycolipid Stimulation of Invariant NKT Cells Expands a Unique Tissue-Resident Population of Precursors to Mature NK Cells Endowed with Oncolytic and Antimetastatic Properties. *The Journal of Immunology*. 2019; 203(7): 1808-1819. DOI: 10.4049/jimmunol.1900487

4. **Rudak PT**, Gangireddy R, Choi J, Burhan AM, Summers KL, Jackson DN, Inoue W, Haeryfar SMM. Stress-elicited glucocorticoid receptor signaling upregulates TIGIT in innate-like invariant T lymphocytes. *Brain, Behavior, and Immunity*. 2019; 80: 793-804. DOI: 10.1016/j.bbi.2019.05.027

5. Khadir F, Shaler CR, Oryan A, **Rudak PT**, Mazzuca D, Habibzadeh S, Haeryfar SMM, Rafati S. Therapeutic control of Leishmaniasis by inhibitors of the

mammalian target of rapamycin. *PLoS Neglected Tropical Diseases*. 2018; 12(8): e0006701. DOI: 10.1371/journal.pntd.0006701

6. **Rudak PT**, Choi J, Haeryfar SMM. MAIT cell-mediated cytotoxicity: roles in host defense and therapeutic potentials in infectious diseases and cancer. *The Journal of Leukocyte Biology*. 2018; 104(3): 473-486. DOI: 10.1002/JLB.4RI0118-023R

7. Haeryfar SMM, Shaler CR, **Rudak PT**. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunology, Immunotherapy*. 2018; 67(12): 1885-1896. DOI: 10.1007/s00262-018-2132-1

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. MAIT cells launch a rapid, robust, and distinct hyperinflammatory response 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*. 2017; 15(6): e2001930. DOI: 10.1371/journal.pbio.2001930

9. Szabo PA, **Rudak PT**, Choi J, Xu SX, Schaub R, Singh B, McCormick JK, Haeryfar SMM. 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 Diseases*. 2017; 215(5): 824-829. DOI: 10.1093/infdis/jiw646

10. **Rudak P**, Su B, Yin C. The Metabolism of L-Arginine and its Importance in Immune Function. *Meducator*. 2014; 26: 15-18.

Manuscripts Under Review:
* denotes co-primary authorship

1. Yao T*, **Rudak PT***, Laumont CM, Michaud AR, Knier NN, Foster PJ, McWilliam HEG, Villadangos JA, Nelson BH, DiMattia GE, Shepherd TG, Haeryfar SMM. MAIT cells accumulate in ovarian cancer-elicited ascites where they retain their capacity to respond to an MR1 ligand, IL-12 and IL-18. *Cancer Immunology, Immunotherapy* (Under review).

2. **Rudak PT**, Haeryfar SMM. *In Vivo* Cytotoxicity by α-GalCer-transactivated NK cells. Invited book chapter for *Methods in Molecular Biology* (Under review).

Awards and Distinctions:

2018 – 2021 Alexander Graham Bell Canada Graduate Scholarship-Doctoral (CGS D) – $35,000 per annum (national award)
Natural Sciences and Engineering Research Council of Canada (NSERC)

2020	Dr. John A. Thomas Award – $1200 (institutional award) University of Western Ontario, London, ON, Canada
2015 – 2020	Western Graduate Research Scholarship – $3500-4500 per annum (institutional award) University of Western Ontario, London, ON, Canada
2019	Poster Award – $150 (national award) 32nd Annual Canadian Society for Immunology Meeting, Banff, AB, Canada
2019	Graduate Student Travel Award (First Prize) – $1000 (national award) 32nd Annual Canadian Society for Immunology Meeting, Banff, AB, Canada
2019	Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology – $2000 (institutional award) University of Western Ontario, London, ON, Canada
2018 – 2019	Ontario Graduate Scholarship – $15,000 (Declined) (provincial/institutional award) University of Western Ontario, London, ON, Canada
2018	First Place Feature Platform Presentation Award – $650 (institutional award) London Health Research Day 2018, London, ON, Canada
2018	Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology – $2000 (institutional award) University of Western Ontario, London, ON, Canada
2017 – 2018	Queen Elizabeth II Graduate Scholarship in Science and Technology – $15,000 (provincial/institutional award) University of Western Ontario, London, ON, Canada
2017	Dr. John Robinson Graduate Scholarship – $1400 (institutional award) University of Western Ontario, London, ON, Canada
2017	Poster Award – $100 (national award) 30th Annual Canadian Society for Immunology Meeting, Banff, AB, Canada
2017	Graduate Student Travel Award (Second Prize) – $500 (national award) 30th Annual Canadian Society for Immunology Meeting, Banff, AB, Canada

2017	Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology – $1000 (institutional award) University of Western Ontario, London, ON, Canada
2017	Pass with Distinction, PhD. Candidacy Evaluation (institutional distinction) MICROIMM 9950Y, University of Western Ontario, London, ON, Canada
2016	Graduate Student Teaching Assistant Award Nominee (institutional award) University of Western Ontario, London, ON, Canada
2016	Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology – $1000 (institutional award) University of Western Ontario, London, ON, Canada
2015	Graduated with *Summa Cum Laude* Distinction (BSc.) (institutional distinction) McMaster University, Hamilton, ON, Canada
2013 – 2014	Dean's Honour List (institutional distinction) McMaster University, Hamilton, ON, Canada
2010	Entrance Honour Award, Level 3 (>90% high school average) – $2000 (institutional award) McMaster University, Hamilton, ON, Canada

Employment Experience:

Sept 2014 – Dec 2014	Co-Op Student, Target Validation Division Centre for Drug Research and Development (now adMare BioInnovations) Vancouver, BC, Canada
May 2014 – Aug 2014	Research Intern, Laboratory of Dr. Guy Sauvageau Institute for Research in Immunology and Cancer University of Montreal, Montreal, QC, Canada
Jan 2013 – Aug 2013	Co-Op Student, Microbiology Laboratory Centre for Probe Development and Commercialization Hamilton, ON, Canada

Conference Attendance:
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Oral Presentations

Rudak PT*, Choi J, Haeryfar SM. Elevated glucocorticoid release during psychological stress diminishes antitumor immunity promoted by invariant NKT cells. 31st Annual Canadian Society for Immunology Meeting. London, ON, Canada, June 1-4 2018.

- Selected for 1 of 32 platform presentations out of 148 accepted abstracts

Rudak PT*, Choi J, Haeryfar SM. Endogenous glucocorticoids released during prolonged psychological stress impair anti-tumour immunity initiated by invariant natural killer T cells. London Health Research Day 2018. London, ON, Canada, May 10 2018.

- Selected for 1 of 40 platform presentations out of 391 accepted abstracts

Rudak PT*, Shaler CR, Haeryfar SM. Pathway-dependent regulation of mucosa-associated invariant T cell responses by adrenergic receptor signalling. 12th Annual Infection and Immunity Research Forum. London, ON, Canada, October 27 2017.

- Selected for 1 of 10 platform presentations out of 56 accepted abstracts

Rudak PT*, Choi J, Haeryfar SM. Potent suppression of invariant NKT cell responses in a model of chronic psychological stress. 30th Annual Canadian Society for Immunology Meeting. Banff, AB, Canada, April 7-10 2017.

- Selected for 1 of 32 platform presentations out of 110 accepted abstracts

Rudak PT*, Jackson DN, Summers K, Haeryfar SM. Chronic stress suppresses invariant NKT cell-mediated immune responses. 11th Annual Infection and Immunity Research Forum. London, ON, Canada, September 23 2016.

- Selected for 1 of 9 platform presentations out of 49 accepted abstracts

Rudak PT*, Shaler CR, Haeryfar SM. Neurotransmitters associated with stress modulate invariant NKT cell responses *in vivo*. London Health Research Day 2016. London, ON, Canada, March 29 2016.

- Selected for 1 of 32 platform presentations out of 424 accepted abstracts

Poster Presentations

Rudak PT*, Choi J, Haeryfar SM. Invariant NKT cells are unusually refractory to glucocorticoid-induced apoptosis but exhibit impaired functional fitness and fail to trigger antitumor immunity following physiological stress. 104th Annual Meeting of The American Association of Immunologists: IMMUNOLOGY 2020. Honolulu, Hawaii, USA, May 8-12 2020.

- Note: This abstract was accepted and published (*The Journal of Immunology* 2020, 204 [1 Supplement] 77.6), however the IMMUNOLOGY 2020 meeting was cancelled due to the COVID-19 pandemic

Rudak PT*, Choi J, Haeryfar SM. Stress-triggered glucocorticoid receptor signaling upregulates TIGIT in iNKT and MAIT cells. CD1-MR1: beyond MHC restricted lymphocytes. Oxford, United Kingdom, September 1-5 2019.

Shaler C, Choi J, **Rudak PT**, Haeryfar SM*. Superantigens hyperactivate MAIT cells before rendering them hyporesponsive to cognate bacterial challenges. CD1-MR1: beyond MHC restricted lymphocytes. Oxford, United Kingdom, September 1-5 2019.

Rudak PT*, Choi J, Haeryfar SM. Sustained psychological stress impairs antitumour immunity orchestrated by invariant NKT cells via intrinsic glucocorticoid receptor signaling. 32nd Annual Canadian Society for Immunology Meeting. Banff, AB, Canada, April 12-15 2019.

Choi J*, **Rudak PT**, Mullins-Dansereau V, Lesage S, Haeryfar SM. iNKT cell activation expands resident pre-MNK cells and skews their responses towards an anti-metastatic phenotype. 32nd Annual Canadian Society for Immunology Meeting. Banff, AB, Canada, April 12-15 2019.

Rudak PT*, Choi J, Haeryfar SM. Psychological stress-induced production of glucocorticoids and interleukin-10 compromise the ability of invariant NKT cells to participate in antitumor immune surveillance. 13th Annual Infection and Immunity Research Forum. Stratford, ON, Canada, October 11 2018.

Rudak PT*, Choi J, Haeryfar SM. Elevated glucocorticoid release during psychological stress diminishes antitumor immunity promoted by invariant NKT cells. 31st Annual Canadian Society for Immunology Meeting. London, ON, Canada, June 1-4 2018.

Rudak PT*, Choi J, Haeryfar SM. Glucocorticoid receptor signaling during prolonged psychological stress compromises the ability of invariant NKT cells to participate in antitumor immune surveillance. 102nd Annual Meeting of The American Association of Immunologists: IMMUNOLOGY 2018. Austin, Texas, USA, May 4-8 2018.

- Published in *The Journal of Immunology* 2018, 200 (1 Supplement) 57.48

Gangireddy RR*, **Rudak PT**, Haeryfar SM. Characterization of adrenergic receptor-mediated immunoregulation of invariant NKT cells. 12th Annual Infection and Immunity Research Forum. London, ON, Canada, October 27 2017.

Rudak PT*, Choi J, Haeryfar SM. Potent suppression of invariant NKT cell responses in a model of chronic psychological stress. 30th Annual Canadian Society for Immunology Meeting. Banff, AB, Canada, April 7-10 2017.

Rudak PT*, Haeryfar SM. Glucocorticoid-mediated suppression of invariant NKT cell responses in a model of chronic psychological stress. London Health Research Day 2017. London, ON, Canada, March 28 2017.

Rudak PT*, Shaler CR, Haeryfar SM. Mediators of stress modulate invariant NKT cell-mediated immune responses to α-Galactosylceramide. 29th Annual Canadian Society for Immunology Meeting. Ottawa, ON, Canada, April 1-4 2016.

Shaler CR*, **Rudak PT**, Choi J, Memarnejadian A, Tun-Abraham ME, McCormick JK, Lantz O, Hernandez-Alejandro R, Haeryfar SM. Human mucosa-associated invariant T cells are hyper-responsive to bacterial superantigens, rapidly becoming anergic and unresponsive to secondary bacterial challenge. 29th Annual Canadian Society for Immunology Meeting. Ottawa, ON, Canada, April 1-4 2016.

Rudak PT*, Shaler CR, Haeryfar SM. Modulation of invariant natural killer T cell responses by sympathetic nervous system neurotransmitters associated with stress. 10th Annual Infection and Immunity Research Forum. London, ON, Canada, November 6 2015.

Teaching Experience:

Sept 2019 – Apr 2020	Immediate Supervisor for Brandon Brower (Biochemistry 4486E – Biochemistry Research Project student) Thesis title: "Optimization of an IFN-γ ELISPOT assay for MAIT cells" Haeryfar Laboratory, University of Western Ontario, London, ON, Canada
Sept 2019 – Dec 2019	Instructor MICROIMM 4300A – Clinical Immunology University of Western Ontario, London, ON, Canada - Delivered three one-hour lectures entitled "Immunology & Immunity Simplified: A Refresher/Primer" and prepared the corresponding examination materials
Sept 2019 – Dec 2019	Senior Teaching Assistant MICROIMM 4300A – Clinical Immunology University of Western Ontario, London, ON, Canada
Sept 2018 – Dec 2018	Teaching Assistant MICROIMM 4300A – Clinical Immunology University of Western Ontario, London, ON, Canada
May 2018 – Aug 2018	Immediate Supervisor for Rakshith Gangireddy (NSERC Undergraduate Student Research Award recipient) Haeryfar Laboratory, University of Western Ontario, London, ON, Canada

Jan 2018 – Apr 2018	Senior Teaching Assistant MICROIMM 3620G – Immunology Laboratory University of Western Ontario, London, ON, Canada
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Jan 2017 – Apr 2017	Teaching Assistant MICROIMM 3620G – Immunology Laboratory University of Western Ontario, London, ON, Canada
Jan 2016 – Apr 2016	Teaching Assistant MICROIMM 3620G – Immunology Laboratory University of Western Ontario, London, ON, Canada

Administrative Experience:

2018	Treasurer & Planning Committee Member 13th Annual Infection and Immunity Research Forum Hosted by Dept. Microbiology and Immunology, Western University
2017	Treasurer & Planning Committee Member 12th Annual Infection and Immunity Research Forum Hosted by Dept. Microbiology and Immunology, Western University