Psychological Trauma Alters T-lymphocyte Inflammation and Redox Through Sympathetic Mechanisms

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PSYCHOLOGICAL TRAUMA ALTERS T-LYMPHOCYTE INFLAMMATION AND REDOX THROUGH SYMPATHETIC MECHANISMS

by

Safwan K. Elkhatib

A DISSERTATION

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in Partial Fulfillment of the Requirements
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Under the Supervision of Professor Adam J. Case

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Abstract

PSYCHOLOGICAL TRAUMA ALTERS T-LYMPHOCYTE INFLAMMATION AND REDOX THROUGH SYMPATHETIC MECHANISMS

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University of Nebraska Medical Center, 2022

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Posttraumatic stress disorder (PTSD) is a devastating psychiatric disorder characterized by distinctive symptom clusters, including intrusive memories (i.e., flashbacks), avoidance of related stimuli, affective changes, and hyperarousal. Strikingly, patients with PTSD face a significantly increased risk for a number of inflammation-driven pathologies, ranging from cardiovascular to autoimmune disease. Yet, the exact etiology of this increased risk remains unknown. The immune system is known to be strongly influenced by the sympathetic nervous system, and sympathetic overactivity is a hallmark of PTSD. Lymphoid organs, such as the spleen, are richly innervated by sympathetic nerve fibers which terminate near adaptive immune cells, namely T-lymphocytes. Importantly, T-lymphocytes are able to receive and synthesize catecholamines (dopamine, norepinephrine, epinephrine; neurotransmitter effectors of the sympathetic nervous system), resulting in altered function. The work herein assesses the causal role of this neuroimmune connection in the inflammation seen following psychological trauma. Firstly, a murine model of PTSD known as repeated social defeat stress (RSDS) was adapted and validated. RSDS resulted in robust increases in systemic inflammation as measured by elevated pro-inflammatory cytokines, which were also strongly linked to anxiety-like...
behavior. Within the spleen, measures of sympathetic tone were elevated, and splenic T-lymphocytes demonstrated increased mitochondrial superoxide specifically, which is a critical regulator of T-lymphocyte activation and inflammation. Within purified T-lymphocytes, RSDS increased expression of pro-inflammatory cytokines, which were strongly correlated with expression of tyrosine hydroxylase (TH; rate-limiting enzyme in catecholamine synthesis). In order to further deduce the role of catecholamines, a method for specific, sympathetic denervation (Dnx) of the spleen was developed. Dnx resulted in attenuation of T-lymphocyte-specific RSDS-induced cytokines, and reversal of mitochondrial superoxide and pro-inflammatory gene expression. Lastly, the effect of T-lymphocyte-generated catecholamines was assessed by generation of a T-lymphocyte-specific TH knockout (KO) mouse. T-lymphocyte TH KO resulted in decreased T-helper 17 (Th17) T-lymphocyte profiles after RSDS, however, the loss did not affect mitochondrial redox changes elicited by RSDS. Collectively, this work demonstrates psychological trauma works in part through sympathetic effectors—generated from both sympathetic nerves and T-lymphocytes—to regulate the T-lymphocyte redox environment and inflammation.
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List of Abbreviations

2-HG  2-hydroxyglutarate
ACE  angiotensin converting enzyme
Ach  acetylcholine
AChE  acetylcholinesterase
AIDS  acquired immunodeficiency syndrome
AngII  angiotensin II
APC  Antigen-presenting cell
AR  adrenergic receptor
ATP  adenosine triphosphate
AUC  area under the curve
AV3V  anteroventral 3rd ventricle
BH4  tetrahydrobiopterin
BM  bone marrow
BuChE  butyrylcholinesterase
Ca\(^{2+}\)  calcium
cAMP  cyclic adenosine monophosphate
CarAT  carnitine acetyltransferase
CD  cluster of differentiation
CHAT  choline acetyltransferase
ChT  Na\(^+\)-dependent choline transporter
CNS  central nervous system
CoA  coenzyme A
COMT  catechol-O-methyltransferase
ConA  concanavalin A
CpG  5'-Cytosine-phosphate-guanine-3'
CRAC  calcium-release activated Ca\(^{2+}\)
CRH  corticotrophin-releasing hormone
CT  cycle threshold
DAG  diacylglycerol
DAT  dopamine transporter
DC  dendritic cell
DMN  dorsal motor nucleus
Dnx  splenic denervation
DOCA-salt  dextro corticosterone-acetate salt
DOPA  L-3,4-dihydroxyphenylalanine
DR  dopamine receptor
DREADD  designer receptors exclusively activated by designer drugs
DSM  *Diagnostics and Statistical Manual*
DβH  dopamine beta hydroxylase
EAE  experimental autoimmune encephalitis
EDTA  ethylenediaminetetraacetic acid
ER  endoplasmic reticulum
ERK  extracellular signal regulated kinase
EZM  elevated zero maze
FBS  fetal bovine serum
FMO  fluorescence minus one
GFP  green fluorescent protein
H3  histone 3
H34K  histone 3 lysine 4
HIV  human immunodeficiency virus
HPA  hypothalamic-pituitary-adrenal
HRP  horseradish peroxidase
ICAM-1  intercellular Adhesion Molecule-1
IFN-γ  interferon-gamma
IgE  immunoglobulin E
IgG  immunoglobulin G
IL  interleukin
IP3  inositol triphosphate
JNK  c-Jun N-terminal kinase
K⁺  potassium
KLF-2  kruppel-like factor-2
KO  genetic knockout
LPS  lipopolysaccharide
M#R  M-number receptor (e.g., M1 receptor is M1R)
MAO  monoamine oxidase
MAP  mean arterial pressure
MAPK  mitogen-activated protein kinase
MFI  mean fluorescence intensity
MHC  major histocompatibility complex
MHPG  3-methoxy-4-hydroxyphenylglycol
MnSOD  manganese superoxide dismutase
MR  muscarinic Receptor
mRNA  messenger ribonucleic acid
nAchR  nicotinic acetylcholine receptor
NE  norepinephrine
NET  norepinephrine transporter
NFAT  nuclear factor of activated T-cells
NF-κB  nuclear factor kappa-light-chain enhancer of activated B-cells
NIGMS  national institute of general medical science
NIMH  national institute of mental health
O₂⁻  superoxide
OVA  ovalbumin
PAL  periarterial lymphoid sheath
PBMC  peripheral blood mononuclear cell
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PHA  phytohemagglutinin
PIP₂  phosphatidylinositol 4,5-bisphosphate
PKA  protein Kinase A
PKC  protein Kinase C
PLC-γ  phospholipase C-γ
PMA  phorbol 12-myristate 13-acetate
PNMT  phenylethanolamine n-methyltransferase
PTSD  posttraumatic stress disorder
PVN  paraventricular nucleus of the hypothalamus
RAAS  renin-angiotensin-aldosterone system
RAG2/-  recombination activating gene 2 knockout
RDoC  research domain criteria
ROC  receiver-operating characteristics
ROS  reactive oxygen species
RSDS  repeated social defeat stress
RVLM  rostral ventrolateral medulla
SCID  severe combined immunodeficiency
SDS-PAGE  sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM  standard error of the mean
SI  social interaction
SSRI  selective serotonin reuptake inhibitor
TCA  tricarboxylic acid cycle
TCR  T-lymphocyte Receptor
TGF-β  tumor growth factor beta
T₉  T-helper T-lymphocyte
TH  tyrosine hydroxylase
THCon  T-lymphocyte-specific tyrosine hydroxylase flanked by loxP control
THKO  T-lymphocyte-specific tyrosine hydroxylase knockout
TNF-α  Tumor necrosis factor alpha
TREG  regulatory T-lymphocyte
VACHT  vesicular acetylcholine transporter
VMA  vanillylmandelic acid
VMAT  vesicular monoamine transporter
VNS  vagal nerve stimulation
WT  wild type
α  alpha
β  beta
γ  gamma
Δ/δ  delta
ε  epsilon
CHAPTER 1: Introduction

Portions of the content covered in this chapter are the subject of published works by Elkhatib et al. in Biological Psychiatry (2021) and Pharmacology Research (2019).

Prologue

A Forgotten Rose: Early Origins of Psychoneuroimmunology

In 1885, a young woman presented to the office of Dr. John Noland Mackenzie with a peculiar chief complaint: an allergy to roses so severe that she couldn’t even be in the same room with the flower (a condition known at the time as “rose cold”). Dr. Mackenzie was skeptical of the woman’s claims but, nevertheless, agreed to see her. After a brief conversation, he conducted a physical exam and found no evidence of an allergic reaction. As they continued speaking, he then produced a rose that he had hidden behind a screen in the room, no more than a few feet away. The woman almost immediately reported the onset of severe symptoms, including itching in her throat and difficulty breathing. Dr. Mackenzie quickly performed another exam and was shocked to observe physical signs of allergy—shocked, because the rose was not a rose at all, but an artificial flower that had been hand-made for this encounter. The woman’s symptoms were not triggered by an actual environmental allergen but simply by the perceived presence of one.

The case is as striking now as it was then because it contradicts some of our most deeply ingrained beliefs about human physiology. The Roman physician Galen was one of the first to link form and function, connecting anatomy with physiological processes and thereby debunking the model of four humors that dated back to Hippocrates. Galen’s ideas
stood for more than a millennium and served as the foundation for later work by Ibn al-
Nafis, Vesalius, and others who offered more nuanced anatomical-physiological
correlations. Over the ensuing centuries, both scientific inquiry — and, eventually, entire
fields of medicine — were largely organized around specific organ systems.

Some of these systems were anatomically discrete, such as the gastrointestinal and
pulmonary systems, while others were more diffuse. For example, coordinated cells
throughout the body that fought off invaders were understood to be part of the immune
system. Diseases of the brain posed greater challenges to researchers due to the relative
inaccessibility of tissue and the lower validity of animal models. Pioneers in this space —
from Charcot to Jackson to Freud — ultimately split the field in two: diseases that could be
localized to the brain became the purview of Neurology and the seemingly more intangible
diseases of the mind became the field of Psychiatry. This insidious dualism continues to
propagate stigma and undermine scientific progress today.

Generally speaking, the divide and conquer approach has been a successful first step
to navigating the overwhelming complexity of the human body. This approach has yielded
innumerable scientific and medical advances. But there remain enigmas — cases like the
“rose cold” study — that do not readily fit this framework. And so, as the rest of medicine
evolved, Dr. Mackenzie’s case report was largely ignored—lost in the annals of medicine,
forgotten for nearly a century.

Fast forward to 1975. Two scientists, Robert Ader and Nicholas Cohen, were
quietly studying the phenomenon of taste aversion in rats, unaware that they were about
to stumble onto a paradigm-shifting discovery. Using a classical conditioning model
(similar to Pavlov’s dogs salivating in response to a bell), they paired an artificial flavor in
the rats’ drinking water with a nauseating immunosuppressant medication. Unsurprisingly,
the rats quickly learned to avoid the drink. The extraordinary finding was what happened
next. After the classical conditioning, when Ader and Cohen administered just the sweetener to the rats they found that some of the animals became ill and even died. The cause of death was reduced immune function. The paper astonished the scientific community. The authors had indisputably shown a direct, causal connection between brain centers involved in classical conditioning and the immune system: a psychological process could dramatically alter immune function.

Their work launched an explosion of investigations into what became the new interdisciplinary field of psychoneuroimmunology. Over the ensuing decades, investigators extended early animal research into a range of human studies that recall the case of the “rose cold.” For example, in one study, researchers recruited professionally trained actors and assessed their immune cell function before and after they were asked to portray positive or negative scenes. They found that acting out a negative scene resulted in lower immune cell proliferative responses than a positive one, demonstrating that even fabricated emotion can have physiological effects. Similarly, strong social relationships have been shown to predict better outcomes in immune-mediated diseases such as HIV or cancer, while social stressors can adversely affect immune function. The psychoneuroimmune connection is clearly real, but how exactly does it work?

A first piece of data came from the work of David Felten. Over the course of the 1980s, he painstakingly examined major immune organs (including the lymph nodes, spleen, and bone marrow) at the cellular and molecular level. In elegant detail, he demonstrated that sympathetic nerve fibers were terminating mere nanometers away from immune cells (Figure 1). Moreover, he established that these connections were functionally significant and that the immune cells were responding directly to neurotransmitters.

These findings offered one answer for how signals from the brain could directly influence immunity, but they left open the converse question: in what ways might the
immune system affect individuals’ thoughts, feelings, and behavior? Longstanding dogma in neurobiology was that the brain is “immune-privileged,” insulated from circulating factors by the blood-brain barrier. The idea of immune cells infiltrating the central nervous system was rejected out of hand. And yet many infectious illnesses did seem to impact mood and cognition – how was it that peripheral immune signals could influence the brain?

The first accepted mechanism was through the action of small proteins, released by immune cells, known as cytokines. These can have prominent systemic effects, including by reaching more primitive parts of the brain that are not shielded by the blood-brain barrier (e.g., interleukin 1β and interleukin 6 acting in the hypothalamus to induce fever). Locally-released cytokines can also activate afferent nerves. Both of these
pathways represent mechanisms through which peripheral immune cells can communicate to the brain (Figure 1).

While this is where conventional thinking about immune-brain connectivity stopped, the full picture appears more nuanced. As early as the late 1700s – and then revived in the early 1960s – researchers described a lymphatic network within the meninges that surround the brain. Like the "rose cold" case study, these findings were largely ignored until they were replicated in 2015 using modern techniques. It is now accepted that meningeal lymphatics may represent a nidus wherein immune cells can act locally to affect brain function.

Moreover, research has shown that neuroimmune crosstalk exists at the level of individual cells: immune cells are able to produce their own neurotransmitters and neurons can communicate independently via cytokines. Even the most basic molecular signaling systems defy our conventional neuro/immune categorization (Figure 1).

The implications of this work are immediately relevant to patient care. Many immune treatments (e.g., corticosteroids or interferon) can affect psychiatric functioning and many illnesses (e.g., systemic lupus erythematosus and auto immune encephalitides) have known psychiatric manifestations. Modern psychiatrists must be fluid in their ability to formulate cases across the full spectrum of etiologies. There are also a range of treatment implications: the use of immune agents may offer novel avenues for psychiatric conditions (such as the use of cyclooxygenase-2 inhibitors for depression, and, in a fascinating study, researchers leveraged Ader and Cohen’s work and used behavioral conditioning to reduce the dose of immunosuppressant medications in transplant patients).

Over 100 years ago, a simple case study defied the dogma of contemporary medicine. Even today, the story continues to challenge our intuition. Was this a classic allergic reaction? Is the patient’s condition best described as a psychiatric, neurologic, immune,
or psychosomatic disorder? Embracing the complexity of neuroimmune functioning represents a crucial step towards better understanding the pathophysiology of psychiatric illness and a much-needed opportunity to improve the lives of patients. While our understanding of the dynamic interconnectivity between immune and nervous functioning remains nascent, the field is ripe for progress and further investigation as outlined and completed herein. In order to fully harness the complexities of neuroimmunity, we must first more fully elucidate the mechanism by which the nervous and immune systems interact.

To this end, the following introductory chapter will provide a thorough overview of 1) autonomic neurotransmission, 2) T-lymphocyte biology, 3) interactions of autonomics and T-lymphocytes, and 4) the role of neurotransmission in T-lymphocytes in the context of the psychiatric condition posttraumatic stress disorder.

**Historical Introduction to Neurotransmission**

“The night before Easter Sunday of [1920] I awoke, turned on the light and jotted down a few notes on a tiny slip of thin paper. Then I fell asleep again. It occurred to me at 6:00 o’clock in the morning that during the night I had written down something important, but I was unable to decipher the scrawl. The next night, at 3:00 o’clock, the idea returned. It was the design of an experiment to determine whether or not the hypothesis of chemical transmission that I had uttered 17 years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart…”

From the autobiography of Nobel Laureate Otto Loewi\(^\text{12}\), this passage details the initial experiment that opened the door for our understanding of chemical signal transmission between neurons. This “simple experiment” involved two frog hearts beating in perfusate—one vagally denervated, and one with the vagus nerve yet intact. Dr. Loewi
stimulated the intact vagus nerve and found a decrease in the heart rate; a well-known phenomenon even in 1920. The addition of the perfusate from the vagally-intact heart to the second denervated heart still showed a decrease in heart rate, as if its vagus nerve had been stimulated. He called this unknown, humoral factor “Vagustoff”. The discovery earned Otto Loewi and Henry Dale the Nobel Prize in Medicine in 1936, and introduced the world of neuroscience to acetylcholine (Ach). This insight also served as fodder for a greater debate raging within the field of neuroscience. Dubbed “the war of sparks and soups”, neuroscientists familiar with denervation studies argued for purely electrical transmission while early pharmacologists believed there to be a chemical messenger\textsuperscript{13}. The integral link between ligand binding and electrical potential changes in post-synaptic neurons was yet to be elucidated.

It is now evident that neurons communicate through both chemical and electrical signal transmission. Experiments on giant squid axons earned Hodgkin and Huxley the Nobel Prize in 1963 while answering many questions about the nature of electrical synapses\textsuperscript{14}. Yet, the breadth and depth of variation in the chemical messengers is something that, almost a century later, continues to elude the full understanding of physiologists. One area in particular that remains elusive is the role of neurotransmitters within the immune system. These mobile defensive cells share uncanny similarities to their neuronal counterparts in that they possess the ability to both produce and respond to neurotransmitters. Experimental evidence has suggested their responsiveness to neurotransmitters may play a potential role in various inflammatory diseases, such as cardiovascular disease, but the mechanisms of neurotransmission in immune cells remains unclear. Within this introduction, we discuss the known effects of autonomic regulation on a specific class of immune cell (i.e. T-lymphocytes), and further examine the interplay of these cells in health and disease.
**Autonomic Neurotransmitters: The Basics**

**What defines a neurotransmitter?**

The term neurotransmitter generally describes a heterogeneous, yet essential, signaling molecule. While there does not exist a single, explicitly stated definition, the traditional one is predicated on the neurotransmitter meeting a few criteria; it must be a molecule or peptide, synthesized in neurons, that is released at the synaptic cleft to bind a respective receptor\(^{15}\). Recent discoveries across a number of fields have begun to redefine this relatively limited definition of neurotransmitters. Steroid molecules have been shown to have neuromodulating properties\(^{16}\), while small, ubiquitous molecules like adenosine triphosphate (ATP) can function as effector molecules when co-released with neurotransmitters\(^{17}\). In cancer biology, neurotransmitters released from non-neuronal cancers are being investigated for their ability to dramatically alter the tumor microenvironment\(^{18}\). Even pathways of “neurotransmitters” that are synthesized centrally, yet act distant sites (like vasopressin and oxytocin), do not fit neatly into traditional definitions of neurotransmitters.

While our understanding of neurotransmitter physiology may be incomplete, the clinical usage of neurotransmitter modulation serves as the foundation of pharmacotherapy in a broad array of pathologies, ranging from cardiovascular disease to psychiatric disorders. The diverse range of their pharmacological targets, and thus effects (e.g., elements that make neurotransmission-modulating therapies most useful) also is their downfall. The dose-limiting side effects of these drugs continue to be a major barrier to effective treatment of many disease states. It is well known that the majority of anti-depressive, anti-psychotic, and anti-epileptic drugs have off-target effects, with long-term sequelae being discovered regularly.


**Synthesis of neurotransmitters**

**Catecholamine Synthesis**

While the discovery of epinephrine is decidedly much less elegant than that of Ach, it is an equally rewarding look into the past. Oliver and Schafer injected adrenal extracts into a variety of animals and found changes in heart and respiration rates; all changes now classically associated with sympathetic activation\(^{19}\). They found that this phenomena was unique to injection of the medullary portion of the adrenal gland, not when extract from the cortex was injected\(^{19}\). The molecule in this extract, later termed and trademarked adrenaline/epinephrine, is one of several biogenic amines.

Biogenic amines (also termed monoamines) are a crucial class of neurotransmitters that can be further subdivided into three groups: catecholamines (epinephrine, norepinephrine, and dopamine), indolamines (serotonin and melatonin), and histamine, but this review will focus specifically on catecholamines. The catecholamines are a class of neurotransmitters based on their namesake in that they all contain a hydroxylated phenol ring known as a catechol moiety. This electron rich structure has been shown to participate in redox chemistry, with both enzymatic and non-enzymatic reactions forming cytotoxic free radicals that have been proposed to contribute to catecholamine-induced neurotoxicity\(^{20-23}\). This is in addition to evidence of catecholamine-receptor binding-induced changes in the cellular redox environment\(^{24-26}\), which is further discussed later within this chapter. All catecholamines are synthesized from the non-essential amino acid L-tyrosine, which enters the cytosol of a catecholaminergic neuron through Na\(^+\)-dependent transporters. The first step in this pathway is the hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (Figure 2). This reaction
requires molecular oxygen, iron, and the reduced co-factor tetrahydrobiopterin (BH4). This is the rate-limiting enzyme for catecholamine synthesis that is subject to negative feedback by its downstream products, and has complex regulation which has been reviewed elsewhere\textsuperscript{27}.

The next step in the catecholamine synthetic pathway is mediated by DOPA decarboxylase, which results in the generation of dopamine (Figure 2). Dopamine’s central production in the substantia nigra of the basal ganglia in the brain plays an essential role in the neuro-circuitry involved in voluntary movement, and decreased production of this neurotransmitter in this nuclei leads to Parkinson’s disease\textsuperscript{28}. Modulation of dopamine levels (with precursors able to cross the blood brain barrier) are the mainstay of pharmacotherapy in Parkinson’s disease. The role of dopamine in Parkinson’s provides insight into the complex chemistry of dopamine synthesis and metabolism, where dopamine is both central to treatment of the disease and implicated in its pathophysiology\textsuperscript{29}. Dopamine is also applied clinically in the management of shock because of its selective ability to dilate the renal vasculature in rabbits, while providing both ionotropic and chronotropic support through an indirect noradrenergic mechanism\textsuperscript{30}.

Dopamine is then transported into monoamine storage vesicles by the vesicular monoamine transporter (VMAT), where the second carbon of the ethylamine side chain

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{catecholamine_synthetic_pathway.png}
\caption{Catecholamine synthetic pathway.}
\end{figure}

The catecholamines (dopamine, norepinephrine, and epinephrine) all originate from the amino acid tyrosine. Classically, dopamine $\beta$-hydroxylase is membrane-bound, which is indicated with a circle.
on dopamine is hydroxylated by the vesicular-membrane bound dopamine β-hydroxylase (DβH; Figure 2), forming norepinephrine (NE) within the vesicle\textsuperscript{31}. NE is the major neurotransmitter used at the sympathetic post-ganglionic synapse. Action potentials mediate the influx of calcium (Ca\textsuperscript{2+}), which cause vesicular translocation and expulsion of vesicular NE into the synapse\textsuperscript{32}, where it perpetuates signal transmission via binding to one of the respective adrenergic receptors.

The final step in catecholamine synthesis is the formation of epinephrine, which mainly occurs in the adrenal medulla (Figure 2). Sympathetic preganglionic neurons terminate at neuroendocrine chromaffin cells in the adrenal medulla that contain the cytosolic enzyme phenylethanolamine-N-methyltransferase (PNMT) that methylates the amine group on NE to produce epinephrine\textsuperscript{33}. These neuroendocrine cells bear resemblance to sympathetic post-ganglionic neurons, only different in that they release their vesicular contents into the systemic circulation by way of a rich vascular network. Within the cortex of the adrenal gland, glucocorticoids released from the fasciculata layer increase the synthesis of epinephrine by increasing the half-life of PNMT mRNA and protein\textsuperscript{34}. Both epinephrine and NE are stored in chromaffin cell vesicles and released into the circulation, although the ratio of release into the bloodstream in humans and rats is approximately 4:1, respectively\textsuperscript{35}.

**Cholinergic synthesis**

The molecule Ach was synthesized in a German lab by Adolf von Baeyer in 1867, decades before its physiological role was ever identified\textsuperscript{36}. After its identification by Otto Loewi in 1921, Ach was characterized as a natural product after extraction from spleens of horses and oxen by Dale and Dudley in 1929\textsuperscript{37}. Ach is now known to be a crucial neurotransmitter, serving as the major chemical signal in the neuromuscular junction,
autonomic ganglia, parasympathetic nervous system, as well as the cholinergic anti-inflammatory reflex during sepsis.

Choline is an essential nutrient that circulates in human plasma. At cholinergic nerve terminals, choline is transported from the extracellular space by a Na⁺-dependent choline transporter (ChT) that serves as the rate-limiting step in the synthesis, with the majority of choline utilized in Ach synthesis derived from recycled choline from Ach metabolism at the synaptic cleft. The mitochondrial-derived and Krebs cycle substrate acetyl coenzyme A (CoA) serves as a substrate for the acetylation of choline in the cytoplasm by choline acetyltransferase (CHAT) to form Ach. Ach is then transported into synaptic vesicles by vesicular acetylcholine transporter (VACHT), which functions as an antiporter to couple the translocation of Ach into the vesicle for the efflux of protons. Ach is released from the synaptic vesicle to bind its respective receptor subtypes, which will be discussed in detail later within this chapter.

**Reception of neurotransmitters**

**Adrenergic receptors**

The adrenergic receptors (ARs) were initially distinguished in 1948 by Raymond Ahlquist, who divided them into alpha (α) and beta (β) receptors based on the physiological response observed when one of six adrenergic agonists were used. He ranked the effects of agonists on various tissues, and divided them into two groups (i.e. α and β) for convenient notation based on the opposing excitatory and inhibitory effects of each purported. This designation of convenience remains with us today. Catecholamines can bind to ARs of either the α or β isoform, and to date 6 subtypes of α receptors (α₁A, α₁B, α₁D, α₂A, α₂B, and α₂C) and 3 subtypes of β receptors (β₁, β₂, and β₃) have been identified. The location, density, and conformational state of these receptor subtypes depends on a number of physiological inputs.
Alpha-1 receptors are linked to \( G_\text{q} \) heterotrimeric G-proteins, which result in the activation of phospholipase C, leading to inositol triphosphate (IP3) formation from phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) causing intracellular Ca\(^{2+}\) release from the endoplasmic reticulum\(^4\). Diacylglycerol (DAG) is also released, activating protein kinase C (PKC), which may act on a number of different effectors, namely by phosphorylating cellular proteins\(^4\). \( \alpha_2 \) post-synaptic receptors are linked to \( G_\text{i} \) and \( G_\text{o} \) proteins which function to decrease the production of cyclic adenosine monophosphate (cAMP) through inhibition of adenylyl cyclase, resulting in a decreased signaling cascade\(^4\). \( \alpha_2 \) receptors are unique in that they have been shown to be present on the pre-synaptic nerve terminal as well\(^4\). These receptors also activate \( G_{\beta\gamma} \) proteins, coupled to inward Potassium (K\(^+\)) channels that result in the hyperpolarization of cellular membranes, which inhibits voltage-gated Ca\(^{2+}\) channels, thus inhibiting post-synaptic signals\(^4\). This uniquely opposite effect of the activation of the \( \alpha_2 \)-AR has been utilized pharmacologically by the \( \alpha_2 \)-AR agonist clonidine. An early anti-hypertensive, clonidine is also now used off-label for diseases associated with increased sympathetic outflow and hyperarousal, such as post-traumatic stress disorder\(^4\).

The family of \( \beta \) receptors are all coupled to \( G_\text{s} \) G-proteins, which associate with adenylyl cyclase to increase cAMP production (for an excellent review of ARs and their ligands, see Tank et al.\(^4\)). This elevation in cAMP activates protein kinase A (PKA), allowing for the phosphorylation of many downstream targets. This canonical pathway is of important note as we discuss AR interactions and non-traditional G-protein coupled receptor signaling in T-lymphocytes.

**Dopaminergic receptors**

Dopamine, as evident by its place along the catecholamine synthetic pathway, is structurally related to NE and epinephrine. This allows it to interact with \( \alpha \) and \( \beta \) receptors
at differing affinities, despite having a greater affinity for its respective dopamine receptors (i.e., D1-D5). These receptors are best classified by their intracellular effects into D1-like and D2-like families; coupled to Ga subunits resulting in increased cAMP and coupled to Gi resulting in decreased cAMP synthesis, respectively\(^4\). However, it has been shown that this exact coupling is not always true\(^4\), which could explain the differences in cellular effects between cell types expressing the same dopamine receptor subtype.

This complexity is further convoluted in that NE has been shown to interact with dopamine receptors, although the effects of this interaction are less clear\(^4\). Dopamine receptors are involved in a diverse array of physiological functions within a variety of systems. As we will discuss further in this chapter, the existence of dopamine receptors on immune cells is not merely a coincidence. Their responses to dopamine agonism and antagonism are complex and physiologically relevant and pose significant clinical ramifications during the catecholamine-targeted pharmacotherapy of several conditions.

**Nicotinic Ach receptors**

Ach is unique in that it is able bind muscarinic receptors and nicotinic receptors, which are metabotropic and ionotropic receptors, respectively. The nicotinic Ach receptor (nAchR) is an ionotropic receptor where ligand binding results in the opening of a water-filled central pore that allows Na\(^+\) influx and K\(^+\) efflux, down their respective concentration gradients. It is structurally composed of 5 protein subunits, but various combinations of subunits can result in a significant amount of receptor diversity depending on location and cell type\(^4\). These subunit types can be classified as \(\alpha\), \(\beta\), \(\gamma\), \(\delta\), and \(\epsilon\) based on structure. The always present \(\alpha\)-subunit is defined by an extracellular pair of cysteine subunits that are required for ligand binding\(^5\).
Nicotinic muscarinic receptors

The muscarinic receptor (MR) is another class of receptor for which Ach is a ligand, with subtypes M1-M5. It was originally discovered by Henry Dale, who found its physiological effects on blood pressure to be similar to those seen with the exogenous administration of extracts from the eponymous mushroom, *Amantia muscaria*. MR differs from the nAchR in its intracellular effects upon ligand binding, and thus, its functional effects. The M1, M3, and M5 receptors are coupled to a Gq protein that result in the signaling cascade involving IP3 and DAG, similar to that of the α1-AR. The M2 and M4 receptors are coupled to G_i and G_o subunits which results in a decrease in intracellular cAMP and downregulation of PKA. These receptors also regulate inward K^+ currents that contribute to membrane depolarization and action potential propagation; an effect allowing M2 receptors to negatively regulate chronotropy in the heart. A relative lack of selective ligands for the M5 receptor has limited our ability to selectively study their contributory effects to peripheral physiology, but there is burgeoning interest in its localization and effects in the brain.

Neurotransmitter metabolism and reuptake

Catecholamines

Catecholamines are subject to re-uptake and recycling for future use at the synaptic cleft. The major mechanism of signal termination is this reuptake process, with simple diffusion away from available receptors serving as a more minor mechanism. There are a variety of transporters responsible for transport of endogenous catecholamines, with differential specificities for substrates and varying distribution in tissues. Two transporters dominate neuronal reuptake: the NE transporter (NET) and dopamine transporter (DAT). Each has a greater affinity for their eponymously named catecholamine, but all able to transport dopamine, NE, or epinephrine. NET cotransports an extracellular Na^+ as the
driving force for transporting catecholamines against their concentration gradient across to the cytoplasmic side. DAT translocates two Na⁺ and a Cl⁻ in order to energetically favor the transport of catecholamines into nerve terminals. Attenuation of catecholamine reuptake by inhibiting these transporters are the mechanism of drugs such as tricyclic antidepressants and amphetamines.

Catecholamine metabolism is a complex process that has been reviewed extensively by Eisenhofer. In brief, monoamine oxidase (MAO) is a flavin-containing enzyme that catalyzes the oxidative deamination of all catecholamines to their respective aldehyde forms. MAO is located on the outer membrane of the mitochondria, and consists of two isoforms: MAO-A and MAO-B. The majority of metabolism of biogenic amines takes place in the cytoplasm (in close proximity to the mitochondria) of sympathetic nerve terminals, secondary to dynamic outward leakage from vesicles. This complex process is balanced with rapid transport by VMAT back into the monoamine vesicles. The majority of circulating NE is transported by the higher affinity VMAT rather than metabolized by MAO. Yet, the inhibition of MAO proved to be a useful therapy in treating depression during the early 1950’s. As we have developed more targeted approaches to modulate monoamine levels, namely through inhibition of reuptake, MAO inhibitors have been mostly abandoned due to extensive side effect profiles.

The deaminated aldehydes generated by MAO are further metabolized in a series of reactions catalyzed by aldehyde reductases, aldehyde dehydrogenases, MAO, and catechol-O-methyltransferase (COMT). This can produce 3-methoxy-4-hydroxyphenylglycol (MHPG), which is subsequently oxidized to produce vanillylmandelic acid (VMA), representing the major-end product of NE, epinephrine, and dopamine metabolism. This MHPG can also be conjugated to sulfate or glucuronide in order to facilitate renal excretion. The more minor pathway, responsible for extraneuronal and adrenally-derived catecholamines, involves the O-methylation by COMT. The subsequent
metabolites are then further catabolized by a complex array of the same enzymes, resulting in VMA formation by the liver. VMA and these conjugates of MHPG represent 90% of all catecholamine metabolites in the urine\textsuperscript{29}.

**Acetylcholine**

Ach metabolism is primarily mediated by two enzymes: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), also known as pseudocholinesterase. In the synaptic cleft, AChE is responsible for the rapid hydrolysis of Ach into choline and acetate. This choline can then be transported back into the cytosol of the nerve terminal by the aforementioned ChT\textsuperscript{38}. In the circulation, BuChE synthesized in the liver is able to hydrolyze Ach, along with many other choline-containing esters ranging from aspirin to cocaine—an area of interest to potentially rescue patients during acute cocaine intoxication\textsuperscript{61}. Some individuals are homozygous for an inactivating mutation in BuChE and show complications during surgery due to the inability to hydrolyze succinylcholine, a common surgical neuromuscular-blocking agent. Nerve transmission mediated by Ach is different from that of the catecholamines in that there is no major reuptake process, so metabolism followed by reuptake of the building block choline is the predominant method.

**Autonomic Neurotransmitters and T-lymphocytes**

**Introduction to T-lymphocytes**

T-lymphocytes play a central role in the orchestration of the immune system, as most evidenced by the clinical presentation of deficient disease states such as severe combined immunodeficiency (SCID) and acquired immunodeficiency syndrome (AIDS). This functional role of T-lymphocytes in orchestrating the adaptive immune response makes them an ideal cell type to serve as a conduit for signal generation and reception. As a functional cell designed to coordinate the destruction and clearance of tolerance-failing
antigens, T-lymphocyte diversity is most crucially determined by their antigen specificity (or idiotype), as decided during thymic development by their T-lymphocyte Receptor (TCR). T-lymphocytes are able to respond to pre-processed antigens only upon their discrete presentation by antigen-presenting cells (APCs); macrophages, dendritic cells, or B-lymphocytes. These processed antigens are loaded into the major histocompatibility complex (MHC) on these APCs, which together serve as a ligand for the TCR. This resultant interaction is the 1st signal, with a subsequent 2nd signal between the T-lymphocyte and APC being necessary for T-lymphocyte activation and immune system coordination to target the respective antigen. It is an unlikely circumstance that these TCRs do in fact meet their respective epitope; around 1 in 10-100 million, depending on the T-lymphocyte subtype\textsuperscript{62, 63}. This low likelihood demands that T-lymphocytes are able to proliferate rapidly upon correct antigen presentation, as well as a need for their preservation even after clearance of the antigen.

T-lymphocyte activation is incredibly complex, and has been reviewed exceptionally elsewhere\textsuperscript{64}. In brief, canonical T-lymphocyte activation depends on the TCR-MHC interaction that triggers the activation of phospholipase C-γ (PLC-γ), which generates DAG and IP\textsubscript{3}, similar to the cascade discussed previously herein. DAG is responsible for the activation of PKC and the subsequent activation of the mitogen-activated protein kinase/extracellular signal regulated kinase (MAP/ERK) pathway, both of which result in nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB) activation, resulting in the transcription of genes crucial to activation and proliferation. Inositol 1,4,5-trisphosphate (IP\textsubscript{3}) allows for the release of Ca\textsuperscript{2+} from the endoplasmic reticulum (ER). This change in Ca\textsuperscript{2+} results in numerous events, one of which is the dephosphorylation and thus activation of nuclear factor of activated T-cells (NFAT). NFAT translocates to the nucleus, where it controls the cytokine interleukin 2 (IL-2) transcription, the principal T-lymphocyte growth factor. A milieu of other cytokines (considered the 3rd signal for T-
lymphocyte activation) are needed for full T-lymphocyte activation, function, and differentiation, but T-lymphocytes respond very differently to cytokines dependent upon their specific lineage.

T-lymphocytes themselves represent a heterogeneous subtype of lymphocytes, most practically categorized by their display of specific cluster of differentiation (CD) markers. The binary divide of CD4+ and CD8+ T-lymphocytes occurs during their selection in the thymus. CD8+ T-lymphocytes interact with MHC I, which are present on all nucleated cells and platelets. CD8+ T-lymphocytes, also termed cytotoxic T-lymphocytes, respond to cells expressing aberrant intracellular proteins within their MHC I. These could be the result of tumor cells or cells infected with an intracellular pathogen. CD8+ T-lymphocytes release cytotoxic granules that can damage membranes and activate apoptotic pathways, while also secreting cytokines (like interferon-γ (IFN-γ)) that aid in the mounting of a coordinated immune response to intracellular pathogens or tumor cells.

The other division of T-lymphocytes is the CD4+ T-lymphocytes, which communicate with MHC II, found on APCs. CD4+ T-lymphocytes then respond to the presence and/or absence of specific APC-generated cytokine signals that result in further differentiation of CD4+ T-lymphocytes (Figure 3). This too is a highly studied area, and has been reviewed elsewhere. In brief, naïve CD4+ cells can polarize to effector cell subtypes, with the most prominent types known as T-helper type 1 (T_H1) cells, T-helper type 2 (T_H2) cells, or T-helper type 17 (T_H17) cells. IL-12 secretion by dendritic cells (DCs) results in polarization to T_H1, which are characterized by IL-2, IFN-γ, and TNF-α secretion. These favor classical cell-mediated immunity, thus making them highly involved in the destruction of intracellular pathogens, tumors, and activation of cytotoxic T-lymphocytes. The absence of IL-12 and presence of IL-4 favors the T_H2 subtype, which functions to effectively activate B-lymphocytes and result in isotype switching to immunoglobulin E (IgE) and eosinophil.
activation. Together, these cells are involved in the effective elimination of helminths and parasites, as well as type 1 hypersensitivity reactions and asthma. TH17 polarization results from DC secretion of IL-23, Tumor growth factor-beta (TGF-β), and IL-6, and these TH17 cells are characterized by their high levels of IL-17 secretion. TH17 cells have been widely implicated in the pathogenesis of autoimmune diseases. In addition to polarization to effector subtypes, CD4+ T-lymphocytes can differentiate to regulatory T-lymphocytes (TREGs), which have been antithetically implicated in autoimmune diseases as well. These are phenotypically characterized by their expression of CD25 and the transcription factor FOXP3, as well as secretion of IL-10, resulting in the suppression of antigen-specific effector T-lymphocytes. Additional CD4+ subtypes such as TH9, TH22,
T_{FH} have also recently been identified, but little is known to date regarding the role of these cells in the adaptive immune response\textsuperscript{73-75}.

**Neurotransmitter Receptors on T-lymphocytes**

**Adrenergic Receptors**

Catecholamines may interact with mature T-lymphocytes most readily in three locations in the human body relevant to our discussion: intravascular space, lymphoid organs (e.g., bone marrow, spleen), and the central nervous system. In the plasma, all three catecholamines exist, with NE found at about 20% of the quantity of epinephrine and dopamine\textsuperscript{76}. In the intravascular space, T-lymphocytes exist as approximately 70% of peripheral blood mononuclear cells (PBMCs)\textsuperscript{77}. In the spleen, sympathetic innervation is regional and specific. Innervation is found in the white pulp that surrounds central arteries, as periarterial lymphoid sheaths (PALs), that are mostly composed of T-lymphocytes\textsuperscript{78, 79}. Catecholamines may also interact with T-lymphocytes in the central nervous system, as inflammation reduces the fortitude of the blood brain barrier, allowing T-lymphocytes to penetrate and possibly directly interact with catecholamines (a thorough review of this topic can be found here\textsuperscript{80}).

In order to best understand the functional effect of catecholamines on T-lymphocytes, we must begin a nuanced discussion of AR expression. A brief dive into the literature on the expression of ARs on T-lymphocytes will immediately appear contradictory. A number of studies have shown that T-lymphocytes exclusively express β_{2}-AR\textsuperscript{81-88}. However, this has been contradicted by other works as well, with reports of expression of β_{1}-ARs, α_{1}-ARs, and α_{2}-ARs on T-lymphocytes. To better dissect this complex discussion, we will review each AR subtype individually.
α-adrenergic receptors

Early studies utilized α agonists and found no changes in T-lymphocyte proliferation, which served as proof for lack of α-ARs in T-lymphocytes. Additionally, there was a reported absence of α1-AR mRNA in PBMCs, while more recent studies have shown that α1-AR mRNA is present in PBMCs by in-situ hybridization. Heilig et al. found that H3-thymidine incorporation was reduced in a dose-response fashion to the α1-AR agonist phenylephrine, with this relationship disappearing with the α-AR antagonist phentolamine. Others have found no expression of α1-ARs on T-lymphocytes until activation with the mitogens (molecules that result in the activation and mitosis of T-lymphocytes, regardless of clonal specificity) phytohemagglutinin (PHA) or lipopolysaccharide (LPS), both of which resulted in increased expression of all three α1-AR. In the same study, addition of NE resulted in increased ERK activation, which could be diminished by selective α1-AR antagonism. In another study, PBMCs isolated from patients with juvenile rheumatoid arthritis were treated with phenylephrine, which resulted in cytokine changes such as increased IL-6 compared to healthy control PBMCs. In relation to T-lymphocyte proliferation, non-specific activation with the mitogen concanavalin A (ConA) followed by phenylephrine did not show any effect on proliferation, IL-4, or IFN-γ production. In an elegant study by the same group, they found that ConA-activated T-lymphocytes, treated with an MAO inhibitor, would polarize to T1,2 and produce more IL-4. This effect could be blocked by α1-AR and β2-AR antagonists, but not α1-AR or β1-AR antagonists. This shows a role for endogenous catecholamines binding α1-ARs, but not in all temporal relationships to activation. Our group has additionally observed modulation of the intracellular redox environment with α1 agonism and antagonism, further supporting the presence of this receptor on T-lymphocytes. As discussed previously, α2-AR represents a different mechanism by nature of its downstream intracellular cascade.
α2-AR agonism by clonidine of ConA-activated T-lymphocytes resulted in inhibited proliferation, as well as decreased IFN-γ and IL-4 production. This effect could be blocked by α2-AR antagonism, and partially attenuated by inhibition of PLC or PKC, indicating a role for this pathway in α2-induced T-lymphocyte inhibition95. We have also identified a role for α2-AR and the T-lymphocyte redox environment, furthering the complexity of AR signaling in these adaptive immune cells24.

As can be seen from this small sample of the literature, T-lymphocyte expression of α-AR is complex with a disparity in findings from the literature. An excellent review by Kavelaars highlights the general thematic immune changes mediated by α1-ARs on immune cells, particularly PBMCs97. In summary, in vitro, α1-AR agonism causes increased activation of T-lymphocytes, as evidenced by increased cytokine production and intracellular redox, which can be modulated with α1-AR antagonism. On the other hand, α2-AR agonism results in inhibition of T-lymphocytes through a specific pathway partially mediated by canonical changes in PLC and PKC. The functional changes represent interesting results in vitro, with little work done to discuss the possibly of these mechanisms in vivo, which could be further extended to the discussion of patients taking systemic α-AR modulating drugs.

β-adrenergic Receptors

β-AR expression was originally noted in T-lymphocytes indirectly. Application of catecholamines resulted in increased cAMP81, 98, 99, indicative of β-AR expression before any physical receptors were actually detected (reviewed by Wolfe et al.100). We will discuss each β-AR subtype individually, with the focus largely on β2-AR differential expression and function.

In terms of the β1-AR, there is little evidence of its expression, but reports do exist regarding a functional role of this receptor in effector T-lymphocytes. A study by
Takayanagi et al. found that NE suppressed IFN-γ and TNF-α production in murine intestinal intraepithelial T-lymphocytes through β₁-AR, as evidenced by selective pharmacological activation and blockade. Other reports have focused on β₁-AR expression in TRGS. Freir et al. noted a greater expression of β₁-ARs on Tregs versus CD25- T-lymphocytes, which were both isolated from healthy human patients undergoing an acute physical stressor. β₃-AR mRNA has been detected in ConA stimulated T-lymphocytes, but without any functional changes upon β₃-AR agonism. This could be due to the fact that β₃-AR agonism has been shown to generate less cAMP than other β-AR subtypes. Recently, β₃-ARs on brown adipose tissue have demonstrated a critical role in psychological stress-induced IL-6 production.

In contrast, a number of sources demonstrate functional β₂-AR expression on T-lymphocytes. For example, naïve CD4+ T-lymphocytes strongly express high affinity, saturable β₂-ARs (exhaustively reviewed in and 88), and this expression is tightly regulated as T-lymphocytes differentiate. Work by Sanders and colleagues has shown that upon polarization to the Th1 and Th2 lineages, the expression level of the β₂-AR is increased or decreased, respectively. The mechanism of this repression in Th2 cells is driven by methylation of CpG islands in the β₂-AR gene promoter, decreased histone 3 (H3) and H4 acetylation, and decreases in histone 3 lysine 4 (H34K) methylation. Th1 cells express opposite responses to upregulate expression of the β₂-AR. When naïve CD4+ T-lymphocytes are exposed to NE or selective β₂-AR agonists, IL-2 and IFN-γ production upon subsequent activation is reduced. This result is consistent with the traditional view that β₂-AR activation results in increased cAMP, resulting in PKA activation and overall suppression of T-lymphocytes. This cytokine response varies temporally with the addition of NE; Th1 polarization and activation followed by NE results in increased IFN-γ production from these cells. We have recently proposed that this complex regulation allows NE to play both suppressive and activating roles in the T-lymphocyte inflammatory response.
This may be due to the intracellular role of cAMP during different activation states, or may even be due to non-canonical β-AR mechanisms\textsuperscript{111}. Further investigation into the intracellular signaling pathways is warranted to elucidate these complex interactions.

\textit{Dopamine receptors}

Dopamine receptors (DRs) of all subtypes (D1-D5) have been described on T-lymphocytes\textsuperscript{112}. The expression levels of each type of receptor are dynamic and context-dependent, thus, creating a complex interplay between receptor expression and function. Therefore, it is simpler to group these DRs by their intracellular effects. In theory, D1-like family-mediated increases in cAMP should result in changes in the intracellular signaling cascade such as inhibition of ERK, c-Jun N-terminal kinase (JNK), and NF-κB activation; all of which would inhibit T-lymphocyte activation, proliferation, and cytokine production (reviewed further by Pacheco \textit{et al.}\textsuperscript{113}). Conversely to this, D2-like family agonism that functions to decrease adenylyl cyclase activity would result in increased T-lymphocyte activation. However, these global assumptions do not always appear to be true experimentally. In order to further elucidate this mechanistic control, we must look at receptors within each DR grouping more closely.

D1-like receptor agonism with physiological concentrations of dopamine \textit{in vitro} has been shown to impair the cytotoxicity of CD4+ and CD8+ cells, as well as reduce their proliferation by IL-2 induction\textsuperscript{114}. This stimulation of the D1-like family has also been implicated in the polarizing of naïve CD4+ T-lymphocytes to T\textsubscript{H}2 by a DC-mediated dopamine dose-dependent increase in cAMP levels\textsuperscript{115}. In another study by Nakano \textit{et al.}, a small molecule D1-like receptor antagonist resulted in DC-mediated IL-23 production that resulted in T\textsubscript{H}17 polarization\textsuperscript{116}; a result that has been shown both \textit{in vitro} and \textit{in vivo}\textsuperscript{117}. In addition to this, it has been shown that activation of the D1-like receptors on T\textsubscript{REGS} results in decreased IL-10 and TGF-β, as well as their decreased proliferation\textsuperscript{102}. 
These cytokines are the primary mechanism by which T\textsubscript{REGs} are able to inhibit effector T-lymphocyte proliferation\textsuperscript{72}, thus dopamine appears to inhibit those processes. Dopamine at high concentrations (10-100 µM) can also effectively inhibit already activated effector T-lymphocytes. \textit{Ex vivo} studies have shown that mitogen-activated T-lymphocytes treated with dopamine showed reduced proliferation and synthesis of IL-4 and IFN-γ\textsuperscript{118}. Another study by Ghosh \textit{et al.} showed that 3-5 ng/mL of dopamine added to human T-lymphocytes, activated with anti-CD3 antibody, showed significantly reduced proliferation and secretion of IL-2, IFN-γ, and IL-4\textsuperscript{119}. In summary, the activation of D1-like receptors results in decreased functionality of CD4+ T-lymphocytes as well as decreasing the ability of T\textsubscript{REGs} to suppress effector T-lymphocytes.

D2-like family reception is less consistent than the D1-like family between receptor subtypes. Levite \textit{et al.} showed that selective agonism of D2R and D3R on T-lymphocytes from healthy humans resulted in the induction of adhesion to fibronectin. This high molecular weight glycoprotein, found in the extracellular matrix, functions to promote cellular trafficking and adhesion\textsuperscript{120}. In naïve CD8+ T-lymphocytes from both humans and mice, dopamine agonism at D3R-induced adhesion to fibronectin and Intercellular Adhesion Molecule 1 (ICAM-1/CD54)\textsuperscript{121}, as well increased T-lymphocyte proliferation\textsuperscript{122}. In addition to this, agonism at D2R and D3R resulted in increased expression (mRNA and protein) of IL-10 or TNF-α, respectively\textsuperscript{123}. This enhanced production of IL-10 would function to inhibit effector T-lymphocytes, while D3R-mediated chemotaxis would promote increased function of these CD8+ cells—a contradictory and complex observation. This overall phenotype promotes cellular immunity, with predominant polarization to T\textsubscript{H}1 in the CD4+ population\textsuperscript{123}. On the other hand, D4R stimulation mediates quiescence by inhibiting the phosphorylation of ERK1/ERK2, thus resulting in the up-regulation of Kruppel-like factor 2 (KLF-2)\textsuperscript{124}. This suppressive effect of D4R mimics the D1-like family activity even though these receptors act similarly to the D2-like family.
In total, it should be realized that the role of dopamine in the physiology of T-lymphocytes is decidedly complex. Dopamine can increase the homing and chemotaxis of CD8+ cells, alter the polarization of CD4+ cells, inhibit activated effector T-lymphocytes, and suppress T\textsubscript{REG} cells. These effects are mediated by the varying dopamine receptor subtypes, which have varying affinities for dopamine itself. This complex interaction is further muddied by the direct interaction of T-lymphocytes with other cell types, like DCs, which also utilize dopamine in their regulation\textsuperscript{113}.

**Acetylcholine reception**

It was originally discovered that T-lymphocytes can bind Ach through radiolabeled binding studies of MRs and nAchRs\textsuperscript{125, 126}. An excellent review by Kawashima et al.\textsuperscript{126} chronicles the history of both Ach receptors in T-lymphocytes and thymocytes. All five subtypes of MRs have been found on human T-lymphocytes at varying densities\textsuperscript{127}. The highest expressed is the M3 subtype, followed by the M4 and M5 subtype, with varying expression between individuals of the M1 and M2 receptor\textsuperscript{128}.

High expression of the M3 receptor (M3R) on T-lymphocytes does not necessarily substantiate claims that it is crucial to T-lymphocyte function. Selective agonism of the M3R in human leukemia T-lymphocytes induced transient rises in intracellular Ca\textsuperscript{2+} with a subsequent rise in extracellular Ca\textsuperscript{2+} oscillations\textsuperscript{129}. This M3R-induced Ca\textsuperscript{2+} current, accompanied by Ca\textsuperscript{2+} influx through calcium-release activated Ca\textsuperscript{2+} (CRAC) channels, results in eventual increased c-fos action and IL-2 gene expression. This was demonstrated by CRAC blockade abolishing the increased IL-2 expression seen with muscarinic agonism by the parasympathomimetic oxotremorine\textsuperscript{130}, while also increasing c-fos mRNA overall DNA synthesis\textsuperscript{131}; an effect that could be subsequently abolished by nonselective MR antagonism\textsuperscript{129}. This data demonstrates the positive effect of M3R agonism on T-lymphocyte proliferation.
M3R is also crucial in allowing for optimal polarization and respective cytokine production from CD4+ T-lymphocytes when presented with an antigenic challenge expected to elicit a T\textsubscript{H}1 or T\textsubscript{H}2 response. Darby \textit{et al.} found that M3R KO (M3R\textsuperscript{-/-}) mice infected with the helminth \textit{Nippostrongylus brasiliensis}, which is commonly used to elicit a predominantly T\textsubscript{H}2 immune reaction, had an attenuated immune response when compared to WT controls\textsuperscript{132}. This reduced T\textsubscript{H}2 response previously seen in the M3R\textsuperscript{-/-} group could be recapitulated in the WT group when M3 antagonists were administered. A similar deficiency in adaptive immunity was found in the M3R\textsuperscript{-/-} cohort in response to \textit{Salmonella enterica}, which elicits primarily a T\textsubscript{H}1 response. WT mice increased IFN-\gamma production in response to M3R agonists, while antagonism resulted in an attenuation of IFN-\gamma production\textsuperscript{132}. Taken together, these data show a direct role for M3R in the mounting of an appropriately polarized immune response to helminth or bacterial infection.

M1R and M5R mediate a similar intracellular cascade to M3R, which may partially explain their effects. In an experiment to test the involvement of M1R and M5R specifically, Fujii \textit{et al.} immunized WT and combined M1R/M5R\textsuperscript{-/-} mice with ovalbumin (OVA)\textsuperscript{133}. In the knockout (KO) mice, they found lower levels of the anti-OVA antibodies of the immunoglobulin G\textsubscript{1} (IgG\textsubscript{1}) isotype, but not IgM. In addition, the M1R/M5R\textsuperscript{-/-} group produced less TNF-\alpha and IL-6, while also showing decreased levels of AChE intracellularly\textsuperscript{133}. This decreased cytokine production in the KO group could be related to the ability of M1R, M3R, and M5R to increase intracellular Ca\textsuperscript{2+}; a process which is well-known to be essential to effective T-lymphocyte activation. M1R, despite its lower expression, has been implicated in improved IL-2 production and IL-2R expression after TCR activation\textsuperscript{131}. In fact, M1R has shown important roles in CD8+ T-lymphocyte differentiation. In M1R\textsuperscript{-/-} mice, naïve CD8+ T-lymphocytes did not effectively differentiate into effector CD8+ T-lymphocytes, resulting in decreased killing of P815 mastocytoma cells (a classic target for cytotoxic T-lymphocytes)\textsuperscript{134}. 
Conversely, M2 and M4 linkage to G\textsubscript{o}/G\textsubscript{i} subunits posits a different functional effect upon their activation, but there is a paucity of studies on their effects on T-lymphocytes. There is some discussion of M2R involvement in asthma pathogenesis, with increased expression of M2R and M3R in asthmatic patients\textsuperscript{135}. This is complicated by the fact that major basic protein, released by eosinophils and characteristically increased in asthmatics, can also interact with M2R and alter Ach binding\textsuperscript{136}.

The current literature points toward specifically the G\textsubscript{q} linked M1R, M3R, and M5R as having some involvement in the activation and differentiation of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T-lymphocytes. These interactions are of great interest for pharmacologists and clinicians alike, with the ubiquitous intentional and off-target effects of cholinomimetics and cholinolytics\textsuperscript{137}. The administration of MR agonists might result in increased differentiation and polarization of T-lymphocytes, while antagonists could suppress cholinergic signaling made by and for immune cells.

As discussed previously, Ach can bind not only the MRs on T-lymphocytes, but also nAChRs. This binding of the nAChR mirrors that seen with muscarinic agonism, but differs in mechanism, with nAChR being an ionotropic receptor that allows for direct changes in cation (Na\textsuperscript{+}, Ca\textsuperscript{2+}) flux upon ligand binding (although this fact is convoluted by more recent evidence of nAChR interacting with G-proteins\textsuperscript{48, 138-140}). While this simplistic understanding of all nAChRs resulting in a similar effect is enticing, it is clear this is not the case based on structure alone. The heterogeneity of these receptors is evident in T-lymphocytes, where subunits determine the effect. nAChR subunit mRNA has been detected in human PBMCs, as well as human T-lymphocyte leukemic lines, with varied expression between individuals and cell lines\textsuperscript{128}.

Discussion around the varying detectable subunits has revolved around the expression of α2, α5, α6, α7, α8, α9, and α10 in leukocytes\textsuperscript{141, 142}, with α2, α5, α9, α10, β1, β2, and β4 nAChR mRNA found in freshly isolated splenic T-lymphocytes.
specifically\textsuperscript{143}. Upon \textit{ex vivo} activation of CD4+ T-lymphocytes by TCR cross-linkage, Qian \textit{et al.} found the following: α4 and α7 mRNA became discernably expressed; α5, α10, and β4 were upregulated; and α9 and β2 were downregulated\textsuperscript{143}. In CD8+ T-lymphocytes, α4 and α7 became expressed upon activation as well, along with dynamic expression changes of other nAchR subunits. For α7 nAchR specifically, mRNA was detected only in activated CD4+ and CD8+ T-lymphocytes, with minimal expression from isolated cells that were not activated.

In recent years, the α7 nAChR has been of great interest to immunologists. In T-lymphocytes, stimulation of the α7 nAchR by nicotine has been shown to reduce indices of T-lymphocyte proliferation when exposed to myelin oligodendrocyte glycoprotein (MOG), a commonly used antigen for experimental autoimmune encephalomyelitis (EAE)\textsuperscript{144}. In this same study by Nizri \textit{et al.}, nAchR agonism resulted in an overall shift to T\textsubscript{H2} differentiation with increased IL-4 production and decreases in T\textsubscript{H1} and T\textsubscript{H17} cytokines. In α7\textsuperscript{-/-}-derived T-lymphocytes, nicotine showed no effect on cytokine production, suggesting this variant is the predominant responding isoform to this stimulant. \textit{In vivo} administration of nicotine attenuated EAE outcomes in WT mice, but α7\textsuperscript{-/-} mice did not respond in a similar fashion, with disease severity reduction being related to impaired antigen presentation\textsuperscript{144}. In another study by Qian \textit{et al.}, nicotinergic stimulation of activated CD4+ T-lymphocytes produced upregulation of IFN-γ, while also downregulating IL-10 and IL-17\textsuperscript{143}. This demonstrates the ambiguity in the literature, where nuance such as method of activation can produce seemingly contradictory results. In addition, further differentiation into various phenotypic subtypes can produce differing results. For example, when nAchR is stimulated on T\textsubscript{REGs}, they have an increased ability to suppress T-lymphocytes\textsuperscript{145}. Additionally, α7 nAchR has been found to be crucial to the "inflammatory reflex", which will be discussed further herein.
Overall, the presence of both mAchR and nAchR on T-lymphocytes suggests an important physiological function, but the numerous subtypes of these receptors makes elucidating the functional role of Ach in T-lymphocyte strikingly difficult. Continued research utilizing T-lymphocyte-specific genetic knockouts will hopefully allow researchers to untether these complex interactions in the future.

_T-Lymphocytes synthesize neurotransmitters_

Despite their eponymous association with neurons, it has long been known that many cell types, including immune cells, synthesize and respond to neurotransmitters. Through the 1980's, Blalock _et al._ presented a number of molecular mechanisms by which the neuroendocrine and immune systems integrate to form a more functional unit. It has since been proposed that neurons and immune cells may have a similar evolutionary origin; an idea posited by a few investigators, but not widely discussed. Aforementioned, T-lymphocytes possess an array of neurotransmitter receptors that allow for critical changes in function, but they may also produce their own neurotransmitters. This ability significantly enhances the complexity of neural-immune crosstalk, and research in this field still is in its infancy.

_Catecholamines_

All three catecholamines have been detected in resting T-lymphocytes as well as the variety of activated lineages. Gene expression of all enzymes along the catecholamine synthetic pathway have also been reported, including DβH in lymphocytes and PNMT in splenocytes and thymocytes. TREGs constitutively express TH and have been shown to contain relatively large amounts of dopamine when compared to effector T-lymphocytes. The levels of these catecholamines within T-lymphocytes increases during activation, as does content of the rate-limiting enzyme TH, and is intimately linked to the activation of PKC and increases intracellular Ca²⁺.
upregulation of catecholamine synthesis is mirrored by the reported upregulation of ARs during similar mitogen stimulation or addition of pro-inflammatory cytokines$^{156,157}$. It could be posited that T-lymphocytes could produce catecholamines in a paracrine and autocrine fashion to inhibit growth and proliferation, not unlike reports in macrophages$^{158}$. Studies have shown catecholaminergic-driven inhibition of T-lymphocyte function by driving anti-inflammatory cytokine expression as well as changes to the apoptotic factor milieu to potentiate cell death$^{159}$, which could be reversed by blocking TH$^{155}$. Upon activation, the increase in TH and other crucial enzymes along the catecholamine synthetic pathway can be thought of as a way for T-lymphocytes to finely titrate the proliferation of specific subpopulations. This type of “catecholamine loop” has come to light in recent literature in macrophage populations during the insidious cytokine storm experienced by some patients receiving immunotherapy$^{160}$.

**Acetylcholine**

Ach is a neurotransmitter heavily relied upon to transmit peripheral nervous signals. It is the primary neurotransmitter at the neuromuscular junction, the autonomic pre-ganglion synapese, and the parasympathetic post-ganglionic synapse. At secondary lymphoid organs, there is no evidence of parasympathetic innervation$^{161}$. However, there are significant quantities of Ach in the spleen (as evidenced by the spleen being its first place of its extraction by Dale and Dudley$^{37}$). This raises the question as to the source of this abundant Ach. One potential source of Ach in a secondary lymphoid organ would be from the blood; a topic that was once a matter of debate$^{162}$. However, this is highly unlikely due to the presence of BuChE in plasma, which would lead to its rapid degradation. Interestingly, unlike the catecholamines, Ach is a short-lived molecule, likely due to its labile ester moiety, that can be rapidly hydrolyzed$^{163}$. To this end, the structure of Ach favors its function as an autocrine or paracrine signal produced directly from immune cells,
thus, positing immune cells are the source of Ach in lymphoid organs. Indeed, Kawashima et al. discovered that 60% of the total Ach in blood was located in the PBMCs, while there was no Ach detected in the polymorphonuclear leukocyte or red blood cell layer\textsuperscript{164}. This has since been validated by the presence of Ach in multiple human T-lymphocyte leukemic cell lines\textsuperscript{165, 166} and purified T-lymphocytes\textsuperscript{167}.

If this Ach is produced by T-lymphocytes, by what synthetic mechanism does this occur? This question does not immediately have an obvious answer, since non-neuronal cells also utilize the mitochondrial enzyme carnitine acetyltransferase (CarAT) to synthesize Ach, in addition to the canonical CHAT enzymatic pathway. In a series of experiments on human T-lymphocyte leukemic cell lines (MOLT-3, HSB-2, and CEM), Fujii et al. found that using the CHAT inhibitor, bromoacetylcholine (100 µM), reduced Ach synthesis by 50%, while the application of the CarAT inhibitor bromoacetyl-L-carnitine (100 µM) only reduced the synthesis by 30\%\textsuperscript{165}. This shows that T-lymphocyte Ach is likely synthesized by CHAT, with a lesser extent of production by CarAT. In experiments of T-lymphocyte activation using PHA, CHAT mRNA and activity were increased while CarAT remained unchanged\textsuperscript{168}, thus showing that the T-lymphocyte activation cascade can through some mechanism control CHAT expression and activity\textsuperscript{169}. PHA, via the TCR complex, results in increased PLC-mediated production of IP\textsubscript{3}. In addition to this mechanism, TCR-independent pathways of activation have shown increased CHAT mRNA and activity; such as the calcium ionophore ionomycin, protein kinase C activator phorbol 12-myristate 13-acetate (PMA), and PKA activator dibutyryl cAMP (also called bucladesine)\textsuperscript{170, 171}.

In summation, T-lymphocytes are able to effectively synthesize both catecholamines and Ach and regulate their production. The source of these neurotransmitters is crucial in situations when there does not appear to be a clear anatomical neuroimmune connection, such as the parasympathetic nervous system communicating with splenic T-lymphocytes.
The Anti-inflammatory Reflex: Neuroimmunology in Action

Relevant to this discussion is a recent body of literature which has developed around how NE and Ach are used in T-lymphocyte communication with macrophages; termed the “inflammatory reflex” or “cholinergic anti-inflammatory reflex” (Figure 4). In order to

Figure 4. The dual nature of NE in T-lymphocytes.
Signals arising centrally in the brain—sent through vagal efferents or splanchnic sympathetic efferents—synapse in the celiac ganglion. The splenic nerve arises here and releases NE locally, wherein it is received by splenic T-lymphocytes, possibly by multiple AR subtypes. In naïve T-lymphocytes, NE reception will result in activation and suppression of cytokine production (system dependent) and increased ROS. Additionally, ChAT+ T-lymphocytes receive NE through the β2-AR, which induces the production of Ach that is received by α7 nAchR-bearing macrophages. This results in a suppression of proinflammatory cytokine release by these splenic macrophages (termed the anti-inflammatory reflex).
better illustrate this salient example of neuroimmunology in action, we must first discuss the autonomic innervation of the spleen.

The discussion of autonomic splenic innervation is a complex one, with the literature asserting that the spleen receives only sympathetic efferent fibers (i.e. no parasympathetic or afferent tracts) in the form of the splenic nerve\(^\text{178}\). The splenic nerve travels in a neurovascular bundle, arising from the celiac ganglion. This ganglion receives sympathetic inputs originating from the rostral ventrolateral medulla (RVLM), a synaptic connection in the intermediolateral cell column in the spinal cord\(^\text{179}\). More interestingly, the celiac ganglia has also been reported to receive efferent vagal fibers from the dorsal motor nucleus (DMN)\(^\text{180, 181}\). The splenic nerve itself is exclusively noradrenergic, and thus, has varicosities along its length where synapses en passant release vesicular NE\(^\text{182}\). This NE is released within the spleen, mainly near both B- and T-lymphocytes due to regional distribution and can reach concentrations in the millimolar range in these local environments\(^\text{183}\). Intriguingly, vagal nerve stimulation results in inhibition of splenic TNF-α production during LPS challenge\(^\text{173}\), providing evidence for the interplay between the vagus and the spleen despite lack of definitive parasympathetic innervation to the spleen.

This paradoxical cholinergic anti-inflammatory reflex is believed to work via neurotransmission between neurons and immune cells, but further propagated by neurotransmission solely between immune cells. As a reflex, afferent information must be transmitted initially. Afferent vagal nerve fibers are activated by various systemic cytokines such as IL-1, TNF, IL-6, IFN-γ, in addition to a host of other inflammatory mediators, reviewed nicely by Goehler and colleagues\(^\text{184}\).

In brief, the efferent arc is the stimulation of the vagus nerve causing NE release by the splenic nerve, which then binds β\(_2\)-AR receptors on CHAT+ CD4+ T-lymphocytes. This β\(_2\)-AR stimulation results in the synthesis and secretion of Ach by the aforementioned T-lymphocytes locally within the spleen\(^\text{185}\), which then binds α7 nAchRs on macrophages
and results in a decrease in serum TNF-α, splenic TNF-α, and macrophage TNF-α release during an LPS challenge. This is further demonstrated by the lack of TNF-α suppression in α7 nAchR-/- mice during vagal stimulation.

Conversely, strong evidence exists to the contrary that suggests the vagus nerve plays no role in the anti-inflammatory reflex. Martelli and colleagues have shown that this reflexive response to LPS is mediated solely by the sympathetic splanchnic nerve, as only ablation of this nerve (and not bilateral vagotomy) showed a significant decrease in splenic TNF-α levels. This is further validated by recent study from the same group that has shown that bilateral transection of splanchnic sympathetic nerves prevented the anti-inflammatory effects seen with vagal nerve stimulation, providing more evidence that afferent vagal fibers receive information that is integrated centrally, but only sent through an efferent sympathetic arm.

Recently, Murray et al. elegantly built upon and synthesized these findings to demonstrate that afferent and efferent vagal nerve stimulation respond to LPS through distinct mechanisms. The latter require T-lymphocyte-derived Ach but efferent fibers do not, while both fiber types ultimately rely on intact β2-AR receptors. This paper is an important step in more specifically delineating how vagal nerve tracts could explain the seemingly disparate findings within the literature.

Regardless of the mechanism, these autonomic-immune interactions have far-reaching implications. These experiments demonstrate the importance of neurotransmission in regulating the inflammatory environment, and the impacts it may have on systemic, inflammation-driven diseases. Importantly, the role of these neuroimmune interactions remain yet unexplored in psychiatric disorders. To this end, we will discuss how the pathways between these tools of sympathetic nervous system and T-lymphocytes might work to play a functional role in the pathophysiology of posttraumatic stress disorder.
Autonomics and T-Lymphocytes in Posttraumatic Stress Disorder

In the preceding section, the importance of autonomic neurotransmitters in T-lymphocyte function was discussed thoroughly in a variety of *in vitro* and *in vivo* systems. However, the question remains of how these substantial but yet understood neuroimmune connections may be critical in disease. To say, what targetable diseases demonstrate perturbations in these respective nervous and immune systems to coalesce into the genesis, development, and progression of frank disease with tremendous morbidity? As foreshadowed in the prologue, psychiatric diseases present an area of research wherein there remains a paucity of investigations which have explored disease mechanisms which are clearly influenced by neuroimmune communication.

The subsequent section will present rationale for how the quintessential stress-related psychiatric condition, posttraumatic stress disorder (PTSD), serves as an important disease state which could be further understood through specific and targeted elucidation of autonomic neurotransmission and T-lymphocyte interactions. Following the rationale for these investigations, specific aims designed to address relevant gaps in the literature will be presented.

*Introduction to Posttraumatic Stress Disorder*

Posttraumatic stress disorder is the archetypal mental health disorder which results after experiences of psychological trauma. Across time and culture, PTSD has been a known potential sequela of any severe traumatic event. It has been referenced amongst survivors of the theatre of war for hundreds of years, with allusions to the characteristic cluster of symptoms dating back to antiquity in many works of literature, ranging from The Bard to Homer\(^{189}\).
Throughout the 20th and into the 21st century, we have continued to define and refine our understanding of diagnostic approaches to psychiatric disease, culminating in the diagnostics and statistical manual (DSM), now on its fifth iteration\textsuperscript{190}. PTSD is characterized within the DSM-V as a trauma and stress related disorder, after being previously categorized as an anxiety disorder in the DSM-IV. A PTSD diagnosis necessitates the following specific criteria as evaluated by a mental health specialist; A) exposure to perceived serious injury, violence, or death—either personally or through learning of a traumatic experience of a loved one; B) presence of at least one intrusive symptom, such as distressing memories, dreams, or dissociative events (e.g., flashbacks); C) Avoidance behavior of specific stimuli related to the traumatic experience; D) Negative alterations in mood or cognition; E) marked hyperarousal and/or hyperreactivity; F) Symptoms lasting greater than one month; G) must cause significant functional impairment, and H) symptoms do not have another physiological or pharmacological etiology. While PTSD is a discrete diagnosis with unique symptom clusters and physiologic dysfunctions, it is often co-morbid with other psychiatric issues. As reported in the National Comorbidity survey\textsuperscript{191}, 50% of patients with PTSD have three or more additional mental disorders, and these patients are two to seven times more likely to have concurrent affective and anxiety disorders\textsuperscript{192, 193}.

As a disease state, PTSD has significant incidence and prevalence across the global population. Lifetime prevalence of PTSD has been estimated to be up to 9.2% of the adult population in the United States\textsuperscript{194}. Specifically, veterans of armed conflicts have a greatly increased risk for developing PTSD, with rates ranging from 11-30% depending on the military campaign\textsuperscript{195-198}. Most early research focused exclusively on members of the military, yet over the past half century there has been an increased understanding and emphasis on PTSD outside the theater of war\textsuperscript{199}. Large consortia such as The Grady
Trauma Project have wholly focused on PTSD in the general population and resulted in crucial insights into disease in civilians. Various individual and societal factors alter pre-trauma risk for developing PTSD, such as genetics, gender, age, race, education, socioeconomic status, and among many more others (reviewed exceptionally by Sareen et al.). How these factors coalesce to determine which persons who experience trauma develop PTSD is still poorly understood. Working to explicate what psychological, social, and biological differences might exist at baseline to make certain populations more “susceptible” versus “resilient” to PTSD is a subject of ongoing inquiry in both clinical and preclinical work, and will be discussed throughout.

As with all diagnoses, there are layers of complexity which can be used to further delineate features of a person’s disease, resulting in complex diagnoses to describe variations in the symptom clusters. While these modifiers are useful, they also lay bare one of the most obvious weaknesses of the DSM. As a diagnostic tool, it has been useful to generate discrete, non-obscure classifications of “normal” versus “disease”. While this nosology continues to be hotly debated with each edition of the DSM, utilizing binary demarcations will always grossly oversimplify the complexity of psychiatric disease, especially in a research setting. More recently, the National Institute of Mental Health has emphasized characterizing features along continuous axes which can be used to more fully describe both normal and disease states. Importantly, the DSM was never designed to be a basic science research tool, and so any direct translation from bedside to bench (and back) should be approached judiciously.

Treatment options for patients with PTSD are variable. For most, the distinction between psychotherapy and pharmacotherapy is dependent on patient preference and treatment availability. Cognitive and exposure therapies have been found to be effective in meta-analyses of multiple clinical trials. FDA-approved pharmacotherapy relies on
selective serotonin reuptake inhibitors (SSRIs), but adrenergic antagonists (such as prazosin) are used off-label and have demonstrated some effectiveness\textsuperscript{207}. Notably, PTSD often becomes a chronic condition for patients. Nearly 40% of patients with PTSD are still symptomatic 10 years after the traumatic event, independent of whether they received any form of treatment\textsuperscript{191, 193}. The total cost of PTSD to society is tremendous, and it continues to be a relevant and devastating disease state that transcends historical and cultural barriers. To this end, rational approaches to elucidate its exact pathophysiology more fully are essential.

**Psychology Meets Physiology in PTSD**

As emphasized within the prologue, the mind and body function together synergistically to produce health and disease. Thus, an important aspect of PTSD is the devastating affect it has on physical health (reviewed well by Ryder et al.\textsuperscript{208}). Well-powered clinical studies have clearly demonstrated that patients with PTSD have increased risk for a jarring number of medical conditions, including devastating cardiometabolic disease\textsuperscript{209-213} and various autoimmune disorders\textsuperscript{214-217}, even after controlling for potentially confounding individual factors. This battery of potential conditions ultimately results in a reduced lifespan for patients with PTSD as compared to their lifestyle-matched controls\textsuperscript{218-220}. In order to improve and extend the lives of patients, we must find ways to effectively target systems which coordinate to result in increased mortality. These co-morbid diseases all vary significantly in their pathogenesis and pathophysiology, and it is unlikely there is one singular, common pathway that represents the etiology for their increased risk in PTSD. However, there is a robust body of literature which shows that PTSD demonstrates severe alterations in both the nervous and immune systems, which undoubtedly play a driving role in these diseases.
Nervous Alterations in PTSD

As described in the diagnostic criteria, symptom cluster E (hyperarousal, hypervigilance, et cetera) is an important, definitional feature of PTSD. Over the past 30 years, investigations in neurobiology and PTSD have worked to better delineate the neural circuitry that might further explain these symptoms. To paraphrase work from Liberzon et al.\textsuperscript{221}, PTSD can best be conceptualized as “a state of heightened responsivity to threatening stimuli, and/or a state of insufficient inhibitory control over exaggerated threat-sensitivity”. Downstream from these pathologies in central processing, rostral control centers of sympathetic tone have demonstrated overactivation in neuroimaging investigations\textsuperscript{221}. Moreover, these rostral control centers of sympathetic tone, such as the paraventricular nucleus of the hypothalamus, directly facilitate activation of the sympathetic nervous system, which is more directly measured through various physiologic parameters in both humans and animal models\textsuperscript{222, 223}.

Fundamental human subject work has demonstrated that patients with PTSD have increased activation of the sympathetic nervous system, as evidenced by increases in urinary and cerebrospinal fluid NE content, baroreflex sensitivity, muscle sympathetic nervous activity, and heart rate variability\textsuperscript{224-227}. Importantly, this increase in sympathoexcitation has been shown to correlate with symptom severity in patients\textsuperscript{226, 227}. This increase in urinary NE is specific to those currently experiencing disease, as those with a history of PTSD showed no increase in urinary NE in the Mind Your Heart cohort\textsuperscript{224}. These features of PTSD are extremely relevant considering the aforementioned intimate relationship between effectors of the sympathetic nervous system and immune cells, specifically T-lymphocytes.
**Immune Alterations in PTSD**

From the aforementioned slew of diseases, the immune system plays an important role in the development of most, if not all. There is a robust body of evidence demonstrating changes to the innate and adaptive arms of the immune system in patients with PTSD. While the results of many of these studies are inconsistent or generally contradictory, the large majority demonstrate increases in a generally pro-inflammatory profile in patients with PTSD as compared to matched controls (reviewed thoroughly by Kim *et al.*\(^{228}\)). This has been demonstrated in studies of various serum inflammatory biomarkers\(^{229-232}\), which have demonstrated serum and saliva pro-inflammatory cytokines such as IL-2, IL-6, TNF\(\alpha\), and IL-17. In studying circulating immune cell populations, there has also been work demonstrating an increase in pro-inflammatory signatures, with several focusing on pro-inflammation shifts in T-lymphocytes specifically\(^{233-235}\). This imbalance is an important feature that could significantly contribute to the decreased lifespan experienced by these patients, as well as serve as a salient target due to the inherently plastic nature of immune cells.

Importantly, there remains an overall uncertainty about the causal relationship—and directionality—of psychological trauma and immune dysfunction. As reviewed exceptionally by Sumner *et al.*\(^{236}\), there is no clear answer as to the whether, 1) PTSD results in altered inflammation, 2) dysfunctional inflammation drives to development of PTSD, or 3) the two are simply correlates with yet unknown mediators interacting between them. Briefly, the literature is mixed but seems to indicate that inflammation might precede the development of PTSD, and that PTSD ultimately results in heightened inflammation\(^{236}\). In order to fully investigate these underlying mechanisms in a causal fashion, it is essential to make use of established preclinical models of PTSD.
**Preclinical Approach to Studying Posttraumatic Stress Disorder**

PTSD is definitionally a human disorder. In order to study mechanisms that might drive the disease, numerous preclinical animal models have been developed. The six most commonly used murine models have been reviewed exhaustively elsewhere (discussed thoroughly by Deslauriers *et al.*). Briefly, each of these is able to recapitulate specific aspects of PTSD while also presenting with their own respective limitations. Given the complexity of PTSD, it is not surprising that no murine model is fully able to recapitulate the disease. To this end, researchers seek to select from the currently designed methods to target specific psychological and physiological characteristics of PTSD—be it dysautonomia, immune dysfunction, depression-like behavior, et cetera. Importantly, many of these murine models have been defined in certain facets but have not been robustly investigated in others. Working to define these model systems, as well as how various aspects of the human condition manifest in a simplified system such as an animal model, can provide prudent insight into a discrete pathological element of psychological trauma. As will be discussed further within the chapters which follow, the work presented herein will specifically focus on the use of repeated social defeat stress (RSDS). This is primarily due to preliminary studies which will be presented, which affirmed previous demonstrations of RSDS as a tenable model for inflammatory studies in psychological trauma.

**Specific Aims and Hypotheses**

In summation, there are significant gaps in the literature in elucidating the mechanistic relationship between sympathetic drive and the characteristic inflammatory signature of PTSD. Based on the detailed review of the literature herein, the following research hypotheses and specific aims were developed.
We hypothesize that catecholamines generated from 1) efferent sympathetic nerves and/or 2) T-lymphocytes themselves induce the pro-inflammatory T-lymphocyte profile seen in psychological trauma that is critical to the potentiation of various aforementioned inflammatory conditions that decrease the quality of life and ultimately contribute to the mortality of these patients. We will address this central hypothesis by way of two Specific Aims (Figure 5):

**Specific Aim 1**

*Identify the contribution of neuronally-derived catecholamines in the spleen in driving psychological trauma-induced inflammation.* We have shown that repeated social defeat results in increased splenic catecholamines, but the origin (neuronal or immune) and effect of these neurotransmitters remains elusive. In order to determine the neuronal contribution, we will ablate the splenic nerve to dissect how a lack of sympathetic input to the spleen during trauma may alter the A) PTSD-like behavioral phenotype and B) the inflammatory milieu.
Specific Aim 2

*Determine the influence of T-lymphocyte-generated catecholamines on trauma-induced inflammation.* Immune cells, particularly T-lymphocytes, possess the ability to generate catecholamines. To determine this influence, we have designed and generated a genetic-knockout mouse model lacking tyrosine hydroxylase (TH)—the rate-limiting enzyme in catecholamine synthesis—specifically in T-lymphocytes. By eliminating the synthesis of catecholamines in T-lymphocytes, we will investigate how a lack of immune-derived sympathetic signaling affects T-lymphocyte-driven, stress-induced inflammation.

The knowledge gap in this antecedent relationship between catecholamines and the pro-inflammatory state of T-lymphocytes must be addressed in order to further our understanding of enhanced inflammatory diseases in patients with PTSD.
CHAPTER 2: Peripheral Inflammation is Linked to Elevated Zero Maze Behavior in Repeated Social Defeat Stress

Portions of the content covered in this chapter are the subject of published works by Elkhatib et al. in *Brain Behavior and Immunity*.

**Abstract**

Post-traumatic stress disorder (PTSD) is a psychiatric illness that results in an increased risk for a variety of inflammatory diseases. The exact etiology of this increased risk is unknown, and thus, several animal models have been developed to investigate the neuroimmune interactions of PTSD. Repeated social defeat stress (RSDS) is an established preclinical model of psychological trauma that recapitulates certain behavioral and inflammatory aspects of human PTSD. Furthermore, RSDS has been utilized to subgroup animals into susceptible and resilient populations based on one specific behavioral phenotype (*i.e.*, social interaction). Herein, we conducted an extensive investigation of circulating inflammatory proteins after RSDS and found significant elevations in various cytokines and chemokines after exposure to RSDS. When categorizing animals into either susceptible or resilient populations based on social interaction, we found no inflammatory or other behavioral differences between these subgroups. Furthermore, correlative analyses found no significant correlations between social interaction parameters and inflammation. In contrast, parameters from the elevated zero maze (EZM) demonstrated significant associations and clustering to 5 circulating cytokines. When animals were subdivided into susceptible and resilient populations solely based upon combined EZM performance, significant inflammatory differences were evident between these groups. Strikingly, these circulating inflammatory proteins
displayed a stronger predictive ability of EZM performance compared to social interaction test performance. These findings provide new insights into inflammatory markers associated with RSDS, and the utility of EZM to effectively group RSDS-exposed mice into populations with differential levels of peripheral inflammation.
Introduction

Post-traumatic stress disorder (PTSD) is a devastating psychiatric illness characterized by exposure to a traumatic event, followed by flashbacks, avoidance, affective changes, and hyperarousal\(^\text{190}\). Interestingly, only about 20% of those that experience a traumatic event develop clinical PTSD, which demonstrates trauma exposure is necessary, but not sufficient alone, to cause PTSD\(^\text{244}\). Given this, there has been a continued interest in determining which psychological or biological factors could determine susceptibility or resilience to PTSD\(^\text{245, 246}\). Understanding that patients with PTSD notably face an increased risk for a number of inflammation-driven diseases\(^\text{213, 214, 229, 247, 248}\), investigations into the inflammatory contributions to PTSD have recently garnered significant attention (reviewed exceptionally by Sumner et al.)\(^\text{236}\).

In attempts to elucidate the pathophysiology of PTSD, many preclinical rodent models have emerged\(^\text{237, 249}\). Repeated social defeat stress (RSDS) is a well-characterized murine model that relies upon the aggressive and territorial nature of retired breeder CD-1 mice\(^\text{250}\), while successfully recapitulating several behavioral and inflammatory aspects of PTSD\(^\text{237, 249}\). Furthermore, this model has been reported to produce both susceptible and resilient populations as defined by a single parameter on the social interaction behavioral test (i.e., social interaction ratio <1.0 or \(\geq 1.0\), respectively)\(^\text{239, 250, 251}\).

Recent work from our group utilized the model of RSDS to examine the autonomic and redox profiles of T-lymphocytes after RSDS exposure\(^\text{238}\). Interestingly, while we demonstrated significant and novel changes to T-lymphocyte-driven inflammation, these did not correlate well with behavior assessed by the social interaction test, despite individually showing differences between RSDS and control cohorts\(^\text{238}\). With this
observation, we hypothesized that peripheral inflammation and social behavior are not tightly coupled following RSDS exposure.

In the present study, we sought to identify links between two behavioral tests to assess social and anxiety-like behavior (i.e., social interaction test and elevated zero maze, respectively) and peripheral inflammation using a large-scale assessment of circulating plasma inflammatory proteins following RSDS. In doing so across a large cohort of animals, we found little data to support any inflammatory differences between susceptible and resilient groups as determined by the social interaction ratio. However, we identified significant associations between select cytokines and the elevated zero maze (EZM) behavioral parameters. Sub-grouping animals into susceptible and resilient populations by an EZM-based definition, we found significant differences in circulating inflammatory proteins between the aforementioned EZM populations. Moreover, these circulating cytokines better predicted EZM susceptibility than social interaction susceptibility.

Materials and Methods

Mice

All control and experimental stress animals were 8-12 week-old male wild-type mice of a C57BL/6J background (Jackson Laboratory #000664, Bar Harbor, ME). Seventy-five total animals were used in this study. The RSDS paradigm precludes the use of female mice, thus, sex differences were not examined and not within the scope of the study described herein. All aggressive mice were 4-8 month-old retired breeder male mice of a CD-1 background (Charles River #022, Wilmington, MA). Experimental mice were bred in house to eliminate physical, psychological, and social stressors. Littermates were group housed (≤5 mice per cage) prior to the stress induction protocol to eliminate social isolation stress. Mice were housed with standard corncob bedding, paper nesting material, and
given access to standard chow (Teklad Laboratory Diet #7012, Harlan Laboratories, Madison, WI) and water ad libitum. Experimental mice were euthanized by pentobarbital overdose (150 mg/kg, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) administered intraperitoneally. Daily RSDS and euthanasia occurred between 0700 and 0900 Central Standard Time to eliminate circadian rhythm effects on immune function. Mice were randomized prior to the start of all experiments, and all efforts were made to blind experimenters to the control and stress groups of mice during biological assay and data analyses. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

**Repeated Social Defeat Stress**

An adapted version of the repeated social defeat stress (RSDS) paradigm described by Golden *et al.* was utilized for all studies. First, retired male breeder CD-1 mice (pre-screened thrice for aggressive behavior) were allowed to inhabit standard cages outfitted with two sets of food, water, and bedding three days before the start of an experiment to allow territory establishment by these mice. On day 1, all elements of the cage (except corncob bedding) were temporarily removed, and an experimental mouse was introduced into the cage for 5 minutes to allow for a physical confrontation. After the 5 minute interaction period, the mice were separated within the same cage by a transparent perforated barrier, and all housing elements were replaced back into the cage. The mice were then co-housed with physical separation for the remainder of the 24 hour period, and the process was repeated again by rotating the experimental mouse to a different CD-1 cage for 10 days. Mice were excluded from the study if they showed signs of wounding or lameness after social defeat sessions. Control mice were pair housed using identical separation and barrier housing techniques, but not allowing for any physical confrontation between mice during the 24 hour periods. RSDS was completed in a separate procedure.
room away from general animal housing so as not to affect control animals by auditory, visual, or olfactory stimuli. At the end of the 10 day period (day 11), all mice were assessed for behavioral changes using both social interaction and elevated zero tests. After testing, control and experimental mice remained in their former co-housed barrier cage until the following day (day 12) when they were sacrificed for biological analysis. While animals with visual wounding (>1cm) or lameness due to stress induction are excluded from further study\textsuperscript{250}, none of the 74 animals utilized herein met this threshold for exclusion.

**Behavioral Testing**

Following RSDS or control exposure for 10 days, all animals were behavioral assessed on the social interaction test and subsequently the EZM on day 11 between the hours of 0700 and 1400, with consistent timing between each behavioral test for all mice.

For social interaction testing, an open field (40 x 40 cm, Noldus Information Technology, Leesburg, VA, USA) was outfitted with a transparent enclosure with mesh caging (6.5 x 10 cm). Control and stress mice were introduced into the open field, and allowed to explore their environment with an empty mesh enclosure for 3 minutes. Runs were performed with one mouse at a time, and the chamber was thoroughly cleaned using water followed by 70% ethanol (allowing time for evaporation) to eliminate olfactory variables before the next mouse was tested. After all mice were assessed with an empty mesh enclosure, a different mesh enclosure was introduced into the open field containing a novel CD-1 aggressive mouse. Control and experimental mice were run in the aforementioned manner in the presence of an enclosed CD-1 mouse. A ratio of the time spent in proximity to the enclosure (interaction zone) or distal corners of the maze in the presence versus absence of a novel CD-1 mouse defines the social interaction ratio and corner zone ratio, respectively\textsuperscript{238, 239, 250}. Each run lasted 2.5 minutes, and all sessions were recorded and digitally analyzed by Noldus Ethovision XT 13 software (Leesburg, VA,
USA). Susceptible and resilient populations were defined by social interaction ratio <1 or ≥1, respectively.

For EZM testing, all experimental mice were introduced into a circular elevated maze (50 cm diameter, 5 cm track width, Noldus Information Technology, Leesburg, VA, USA) with 50% enclosed (20 cm wall height) and 50% open arenas. Mice were allowed to freely explore the novel environment for 5 minutes. Runs were performed with one mouse at a time, and the maze was thoroughly cleaned using water followed by 70% ethanol (allowing time for evaporation) to eliminate olfactory variables before the next mouse was tested. Tests were performed within the housing room of mice during the light cycle using approximately 265 lux of ambient lighting at the testing arena. All sessions recorded and digitally analyzed by Noldus Ethovision XT 13 software (Leesburg, VA, USA).

**Plasma Inflammatory Protein Measurement**

Blood was obtained by cardiac puncture immediately after sacrifice using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Plasma was separated by centrifugation, and stored at -80°C until use. Plasma inflammatory protein measurements were obtained by utilizing the Meso Scale Discovery V-Plex Mouse Cytokine 29-plex kit (#K15267D, Rockville, MD, USA). Meso Scale Discovery V-plex plates are tightly validated by the manufacturer to avoid antigenic cross-reactivity. The following inflammatory proteins were measured: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-15, IL-16, IL-17A, IL-17C, IL-17F, IL-17A/F, IL-17E/IL-25, IL-21, IL-22, IL-23, IL-27p28/IL-30, IL-31, IL-33, CXCL10, CXCL1, CCL2, CCL3, CXCL2, CCL20, TNF-α. All experiments were conducted per the manufacturer’s instructions, with samples run on Meso Scale Discovery Quickplex SQ 120 and analyzed using the Mesoscale Discovery Workbench software.
Statistics

A total of 74 animals (34 control, 40 RSDS) were utilized for the following studies. Associations between behavioral assessments and inflammatory proteins were assessed by nonparametric spearman correlation with two-tailed confidence intervals. Familywise error rate for the correlation matrix was conservatively corrected with the Bonferroni procedure for 20 inferential hypotheses (16 detectable inflammatory proteins and 4 behavioral outputs), with statistical significance thus established at \( p<0.0025 \) (\( \alpha/20 \) hypotheses tested). Receiver-operating characteristics (ROC) curves were assessed for classification ability by the area under the curve (AUC), with predicted probability for each subject set at 0.5. Goodness-of-fit was assessed by likelihood ratio test.

For inflammatory protein measurements, individual data are presented, with summary data displayed as mean ± SEM. Each data set was assessed for normality utilizing the Shapiro-Wilk test, followed by appropriate inferential hypothesis testing. For all inflammatory protein and behavioral measurements, Mann-Whitney U test was used to assess differences between control and RSDS. Susceptible and resilient populations by social interaction are subgroups of RSDS, and thus statistical tests were only conducted between the two subgroups. Susceptible and resilient populations by EZM includes both control and RSDS populations, and thus additionally only warrants comparative subgroup comparisons. All statistical analyses were completed in GraphPad Prism 8.4.2 (San Diego, CA, USA).

Results

Susceptibility and resilience by social interaction test does not differentiate inflammatory outcomes.
After RSDS, all mice were examined for behavioral changes by social interaction and EZM tests. Social interaction and corner zone ratios were highly variable, but showed near-significant differences between control and RSDS, respectively (p=0.0520, p=0.0505; Figure 6). EZM parameters—distance moved and time in the open arm—showed significant and robust differences after RSDS (p<0.0001, p<0.0001; Figure 6). When RSDS animals were subdivided into susceptible and resilient groups by social interaction ratio (susceptible = SI ratio <1; resilient = SI ratio ≥1), there was a statistically significant difference between these subgroups in the corner zone ratio of the social interaction test.

**Figure 6.** Susceptible and resilient subgrouping as determined by social interaction ratio does not reflect additional behavioral phenotypes.

Mice were run through the 10-day RSDS paradigm followed by behavioral testing, including **A.** social interaction test and **B.** elevated zero maze. RSDS mice were categorized into susceptible or resilient subgroups by a social interaction (SI) ratio <1.0 or ≥1.0, respectively (**A, left**). N = 35 control, 40 RSDS, with susceptible and resilient as subgroups of RSDS. Statistical significance by parametric Student’s t-test or nonparametric Mann-Whitney U test where appropriate.
interaction test (p=0.0074), but no differences in distance moved or time in open on the EZM (p=0.1895, p=0.0907, respectively; Figure 6).

With peripheral inflammation as our primary biological outcome, we then investigated circulating inflammatory protein levels in RSDS-exposed animals. Of the 29 inflammatory proteins analyzed, 26 were found to have at least several mice consistently above the lower limit of detection. Furthermore, 8 of these proteins showed significant differences between control and RSDS animals after correction for multiple analyses (Figure 7). Next, we further grouped the inflammatory proteins from RSDS animals into susceptible and resilient groups as determined by the social interaction ratio. However, no significant differences in peripheral inflammation were detected between susceptible and resilient groups (Figure 7). Overall, RSDS results in complex and robust changes to peripheral inflammation.

![Figure 7. Susceptible and resilient subgrouping as determined by social interaction ratio does not reflect peripheral inflammation.](image)

Mice were run through the 10-day RSDS paradigm followed by behavioral testing and plasma extraction. RSDS mice were categorized into susceptible or resilient subgroups by a social interaction (SI) ratio <1.0 or ≥1.0, respectively. Circulating levels of 29 cytokines were assessed by Meso Scale Discovery multiplex analysis; 26 were above the limit of detection, and 8 showed significant differences between control and RSDS animals. N = 35 control, 40 RSDS. Statistical significance by nonparametric Mann-Whitney U test.
inflammation. However, these changes do not discriminate between susceptible or resilient populations when separated by social interaction ratio.

**Selected cytokines and EZM parameters demonstrate significant associations and hierarchical clustering.**

In order to conduct a more comprehensive examination of the associations between the social interaction test, EZM, and circulating inflammation, heat maps were generated which represent correlation coefficients (**Figure 8A**) and p-values (**Figure 8B**) between all detectable inflammatory proteins and the aforementioned behavioral parameters across all animals (controls and RSDS combined). Hierarchical clustering additionally provided qualitative associations between discrete clusters of variables. We found that social interaction test parameters (i.e., social interaction ratio and corner zone ratio) did not significantly correlate with any measured inflammatory proteins (**Figure 8A-B**). However, EZM outputs (i.e., distance moved and time in open) associated significantly with 5 inflammatory proteins (**Figure 8C**). Overall, social interaction test parameters do not appear to significantly associate with any measured variable of circulating inflammation, while EZM correlates significantly with 5 tightly clustered cytokines.

**EZM-defined susceptible and resilient populations show significant differences in peripheral inflammation.**

In order to further assess the association between EZM and circulating inflammation, we developed a method for subgrouping all animals (controls and RSDS combined) by performance on the EZM. In order to generate a single metric which utilizes both distance moved and time spent in the open arm, each variable was normalized to the median (due
to the non-gaussian distribution) seen across both control and RSDS animals. These
normalized values were then summed. Animals with a sum greater than the overall median
(median EZM = 2.12) were considered “resilient”, while those below were deemed
“susceptible”. This can be represented mathematically as:

\[
\text{Figure 8. Selected cytokines and EZM parameters demonstrate significant associations and}
\text{hierarchical clustering.}
\]

Mice were run through the 10-day RSDS paradigm followed by behavioral testing, plasma extraction,
and inflammation assessment by Meso Scale Discovery multiplex analysis. Correlation matrices and
cluster analyses were completed for all behavioral and detectable circulating inflammatory proteins
within each animal. A. Heat map representation of correlation matrix of all variables with hierarchical
clustering above. Colors represent Spearman R coefficients (two-tailed test) with accompanying legend.
B. Heat map representation of correlation matrix of all variables with hierarchical clustering above.
Colors represent p-values by Spearman correlation (two-tailed test) with accompanying legend. C.
Individual correlation plots of select significant cytokines and elevated zero maze parameters. N = 35
control, 40 RSDS used for all analyses. Significance by Spearman correlation (two-tailed test).
When peripheral inflammation was assessed upon this EZM-defined susceptibility or resilience, 7 of the 8 original significant cytokines between control and RSDS demonstrated significant differences between EZM resilient and susceptible (Figure 9): IL-2 \((p<0.0001)\), IL-10 \((p<0.0003)\), IL-17A \((p<0.0002)\), IL-22 \((p=0.0003)\), TNF\(\alpha\) \((p<0.0001)\), IL-6 \((p=0.0302)\), and CCL2 \((p=0.0439)\).

Figure 9. Susceptible and resilient subgrouping as determined by elevated zero maze reflects peripheral inflammatory alterations.

Mice were run through the 10-day RSDS paradigm followed by behavioral testing and plasma extraction. Both RSDS and control mice were categorized into susceptible or resilient groups by elevated zero maze (EZM; normalized distance moved + normalized time in open) < median, or > median, respectively. Circulating levels of 29 cytokines were assessed by Meso Scale Discovery multiplex analysis; 26 were above the limit of detection, and 8 showed significant differences between control and RSDS animals. \(N = 35\) control, \(40\) RSDS. Statistical significance by parametric Student’s t-test or nonparametric Mann-Whitney U test.
Selected circulating cytokines predict EZM-defined susceptibility but not social interaction test susceptibility.

Next, multiple logistic regression was utilized to assess the predictive ability of inflammatory parameters in regard to behavioral outputs. From this regression model, ROC curves were generated. Social interaction-defined susceptibility was not significantly predicted by a regression model informed by circulating cytokines (IL-2, IL-10, IL-17A, IL-22, and TNFα; AUC=0.6540, p=0.0972; Figure 10A). However, the aforementioned 5 circulating cytokines were significantly predictive of EZM-defined susceptibility (AUC=0.7596, p=0.0001; Figure 10B). Overall, we demonstrate that peripheral inflammation is a better predictor of EZM susceptibility, while SI susceptibility is not predicted by inflammation.

Discussion

In the current study, we confirm that RSDS results in specific behavioral and peripheral inflammatory alterations. When examining 29 inflammatory proteins in circulation, we found 8 that were differentially extant in the plasma of RSDS mice. However, grouping animals into susceptible and resilient subgroups by social interaction ratio did not meaningfully divide RSDS animals by any measured peripheral inflammatory changes. Furthermore, when categorizing RSDS into susceptible and resilient groups indexed by the social interaction ratio, we did not observe similar grouping by EZM outputs. Indeed, in the original categorization of susceptible and resilient by social interaction ratio by Krishnan et al., the authors also conceded this grouping did not reflect anxiety-like behavior, locomotor activity, stress-induced polydipsia, or despair behavior after RSDS239. In relation to social interaction ratio and peripheral inflammation, previous work by Hodes et al. found significant associations between susceptibility and several circulating
cytokines, with a potential causal role for IL-6 in social interaction behavior. A major experimental difference between our two studies lies in the temporal relationship between RSDS and measurement of inflammation. Hodes et al. measured cytokines 20 minutes or 48 hours after the first social defeat stress bout, whereas the inflammatory data herein was performed after 10 complete days of RSDS. While we recapitulated their observed differences in IL-6, IL-10, and CCL2 levels between control and RSDS, we did not find these to significantly relate to social interaction susceptibility. In our relatively large sample

Figure 10. A specific subset of peripheral inflammatory proteins is more predictive of EZM-determined susceptibility than SI-determined susceptibility.

Mice were run through the 10-day RSDS paradigm followed by behavioral testing, plasma extraction, and inflammation assessment by Meso Scale Discovery multiplex analysis. A. Receiver-operating characteristics (ROC) curves were generated for select inflammatory parameters (IL-2, IL-10, IL-17A, IL-22, and TNFα) predicting social interaction (SI) susceptibility. B. ROC curves generated for select inflammatory parameters (IL-2, IL-10, IL-17A, IL-22, and TNFα together) predicting elevated zero maze (EZM) susceptibility. Prediction ability was calculated by area under curve, with p-value calculated by a two-tailed test with predicted probability of each subject set at 0.5. Goodness-of-fit additionally assessed by Log-likelihood ratio.
size (n=74), we found no significant differences in inflammation when animals were grouped by the social interaction ratio. Furthermore, we demonstrated no significant relationship between social interaction or corner zone ratio and any measured circulating inflammatory proteins, dispelling the likelihood of any potential causal association. While the categorization of mice by the social interaction ratio has indeed uncovered biological differences between these social interaction ratio-derived populations, our findings suggest this grouping is not all-encompassing, and may be limited to only a specific behavioral manifestation that may or may not be related to human disease.

Importantly, in analyzing correlates between behavioral outcomes and inflammation, our approach combined both control and RSDS animals, with all analyses including both behavioral and inflammatory data points from every animal within this study. This approach provides a methodologically-different assessment of the interaction between behavior and peripheral inflammation—especially as it relates to the spectrum of behavior. These data support the sentiment of recommendations by the National Institute of Mental Health *Research Domain Criteria* (RDoC)\(^{203, 204}\), which seeks to represent all behavioral characterizations as continuous variables across multiple domains to allow for more definitive (and complex) analyses. In doing so, we were able to find significant associations between EZM parameters and select circulating cytokines (IL-2, IL-10, IL-17A, IL-22, and TNF\(\alpha\)). Furthermore, when we subdivided animals by a metric that combined two outputs of the EZM (*i.e.*, distance moved and time in open arm) we found significant differences in these newly defined EZ susceptible and resilient populations, as well as the ability of inflammation to accurately predict EZ susceptibility or resilience. Notably, there is prior work demonstrating a causal relationship between circulating inflammatory proteins differentially detected within our paradigm, such as CCL2, and anxiety-like behavior\(^{241}\). This data prompts further query into the potential usage of the
EZM to divide subpopulations of susceptible and resilient from RSDS, especially in showing meaningful differences in measures of systemic inflammation.

Based on our findings, an important aspect for discussion is how to appropriately segment traumatized animals into susceptible and resilient populations. While the social interaction ratio provides a convenient methodology for categorizing RSDS mice, we have presented data herein to shown that it does not effectively mirror changes in systemic inflammation, while EZM behavior appears to be more strongly associated with this parameter. Additionally, recent work has shown that the social interaction test itself appears to involve conditioned learning based on prior exposures to an aggressive white CD-1 mouse, versus true tests of antisocial behavior\textsuperscript{252}. Importantly, RSDS has been shown to generalize to unfamiliar animals during the social interaction test, further complicating this interaction. Overall, these data should serve as unembellished reminders of the inherent complexity of psychological and physiological factors which might influence the development of susceptibility or resilience—even within our more reductionist preclinical models. The development of multifaceted approaches to assess the degree of psychological trauma will be paramount in further elucidation of the pathophysiology of this disease, as well as assessment of the efficacy of potential therapeutics.

The present study and its implications are of course not without limitations. The most fundamental is the lack of examination of sex as a biological variable. Due to the introduction of aggressive retired-breeder male mice in traditional RSDS, it cannot be used to induce psychological trauma similarly in female mice. There have been recent reports of modified female social defeat stress\textsuperscript{253, 254}, which—while potentially effective insofar as stress induction—vary significantly enough in approach, intensity, and duration to serve as serious confounding variables reducing internal validity. For this reason, this was not addressed in the present study. Future examination should include these behavioral and
inflammatory paradigms in the context of newly developed models of female social defeat stress.

Furthermore, while RSDS has shown robust and consistent peripheral inflammatory changes, one potentially problematic aspect of the model is the prospect of wounding. While we take care to exclude all animals with significant wounds or lameness, as previously discussed, this remains a possible confounder in RSDS and other models that incorporate physical stress. We estimate at least 75% of all animals in this study showed no visible signs of wounding, yet virtually all animals displayed a peripheral inflammatory response. These data are suggestive that wounding does not fully explain the inflammatory changes of these mice, but at this time cannot be completely ruled out. Importantly, previous research in preclinical models of PTSD has examined how witnessed social defeat stress can also result in inflammatory changes, which would definitionally be void of potential wounding. Additionally, it should be noted that psychological trauma in humans very often is accompanied by physical trauma. This is especially vital in the forms of trauma that are most likely to result in PTSD—rape, physical assault, and serious injury—and that may also directly impact the immune system.

In finding that peripheral inflammation in RSDS was most tightly-linked with the EZM—a test of anxiety-like behavior—it is worth noting the DSM-IV originally characterized PTSD under the umbrella of anxiety disorders. There is a breadth of clinical literature demonstrating differences in peripheral inflammation in patients with PTSD as well as other anxiety disorders. Importantly, many of the cytokines we found to be differentially elevated in control versus RSDS have also been shown to be elevated in human plasma from patients with PTSD, such as IL-2, IL-6, IL-17, and TNFα (summarized well by Wang et al). While preclinical, our results provide potential new avenues for investigation into understanding how selected circulating cytokines could be involved...
mechanistically in the pathogenesis of disease or approached quantitatively as potential variables within a more complex biomarker panel.

In summary, this current study provides novel findings that suggest a more nuanced approach to determination of susceptibility and resilience only by use of the social interaction ratio. Further, we present a novel method of quantifying the results of EZM performance into newly defined susceptible and resilient subgroups. Moreover, this EZM-based definition strongly correlates with a select peripheral inflammatory panel, which was able to effectively predict EZM susceptibility. Together, these findings call for a more in-depth and mechanistic analysis of psychological trauma in the RSDS model, as well as translational examination of peripheral inflammation in trauma-exposed human individuals.
CHAPTER 3: Autonomic and Redox Imbalance Correlates with T-Lymphocyte Inflammation in Repeated Social Defeat Stress

Portions of the content covered in this chapter are the subject of published works by Elkhatib et al. in Frontiers in Behavioral Neuroscience238

Abstract

Patients diagnosed with post-traumatic stress disorder (PTSD) are at a significantly elevated risk of developing comorbid inflammatory conditions, but the mechanisms underlying this predilection remain unclear. Our previous work has shown that T-lymphocytes exposed to elevated levels of norepinephrine (NE) displayed a pro-inflammatory signature reminiscent of an autoreactive phenotype. With this, we hypothesized that the increased sympathetic tone observed during psychological trauma may be promoting pro-inflammatory T-lymphocytes, which causes a predisposition to comorbid inflammatory conditions. Here, we examined the consequences of psychological trauma on splenic T-lymphocytes using a mouse model of repeated social defeat stress. Social defeat led to anxiety-like and depression-like behavior as has been previously described. The spleens of socially-defeated mice showed significant elevations of NE, tyrosine hydroxylase, and acetylcholinesterase levels, which appeared to be due in part to increased expression within T-lymphocytes. Additionally, T-lymphocytes from stressed animals showed higher levels of pro-inflammatory cytokines and mitochondrial superoxide. Interestingly, in this model system, close associations exist within splenic T-lymphocytes amid the autonomic, inflammatory, and redox environments, but these only weakly correlate with individual behavioral differences among animals suggesting the psychological and physiological manifestations of trauma may not be tightly coupled.
Taken together, this data provides new insights into behavioral and physiological links in model of PTSD which can provide relevant therapeutic targets for psychological trauma and its inflammatory comorbidities.
Introduction

Approximately 70% of adults in the United States have experienced some form of traumatic event, and development of post-traumatic stress disorder (PTSD) in this population is estimated at over 20% or 45 million Americans\textsuperscript{192-194}. PTSD is classified as an anxiety disorder experienced after trauma, and the disease manifests itself in several behavioral changes including intrusion symptoms, avoidance, and negative alterations in cognitions and mood\textsuperscript{190}. PTSD patients also demonstrate significantly elevated risks for the development of comorbid somatic illnesses such as cardiovascular, metabolic, and autoimmune diseases\textsuperscript{212, 215, 248, 260-262}. While PTSD patients frequently partake in activities that independently increase the chances of developing these disorders (e.g. smoking, drug use, poor diet, lack of exercise, etc.), statistical analyses have explicitly shown a consistent and significantly elevated comorbid disease risk even after controlling for these other risk factors. Moreover, it is unclear if treatment of the behavioral manifestations of PTSD impact the development of these comorbid somatic conditions, suggesting the control mechanisms remain elusive.

One characteristic physiological change of PTSD that may partially explain the development of comorbid somatic diseases is elevated sympathetic nervous system activity and norepinephrine (NE) outflow\textsuperscript{225, 227}. Compared to other psychological conditions such as chronic depression, bipolar, or schizophrenia disorders, PTSD patients show significantly elevated NE levels in both urine and cerebrospinal fluid compared to matched controls\textsuperscript{226, 263-265}. Moreover, targeting of NE via pharmacological means using prazosin or clonidine (α1 adrenergic antagonist and α2 adrenergic agonist, respectively), physical manipulation by denervation of the sympathetic chain, as well as anesthetic ganglion blockade have all demonstrated moderate benefits in attenuating the psychological manifestations of the disease\textsuperscript{207, 266-270}. These treatment modalities are
highly suggestive of a sympathetic component contributing to PTSD, however, it remains unclear if this dysregulation of autonomic tone is causal to the development of comorbid somatic diseases.

Inflammation is also a theme of all the comorbid diseases described in PTSD to date, and the immune system, particularly T-lymphocytes, appear to be highly sensitive to the psychobiological and sympathetic changes after trauma. For example, PTSD patients have decreased numbers of naïve and regulatory (anti-inflammatory) T-lymphocytes with concurrent increases in memory T-lymphocytes\textsuperscript{271, 272}. Additionally, circulating levels of various pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 17A (IL-17A) have been shown to be elevated in the PTSD population\textsuperscript{229, 233, 273, 274}. Animal models have corroborated these results showing alterations in both T-lymphocyte populations and cytokine production with various modalities of traumatic stress induction\textsuperscript{239, 242}. We and others have previously demonstrated that exposure to simply elevated levels of NE can have profound effects on T-lymphocyte activation and cytokine production\textsuperscript{24, 110, 275}, and our recent report has elucidated a novel role for the mitochondrial redox environment in NE-mediated T-lymphocyte regulation\textsuperscript{24}. Taken together along with the observation that glucocorticoid levels are often not elevated in patients with PTSD\textsuperscript{263}, we hypothesized that the increased sympathoexcitation observed in PTSD is leading to an increased pro-inflammatory T-lymphocyte phenotype via redox mechanisms, and it is this inflammation that predisposes these patients to increased incidences of comorbid somatic diseases.

To address this hypothesis, herein, we utilized an established and accepted mouse model of psychological trauma known as repeated social defeat\textsuperscript{237, 250}. We show that these animals demonstrated altered behavior, dysregulated autonomic balance with elevated sympathetic tone, and increased T-lymphocyte pro-inflammatory cytokine production concurrent with a disrupted mitochondrial redox environment, which confirms and extends our previous observations using \textit{in vitro} systems\textsuperscript{24}. However, examination of individual
animals identified only a few physiological parameters which associated significantly with specific behavioral phenotypes while showing strong associations to other physiological elements.

**Materials and Methods**

**Mice**

All control and experimental stress animals were 8-12 week-old male wild-type mice of a C57BL/6J background (Jackson Laboratory #000664, Bar Harbor, ME). For further detail on how mice were housed, dissected, and euthanized, refer to the **Materials and Methods section of Chapter 2**. When possible, experimenters were blinded to the control and stress groups of mice until the completion of the study. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

**Repeated social defeat stress (RSDS) paradigm**

An adapted version of RSDS as described by Golden *et al.* was utilized for all studies and is summarized in **Figure 11A**. For further detail on the RSDS paradigm, refer to **Materials and Methods section of Chapter 2**. At the end of the 10-day period (day 11), all mice were behaviorally tested. After testing, control and experimental mice remained in their former co-housed barrier cage until the following day (day 12) when they were sacrificed for biological analysis.

**Behavioral testing**

For further detail on behavioral testing, refer to **Materials and Methods section of Chapter 2**.
**Splenic T-lymphocyte isolation**

T-lymphocytes were isolated from whole spleens as has been previously described\textsuperscript{24}. Briefly, murine spleens were dissected and physically disrupted using ground glass slides until single cell suspensions, which were then filtered with 70 μM nylon mesh filters (Thermo Fisher #352350, Waltham, MA) to remove large debris. Erythrocyte depletion was accomplished through suspension in a lysis buffer (15.5 mM NH₄Cl, 1 mM KHCO₃, 10 μM EDTA), followed by a second filter pass through a 70 μM nylon mesh filter. Subsequently, splenic T-lymphocytes were negatively selected using the EasySep Mouse T-Cell Isolation Kit (StemCell Technologies #19851, Vancouver, BC). Cells were suspended in supplemented RPMI media (RPMI media + 10% fetal bovine serum, 1% GlutaMAX\textsuperscript{TM} (Gibco, #35050061, Grand Island, NY, USA), 1% HEPES, 1% penicillin and streptomycin, and 0.1% 50 μM β-mercaptoethanol). The purity (assessed by flow cytometry) and viability (assessed by a Bio-Rad TC20 Automated Cell Counter using trypan blue exclusion) of the T-lymphocytes was randomly quality controlled and found to be >90%.

**Catecholamine ELISA**

Total catecholamines were assessed in plasma and splenic lysates using the 3-CAT research ELISA (Rocky Mountain Diagnostics #BAE-5600, Colorado Springs, CO) as per manufacturer’s instructions, which utilizes a cis-diol specific affinity gel followed by acylation and subsequent immunoassay. Splenic catecholamine amounts were normalized to starting splenic weights, and then to controls within respective experiments.

**Western blot analysis**

Western blotting for the quantification of proteins was performed as previously described\textsuperscript{276}. Briefly, whole cell soluble lysate (30 μg) was separated by SDS-PAGE and
transferred to a nitrocellulose membrane. Membranes were incubated with antibodies
directed against tyrosine hydroxylase (TH; 1:1000 dilution, EMD Millipore #AB152,
Burlington, MA) and actin (1:1000 dilution, Sigma Aldrich #A2066, St. Louis, MO) followed
by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Thermo
Fisher #31460, Waltham, MA). Densitometric analysis of band intensity was determined
using ImageJ analysis software.

RNA extraction, cDNA production, and quantitative real-time RT-PCR

Assessment of mRNA levels was performed as previously described. Briefly, total
RNA was extracted from purified T-lymphocytes using the RNAeasy mini kit (Qiagen #
74104, Valencia, CA) according to the manufacturer’s protocol. Concentration of RNA was
determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Fisher
Thermo Scientific, Waltham, MA). The High-Capacity cDNA Reverse Transcription Kit with
RNase Inhibitor (Applied Biosystems #4374966, Grand Island, NY) was used to obtain
cDNA from total RNA. Generated cDNA was then subjected to SYBR green (Applied
Biosystems #4385612, Grand Island, NY) quantitative real-time PCR with primers specific
to the coding sequence of the respective genes using intron-spanning gene-specific
oligonucleotide primers (Appendix B). PCR product specificity was determined by thermal
dissociation. A threshold in the linear range of PCR amplification was selected and the
cycle threshold (Ct) determined. Levels of transcripts were then normalized to the 18s
rRNA loading control (ΔCT). For all analyses, 1/ΔCT was utilized to assess levels of
transcripts in a directional manner relative to expression with only normalization to the 18s
rRNA loading control.

Flow cytometric redox assessment

Mitochondrial-specific assessment of superoxide was performed as has been
previously described. Briefly, cells were stained with 1 µM of O₂^- -sensitive mitochondrial-
localized probe, MitoSOX Red (Thermo Fisher Scientific #M36008, Waltham, MA), for 30 minutes at 37°C. Cells were analyzed on a LSRII flow cytometer at 488/610 nm excitation/emission, respectively, and data analyzed using FlowJo software.

**Cytokine analysis**

Analysis of circulating levels of cytokines was performed using a Meso Scale Discovery 35 U-Plex Mouse Biomarker Group (#K15083K-1, Rockville, MD) per manufacturer’s instructions. Samples were assessed using a Meso Scale QuickPlex SQ 120 and analyzed using Meso Scale Discovery software.

**Statistics**

A total of 102 animals (50 control, 52 stress) were utilized in these studies. Not all physiological parameters were able to be run in a single animal, thus, each graph is individually labeled with N values and statistical information utilized for a specific set of experiments. Individual data are presented along with mean ± standard error of the mean (SEM). For two group or three group comparison, significance was assessed using the Mann-Whitney U-test or Kruskal-Wallis test due to the non-parametric distribution of the data. Correlations were performed using linear regression with Pearson correlation coefficient calculations. Differences were considered significant at p<0.05, and exact p-values are displayed on individual graphs.

**Results**

*Repeated social defeat stress increases anxiety-like and depression-like behavior*

Several animal models exist that mimic the behavioral changes of human PTSD, and while repeated social defeat stress (*Figure 11A*) does not recapitulate all of these human PTSD phenotypic changes, it was chosen for this study due to its reproducible impact on
inflammation. Specific behavioral changes were first tested using an elevated zero maze to assess anxiety-like behavior and locomotor activity. Socially-defeated mice demonstrated decreased locomotor activity as evidenced by decreased total distance moved as well as increased anxiety-like behavior due to less time spent in the open arms.

Figure 11. Repeated social defeat stress increases anxiety-like and depression-like behavior.

A. Overview of experimental setup and timeline. B. Upper, representative heat map tracings of the elevated zero maze analysis for control and socially defeated (Stress) animals. Hot colors indicate more time spent in a specific area, while cool colors represent less time. Lower, quantification of elevated zero maze parameters. Distance Moved (N=50 controls, 52 stress; 2-tailed; Mann-Whitney U=655.5), Time in Open Field (N=50 controls, 52 stress; 2-tailed; Mann-Whitney U=832.5). C. Upper, representative heat map tracings of the social interaction test. Color spectrum identical to elevated zero maze. Lower, quantification of social interaction test parameters. Interaction Zone Ratio (N=50 controls, 52 stress; 2-tailed; Mann-Whitney U=926.0), Corner Zone Ratio (N=50 controls, 52 stress; 2-tailed; Mann-Whitney U=1295.0).
of the elevated zero maze compared to control animals (Figure 11B). Additional parameters such as latency to first, body elongation, or head directed towards the open field only trended significance (data not shown). Depression-like and antisocial behavior was also confirmed by the use of a social interaction test, where stressed animals displayed less time spent in the interaction zone and more time in the corner zone relative to controls (Figure 11C). Together, these data support previous reports that repeated social defeat stress increases anxiety-like and depression-like behavior (34, 38). However, these previous reports have suggested that stressed mice may be classified as “susceptible” or “resilient” based on a social interaction ratio threshold of 1.0\(^{239,250}\). When this analysis was performed on our mice, we observed that the “resilient” group only represented approximately 30% of the stressed mice, but moreover, was statistically different from control animals in regard to the average social interaction ratio (Figure 12A). Furthermore, when examining locomotor activity and anxiety-like behavior on the elevated zero maze, no differences were observed between resilient and susceptible groups (Figure 12B). Due to these discrepancies, data were processed using only major group categories (i.e., control versus stress) as well as correlation analyses among all mice to identify dimensional individual differences among behavioral and physiological parameters.

**Increased sympathetic signatures are observed in T-lymphocytes from stressed mice, but do not correlate with behavior**

Aforementioned, sympathoexcitation is a hallmark of PTSD. To assess this in our animal model, we first measured circulating levels of catecholamines at the completion of the stress induction paradigm. Unexpectedly, we did not observe any differences in circulating levels of catecholamines, but identified significant increases within the spleen—a secondary lymphoid organ which receives sympathetic inputs—following social defeat
This lack of increased circulating catecholamines suggested the potential for elevated sympathetic neuronal activity to the spleen. To assess this, we next evaluated (Figure 13A).
the level of TH (the rate limiting enzyme of catecholamine synthesis) protein in the spleen and observed significant increases in stressed animals (Figure 13B). To understand if the increased TH was due to potentiated neuronal expression of the protein, we excluded neurons by performing quantitative real-time RT-PCR analysis for TH mRNA in purified T-lymphocytes. To our surprise, we identified large and significant increases for TH message within T-lymphocytes (Figure 13C). We further evaluated other neurotransmitter synthesis and degradation enzyme mRNA levels within purified splenic T-lymphocytes and identified trending increases in monoamine oxidase A (MAO-A), trending decreases in choline acetyltransferase (CHAT), as well as significant increases in acetylcholinesterase (ACHE; Figure 13C). Together, the level of transcripts of these enzymes displayed a pro-

Figure 13. Splenic T-lymphocyte sympathetic tone is increased with psychological trauma.

Plasma, whole spleens, and purified splenic T-lymphocytes were isolated following the social defeat (Stress) paradigm. A. Quantification of total catecholamines (Cat) in the spleens and plasma. Splenic values were first normalized to spleen weight. All values are displayed normalized to respective controls per experiment. Spleen (N=8 controls, 8 stress; 2-tailed; Mann-Whitney U=5), Plasma (N=8 controls, 8 stress; 2-tailed; Mann-Whitney U=22). B. Left, representative western blot analysis for splenic tyrosine hydroxylase (TH) content. Right, quantification of TH content by western blot. (N=9 controls, 7 stress; 2-tailed, Mann-Whitney U=2). C. Quantitative real-time RT-PCR analysis for various neurotransmission enzyme mRNA levels in purified T-lymphocytes. Data are shown as 1/ΔCT as normalized by 18s rRNA loading control. Monoamine oxidase A (MAO-A); choline acetyltransferase (CHAT); acetylcholinesterase (ACHE). TH (N=36 controls, 38 stress; 2-tailed, Mann-Whitney U=448.5), MAO-A (N=36 controls, 38 stress; 2-tailed, Mann-Whitney U=592.0), CHAT (N=36 controls, 38 stress; 2-tailed, Mann-Whitney U=660), ACHE (N=36 controls, 38 stress; 2-tailed, Mann-Whitney U=338.5).
sympathetic neurotransmission signature within purified T-lymphocytes after stress, which suggested the potential for lymphocyte-specific neurotransmitter production in response to stress.Interestingly, T-lymphocyte expression of genes driving a pro-sympathetic environment did not correlate with individual differences in anxiety-like or depression-like behavior (Figure 14).

**Figure 14.** Pro-sympathetic neurotransmission signatures in splenic T-lymphocytes do not correlate with behavior.

A. Correlation of tyrosine hydroxylase (TH) mRNA levels within splenic T-lymphocytes with anxiety-like and depression-like behavior indices. (N=36 controls, 38 stress. DFn, Dfd= 1,72 for all). B. Correlation of acetylcholinesterase (ACHE) mRNA levels within splenic T-lymphocytes with anxiety-like and depression-like behavior indices. (N=36 controls, 38 stress. DFn, Dfd= 1,72 for all). Black circles indicate control animals; blue squares indicate socially-defeated (Stress) animals. Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line).

Behavioral responses to social defeat and social defeat-induced stress also correlated with increased production of mitochondrial superoxide and pro-inflammatory cytokine expression (IL-6, IL-17A). Our previous work demonstrated that ex vivo T-lymphocytes exposed to NE expressed increased levels of IL-6 and IL-17A that was driven in part due to amplified mitochondrial superoxide production. Understanding that social defeat elevated

**Psychological trauma elicits elevations in T-lymphocyte mitochondrial superoxide and pro-inflammatory cytokine expression**
catecholamine levels in proximity to T-lymphocytes, we examined the effects of stress on T-lymphocyte redox and inflammatory environments. We first observed that social defeat caused a significant decrease in the percentage of splenic T-lymphocytes (Figure 16), which has been previously reported in a similar trauma animal model. Assessment of these remaining splenic T-lymphocytes showed an approximate 2-fold induction of mitochondrial superoxide levels compared to controls (Figure 16B). Mitochondrial superoxide was not altered in circulating T-lymphocytes (data not shown), which further supported the importance of direct interaction with catecholamines. No change was observed in splenic T-lymphocyte nitric oxide levels from socially-defeated animals (Figure 15), demonstrating not all redox signaling is perturbed with psychological stress. IL-6 and IL-17A levels were increased in circulation of socially-defeated animals (Figure 16C-D), and mRNA levels for these cytokines were also specifically and significantly elevated within splenic T-lymphocytes (Figure 16E-F). Overall, these data confirm and extend our previous in vitro findings in a relevant in vivo model of psychological trauma that catecholamines impact T-lymphocyte inflammation likely via redox mechanisms.

**T-lymphocyte mitochondrial superoxide correlates with anxiety-like behavior, while IL-6 expression associates with depression-like behavior**

To assess if the T-lymphocyte mitochondrial redox and inflammatory environments had any impact on behavior, we performed correlation analyses on all animals comparing
individual behavioral indices and these physiological readouts. Intriguingly, a positive correlation was observed between T-lymphocyte mitochondrial superoxide levels and anxiety-like behavior, but not depression-like behavior (Figure 17A). In contrast, splenic T-lymphocyte expression of IL-6 or IL-17A did not correlate with anxiety-like behavior
indices but IL-6 positively correlated with depression-like behavior (Figure 17B-C), which has been previously reported. Together, this data suggest that splenic T-lymphocyte mitochondrial superoxide levels may serve as an indicator of anxiety-like behavior, whereas specific inflammatory components may be more predictive of depressive-like symptoms in the social defeat stress model.
Significant associations exist between the autonomic, redox, and inflammatory signatures of T-lymphocytes

While the biological changes observed with RSDS did not completely associate with individual behavioral phenotypes, we next set out to address if these physiological changes correlated with each other. Strikingly, we observed strong and significant positive correlations among all combinations of biological measures including T-lymphocyte mitochondrial superoxide and IL-6, IL-17A, TH, and AHCE transcript levels (Figure 18 and Figure 19, respectively). These data are highly suggestive of crosstalk between the autonomic, redox, and inflammatory pathways in T-lymphocytes during psychological trauma.

Discussion

In the current study, we identify several previously undescribed links between the autonomic, redox, and inflammatory environments of splenic T-lymphocytes during...
psychological trauma. Together, these provide new insights into the regulatory control of the adaptive immune system during stress, and suggest potential pathways which may lead to the increased incidence of inflammatory comorbidities in diseases like PTSD.

PTSD is a multifaceted disease that has proven difficult to replicate in animal models. A recent review by Deslauriers et al. has elegantly summarized accepted animal models that recapitulate PTSD behavioral and biological phenotypes. There are currently six animal models that are able to mimic the behavioral and biological phenotypes of PTSD, but each has demonstrated at least one phenotype of the human disease that is not able to be fully recapitulated. We chose the repeated social defeat model based on its ability to robustly and consistently produce increased anxiety-like behavior, depression-like behavior, as well as peripheral inflammation. As described in this review, repeated
social defeat is only one of two accepted animal models that produces a peripheral inflammatory response similar to human PTSD, which was the primary focus of this work. However, social defeat is limited in the fact that it does not demonstrate hallmark phenotypes of PTSD such as decreased fear extinction or increased HPA feedback. Additionally, the standard repeated social defeat does not allow for the use of female mice, which precludes examination of sex differences (An alternative version of social defeat using genetically modified CD-1 mice has been reported to be used with females\(^{278}\)). Therefore, while we have identified novel redox and inflammatory findings using a repeated social defeat model, due to the limitations of this model, further investigation is warranted to validate these observations in additional models of PTSD such as

\[ R=0.4739 \quad p<0.0001 \quad F=20.85 \]

\[ R=0.7297 \quad p<0.0001 \quad F=82.02 \]
unpredictable variable stress, predator exposure, inescapable foot shocks, or single prolonged stress.

Repeated social defeat has also previously been reported to generate both “resilient” and “susceptible” phenotypes across stressed animals\textsuperscript{239, 250, 251}. This grouping is based off the social interaction behavior test, and the threshold cutoff is set at 1.0 (the value in which a mouse will enter the interaction zone at the same frequency with and without a CD-1 present). Using these categories, these two groups of mice have been shown to have different physiological phenotypes, but in contrast, also show no differences in many behavioral phenotypes\textsuperscript{251}. When performing this categorization on our animals, we found that approximately 30% of stressed animals were found to be “resilient,” which is within the range (albeit the extreme low end) previously reported by Golden and colleagues\textsuperscript{250}. However, unlike previous reports, the resilient group here was statistically different in regards to social interaction ratio (and other parameters) to control animals. When examining the data closely, this difference is because animals demonstrating low social interaction ratios exist among control animals, but yet are all averaged as one composite group overlooking this natural variation. Therefore, we have pursued an alternative approach examining both group statistics (\textit{i.e.}, control versus stress), and also individual statistics across dimensions of behavior and physiology. By using this type of analysis, we aim to mirror the National Institutes of Mental Health’s Research Domain Criteria (RDoC) method that attempts to limit categorization diagnoses, but instead examine individuals across various dimensions of behavior. In doing so, we find that correlations hold true across all animals dependent upon behavior phenotype, not trauma exposure, which we believe may be more reflective of the human condition.

One of the first intriguing findings of this work is that we show that T-lymphocytes express their own neurotransmission synthesis and degradation machinery that is dysregulated during psychological stress, suggesting the potential for T-lymphocyte-
driven microenvironmental control of inflammation. Indeed, expression of these pro-
sympathetic genes was tightly correlated with both mitochondrial superoxide and pro-
inflammatory cytokine levels within T-lymphocytes, further supporting this pro-
inflammatory hypothesis. However, T-lymphocyte expression of neurotransmission
enzymes is a relatively new observation, and little is known regarding the contribution of
these signaling pathways in these adaptive immune systems. TH expression and
endogenous catecholamine production by T-lymphocytes was first observed in the mid-
1990’s and its function was shown to suppress lymphocyte proliferation and differentiation,
which was suggested as a possible negative feedback mechanism to attenuate
inflammation150. Work from Yu-Ping Peng and Yi-Hua Qiu has also shown an overall
suppression phenotype of TH expression within T-lymphocytes. Several studies from this
group have shown that TH expression and T-lymphocyte catecholamine production leads
to a pro-Th2 phenotype with suppression of Th1279-281. They have more recently
demonstrated that TH expression correlates with the pro-inflammatory Th17 subtype of T-
lymphocytes, however, forced over-expression of TH in T-lymphocytes suppressed the
polarization to the Th17 phenotype282. This data again suggests that T-lymphocytes may
upregulate TH in pro-inflammatory subtypes as an auto-regulatory feedback mechanism
to control inflammation. However, contradicting results exist showing both positive and
negative effects of endogenous catecholamine production on anti-inflammatory regulatory
T-lymphocyte function102,282. Here, we demonstrate significant elevation of TH expression
within T-lymphocytes after repeated psychological trauma. Given the high correlation
between TH and pro-inflammation gene expression levels among T-lymphocytes, these
data support that either TH promotes inflammation within T-lymphocytes, or is upregulated
in a compensatory manner to counteract the pro-inflammatory phenotype. Interestingly,
T-lymphocyte TH levels did not correlate with behavioral changes after stress-induction,
suggesting psychological manifestations after stress may not be directly coupled to autonomic changes.

The cholinergic system has also shown significant regulatory control over T-lymphocytes, yet, lymphoid organs such as the spleen are not cholinergically innervated. Acetylcholine, CHAT, and ACHE are all endogenously produced within T-lymphocytes, and the regulation of acetylcholine on T-lymphocytes is complex, extensive, and has been previously reviewed. Overall, due to the spleen being exclusively innervated by the sympathetic splenic nerve, it is believed the primary source of acetylcholine in this organ is CHAT-expressing T-lymphocytes. Because of this, much work has focused on the role of CHAT-positive T-lymphocytes, while those expressing ACHE have been relatively understudied. Similar to CHAT, ACHE is significantly upregulated during T-lymphocyte activation suggesting a critical regulatory role in modulation of cellular function. Additionally, pharmacological inhibition of ACHE in T-lymphocytes reduced the production of IL-17A, suggesting the degradation of acetylcholine by ACHE may be important in the pro-inflammatory response. Here, we also demonstrate a significant induction of ACHE mRNA in splenic T-lymphocytes after stress, which like TH, correlated significantly with pro-inflammatory cytokine expression and mitochondrial superoxide levels in these cells. Together, these data suggest the potential for a causal relationship between sympathetic autonomic balance and the inflammatory phenotype of T-lymphocytes, but further studies are needed to identify the mechanistic nature of these neurotransmission enzymes during psychological trauma.

While our work demonstrated the effects of psychological stress on T-lymphocytes, it is interesting to note that previous work has shown that peripheral T-lymphocytes may conversely impact behavior. Original studies utilized immunodeficient mice, and could demonstrate that reconstitution with naïve T-lymphocytes restored enhanced cognitive function. Anxiety-like and depression-like behavior has also been improved by the
addition of naïve T-lymphocytes to immunodeficient animals\textsuperscript{291-293}, whereas pro-inflammatory T-lymphocytes potentiate pathological behavior\textsuperscript{294}. However, behavioral changes in response to T-lymphocytes do not appear to be universal, and vary among stress-induction paradigms\textsuperscript{295}. Here, we demonstrate that repeated social defeat stress increases pro-inflammatory gene expression within T-lymphocytes, however, expression of these cytokines only associated with depression-like and not anxiety-like behavior. Correlations between circulating IL-6 levels and depression-like behavior have been previously observed when categorizing socially-defeated animals into susceptible and resilient groups\textsuperscript{239}, but our data suggest individual differences display more as a spectrum as opposed to two separate entities. Additional studies are needed to identify if these correlations are specific to the social defeat paradigm, or may be more broadly applied.

Herein, we identified previously undescribed observations. First, we elucidate that repeated social defeat stress significantly increases mitochondrial superoxide levels within T-lymphocytes. This phenomenon appears specific to splenic T-lymphocytes, in that other cells of the spleen or even T-lymphocytes in circulation did not alter their mitochondrial redox environments after stress (data not shown). We previously observed that exogenous NE applied to T-lymphocytes \textit{ex vivo} could increase mitochondrial superoxide levels, which appeared to regulate both IL-6 and IL-17A expression in these cells\textsuperscript{24}. We posit that the elevated catecholamine levels in the spleens of social defeat animals may be eliciting a similar effect. Additionally, the highly significant positive correlations between mitochondrial superoxide and IL-6 or IL-17A levels with repeated social defeat is suggestive of similar mechanisms at play as well. The underlying cause of the increase in mitochondrial superoxide is currently unknown, but it is hypothesized that this induction may be occurring due to a potentiated metabolic state of the T-lymphocytes that occurs during activation\textsuperscript{296}. However, our previous work would suggest that mitochondrial superoxide plays a critical regulatory role in T-lymphocyte activation and differentiation,
and is not simply a by-product of another cellular process. Another potential source of superoxide within T-lymphocytes could be from the direct oxidation of catecholamines. Utilizing a mouse model of NE infusion along with a combination of adrenergic receptor and catecholamine transport inhibitors, we previously identified that direct intracellular oxidation of catecholamines was not the primary source of superoxide within T-lymphocytes, but this possibility has not been exhaustively tested yet in our psychological trauma model. Last, we observed that T-lymphocyte-specific mitochondrial superoxide levels positively correlated with anxiety-like behavior after social defeat stress. Taken together with the tight positive correlations among mitochondrial superoxide levels, pro-inflammatory cytokine expression, and neurotransmission gene expression in T-lymphocytes, our findings suggest the mitochondrial redox environment of these cells may be causally involved in the pro-inflammatory nature of these cells, which could potentiate pathological anxiety-like behavior similar to what is observed in PTSD. Overall, this research opens a new avenue of investigation into the mechanistic roles of neurotransmission, inflammation, and redox into the long-term consequences of psychological traumatic diseases like PTSD.
CHAPTER 4: Splenic Denervation Attenuates Repeated Social Defeat Stress-induced T-lymphocyte Inflammation

Portions of the content covered in this chapter are the subject of published works by Elkhatib et al. in Biological Psychiatry Global Open Science (in press)

Abstract

Posttraumatic stress disorder (PTSD) is a devastating psychological disorder. Patients with PTSD canonically demonstrate an increased risk for inflammatory diseases, as well as increased sympathetic tone and norepinephrine (NE) outflow. Yet, the exact etiology and causal nature of these physiologic changes remain unclear. Previously, we demonstrated that exogenous NE alters mitochondrial superoxide in T-lymphocytes to produce a pro-inflammatory T-helper 17 (T\textsubscript{H}17) phenotype, and observed similar T\textsubscript{H}17 polarization in a preclinical model of PTSD. Therefore, we hypothesized sympathetic-driven neuroimmune interactions could mediate psychological trauma-induced T-lymphocyte inflammation. Repeated social defeat stress (RSDS) is a preclinical murine model that recapitulates the behavioral, autonomic, and inflammatory aspects of PTSD. Targeted splenic denervation (Dnx) was performed to deduce the contribution of splenic sympathetic nerves to RSDS-induced inflammation. Eighty-five C57BL/6J mice underwent Dnx or sham-operation, followed by RSDS or control paradigms. Animals were assessed for behavioral, autonomic, inflammatory, and redox profiles. Dnx did not alter the antisocial or anxiety-like behavior induced by RSDS. In circulation, RSDS Dnx animals exhibited diminished levels of T-lymphocyte-specific cytokines (IL-2, IL-17A, and IL-22) compared to intact animals, whereas other non-specific inflammatory cytokines (e.g., IL-6, TNF-\textalpha, and IL-10) were unaffected by Dnx. Importantly, Dnx specifically ameliorated the increases
in RSDS-induced T-lymphocyte mitochondrial superoxide, T_{H17} polarization, and pro-inflammatory gene expression with minimal impact to non-T-lymphocyte immune populations.

Overall, our data suggest that sympathetic nerves regulate RSDS-induced splenic T-lymphocyte inflammation, but play less of a role in the behavioral and non-T-lymphocyte inflammatory phenotypes induced by this psychological trauma paradigm.
Introduction

Post-traumatic stress disorder (PTSD) is a stress-related disorder which is characterized by intrusive re-experiences of trauma (e.g., flashbacks), avoidance of reminiscent stimuli, affective changes, hyperarousal, and significant functional impairment. Among patients with PTSD, well-controlled clinical studies have repeatedly demonstrated an increased risk for a variety of inflammation-driven diseases such as rheumatoid arthritis, systemic erythematosus lupus, and cardiovascular disease. Importantly, the relationship and directionality between PTSD and the altered immune milieu remains a crucial question necessary in order to address the inflammation-driven diseases patients with PTSD experience (reviewed thoughtfully by Sumner et al.).

A breadth of studies has established that PTSD is associated with significantly increased activation of the sympathetic nervous system. This is evidenced by reported increases in urinary and cerebrospinal fluid norepinephrine (NE) content, baroreflex sensitivity, and muscle sympathetic nerve activity, many of which have also been found to correlate with PTSD symptom severity. This association between sympathetic tone and PTSD is subject to the same causality dilemma as the increased incidence of inflammatory diseases in these patients. Interestingly, secondary lymphoid organs such as the spleen are exclusively innervated by sympathetic efferent nerves that terminate near T-lymphocyte-rich areas, suggesting NE release may play a role in immune regulation during states of sympathoexcitation. Indeed, work from our lab has established that exogenous NE enhances ex vivo T-lymphocyte production of interleukins 6 (IL-6) and 17A (IL-17A). During investigations probing the intracellular mechanisms responsible, we demonstrated the NE-induced increase in T-lymphocyte production of IL-6 and IL-17A could be partially reversed by scavenging of mitochondrial superoxide in
these T-lymphocytes. Importantly, IL-6 and IL-17A have been heavily implicated in the pathogenesis of a number of inflammation-driven diseases, with human studies demonstrating their increase in patients with PTSD (summarized exceptionally by Wang et al). Moreover, in a preclinical mouse model of PTSD (i.e., repeated social defeat stress; RSDS), we elucidated a tight association between splenic T-lymphocyte NE content, mitochondrial superoxide, and inflammatory cytokine expression. Therefore, we sought to delineate the potential causal relationship between increased splenic sympathetic tone and T-lymphocyte inflammation in the context of RSDS.

In order to mechanistically investigate the role of RSDS-induced splenic sympathoexcitation on T-lymphocyte function, we performed selective sympathetic nerve ablation specifically to the spleen. Denervation of targeted sympathetic nerve beds has recently shown significant clinical utility in the cardiovascular arena by its ability to reduce blood pressure, improve cardiac and renal function, and lower blood glucose long-term with minimal to no adverse effects. Specifically, splenic denervation has also shown promise in a large animal model of inflammation, and is currently being considered for clinical trials of inflammatory conditions. However, to our knowledge, this approach has not been reported in any preclinical or clinical study of PTSD. Herein, we show that splenic denervation has a significant impact on systemic T-lymphocyte-derived inflammation. These results demonstrate the importance of direct neuro-immune interactions in RSDS, and put forth a novel translational finding that may attenuate psychological-trauma induced inflammation.
Materials and Methods

Mice

For further detail on how mice were housed, dissected, and euthanized, refer to the Materials and Methods section of Chapter 2. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Splenic denervation (Dnx)

To selectively chemically ablate the splenic nerve, mice were first anesthetized with 2.5% nebulized isoflurane supplemented with oxygen until appropriate depth of anesthesia was achieved. Mice were then shaved along the left lateral side, and the site was cleaned with surgical grade betadine followed by 70% ethanol. A small incision was made along the left side caudal to the ribs through the skin and peritoneum to visualize the spleen. Under magnification, the splenic artery was carefully dissected away from surrounding adipose and pancreas tissue. A cotton applicator soaked in 10% phenol in ethanol was gently applied to the splenic artery for 5-10 seconds until visual vasodilation was evident, with care taken to avoid contact with the spleen or surrounding tissue. Sham-operated mice were subjected to identical conditions with saline applied to the splenic artery. Following the operation, the muscular and peritoneal layer were sutured closed using 4-0 absorbable vicryl sutures (Ethicon Inc., #VCP304H, Somerville, NJ, USA), followed by skin and external fascia closure with 6-0 prolene sutures (Ethicon Inc., #8307H, Somerville, NJ, USA). Efficacy of denervation was assessed by measurement of splenic NE (discussed below) after euthanasia. Following denervation or sham operation, mice recovered for 7 days before entering RSDS or control protocols (described below).
**Repeated social defeat stress (RSDS)**

For further detail on the RSDS paradigm, refer to [Materials and Methods section of Chapter 2](#), with the paradigm summarized in Figure 11A. Following the 10-day RSDS or control paradigms, mice underwent behavioral testing on day 11 and terminal biologics harvested on day 12. All mice were assessed for visual wounding (>1 cm) or lameness and dutifully excluded (four mice in total were excluded in this study due to these parameters; these mice were not compiled into the final animal count).

**Splenic artery ultrasound**

Mice were anesthetized using 1-3% isoflurane with appropriate oxygen balance. Mice were placed on a heated stage in a lateral decubitus position with their left side facing the probe. Hair was removed using a depilatory cream (Nair & Co., Nair, Bristol, UK). B-mode and color Doppler imaging were used in combination to find the splenic artery using the high frequency Vevo 3100 (FujiFilm VisualSonics Inc., Toronto, ON, Canada) ultrasound machine and the MX550D transducer (center frequency 40MHz, axial resolution 40 µm). Pulsed-wave Doppler was used to measure blood velocity by placing the Doppler gate at the site of maximum velocity. Ultrasound operator was blinded to sham or Dnx status of animals. VevoLab (Fujifilm VisualSonics Inc.) was used for post-imaging analyses and measurements.

**Behavior testing**

For further detail on the behavioral testing, refer to [Materials and Methods section of Chapter 2](#).

**Splenocyte isolation**

For further detail on splenocyte isolation, refer to the [Materials and Methods section of Chapter 3](#).
**T-lymphocyte isolation**

For further detail on T-lymphocyte isolation, refer to Materials and Methods section of Chapter 3.

**T-lymphocyte immunophenotyping**

Splenocytes were incubated at 37°C for 4 hours in RPMI media supplemented with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), ionomycin (0.5 µg/mL), and BD Golgi Plug (containing Brefeldin A; 1 µL/mL; BD Biosciences, 555029, San Jose, CA, USA). Cells were then washed, re-suspended in cold phosphate buffered saline (PBS), and stained for viability for 30 min at 4°C using live/dead fixable UV stain (1:1000; Thermo Fisher, L23105, Waltham, MA, USA). Subsequently, cells were washed and re-suspended in aforementioned RPMI media supplemented with the following fluorescently tagged antibodies targeting extracellular T-lymphocyte proteins for 30 min: CD3ε PE-Cy7 (eBioscience clone 145-2C11, #25-0031-82, San Diego, CA, USA), CD4 Alexa 488 (eBioscience clone GK1.5, #53-0041-82, San Diego, CA, USA), CD8a APC (BD Biosciences clone 53-6.7, #553035, San Jose, CA, USA), CD25 BV605 (BD Biosciences clone PC61, #563061, San Jose, CA, USA). Following this, samples were washed, fixed, and permeabilized using the FOXP3 fixation and permeabilization kit (eBioscience, 00-5521-00, San Diego, CA, USA) per the manufacturer’s instructions. Per protocol, cells were washed again and re-suspended in permeabilization buffer with the following fluorescently-tagged antibodies targeting intracellular T-lymphocyte proteins for 30 min: IL-4 BV 421 (BD Biosciences clone 11B11, #566288, San Jose, CA, USA), IFN-γ APC-Cy7 (BD Biosciences clone XMG1.2, #561479, San Jose, CA, USA), IL-17A PE (BD Biosciences clone TC11-18H10, #561020, San Jose, CA, USA), FOXP3 PE-Cy5
(eBioscience clone FJK-16s, #15-5773-80, San Diego, CA, USA). Cells were washed, re-suspended in PBS, and data acquired using a customized BD LSRII flow cytometer. All flow cytometry experiments were conducted with accompanying single color and fluorescence-minus one (FMO) control tubes. Data was analyzed using FlowJo software.

**Splenocyte immunophenotype and mitochondrial redox analysis**

Freshly isolated live splenocytes were incubated in RPMI media supplemented with the following fluorescently tagged antibodies targeting extracellular proteins: CD3ε PE-Cy7 (eBioscience clone 145-2C11, #25-0031-82, San Diego, CA, USA), CD19 APC-Cy7 (Biolegend clone 6D5, #115530, San Diego, CA, USA), CD11b SB-436 (eBioscience clone M1/70, #62-0112-82, San Diego, CA, USA), CD11c APC (eBioscience clone N418, #17-0114-82, San Diego, CA, USA), and NK1.1 SB-600 (eBioscience clone PK136, #63594182, San Diego, CA, USA). Concurrently, 1 µM MitoSox Red, a mitochondrially-targeted superoxide sensitive probe (Thermo Fisher Scientific #M26008, Waltham, MA, USA), was added and cells incubated for 30 minutes at 37°C. Cells were washed, re-suspended in PBS, and data acquired using a customized BD LSRII flow cytometer. MitoSox Red mean fluorescence intensities (MFI) were normalized to intra-experiment sham-operated control samples. All flow cytometry experiments were conducted with accompanying single color and fluorescence-minus one (FMO) control tubes. Data was analyzed using FlowJo software.

**RNA extraction, cDNA production, and real-time RT-qPCR**

For further detail on the gene expression assessment, refer to the Materials and Methods section of Chapter 3. Generated cDNA was assessed for transcript levels by qPCR using intron-spanning gene-specific oligonucleotides (Appendix B). Gene specific PCR products were validated by thermal dissociation curves. Thresholds were set
objectively to determine cycle thresholds (CT), with 18s rRNA utilized as a loading control to determine \( \Delta CT \). All values were normalized to sham-operated control samples to determine \( \Delta \Delta CT \) values, which were then transformed to generate fold changes by the \( 2^{-\Delta \Delta CT} \) method.

**Immunoblotting**

For further detail on immunoblotting, refer to the Materials and Methods section of Chapter 3. Membranes were probed with primary antibodies targeted against tyrosine hydroxylase (TH, 1:1000 dilution, EMD Millipore #AB152, Burlington, MA, USA) or \( \beta \)-Actin (loading control; 1:1000 dilution, Sigma Aldrich #A2066, St. Louis, MO, USA), and secondary anti-mouse antibodies conjugated to horseradish-peroxidase (1:10,000, Thermo Fisher #31460, Waltham, MA, USA). Quantification was performed by densiometric analysis using ImageJ analysis software.

**Catecholamine assessment**

For further detail on the catecholamine assessment, refer to the Materials and Methods section of Chapter 3. All assays were completed according to the manufacturer’s protocol, with splenic and renal lysate catecholamine concentration normalized to wet tissue weight.

**Circulating cytokine analysis**

For further detail on circulating cytokine analysis, refer to the Materials and Methods section of Chapter 2. All experiments were conducted per manufacturer’s instructions and quantified on a Meso Scale Discovery Quickplex SQ 120, with analyses conducted using Mesoscale Discovery Workbench software (Rockville, MD, USA).
Statistical analyses

A total of 85 animals (38 control, 47 RSDS) were utilized in these studies. All mice were randomized to one of the four cohorts (Sham-Control, Sham-RSDS, Dnx-Control, or Dnx-RSDS), with all efforts made to blind experimenters during biological assay, data acquisition, and data analysis. At least 3 independent experimental repeats were conducted, with animals across each cohort included. Not all biological parameters were able to be run in a single animal, thus, figures are individually labeled with N values and statistical information utilized for a specific set of experiments. Individual data are presented along with mean ± standard error of the mean (SEM). All data were assessed for parametric distribution prior to determine the appropriate statistical analysis. Differences were considered significant at p<0.05.
Results

*Splenic Dnx attenuates RSDS-induced elevations in splenic sympathetic tone*

In order to effectively evaluate the role of splenic innervation in RSDS-induced inflammation, targeted splenic Dnx was performed prior to RSDS paradigm (Figure 20A). Prior to RSDS induction, Dnx was able to significantly reduce levels of tyrosine hydroxylase (Figure 20B) and norepinephrine (Figure 20D).

*Figure 20. Splenic denervation is a robust and specific method to reduce splenic norepinephrine.*

Wild-type (WT) C57BL/6J mice were sham-operated or denervated (Dnx), with biologicals assessed after 7 days before introduction to RSDS. **A** Overall experimental schematic. **B** Representative western blot analysis of splenic tyrosine hydroxylase (TH) in sham and Dnx animals. **C** Quantification of splenic TH normalized to β-actin protein. **D** Norepinephrine (NE) content in tissue lysate by ELISA. Left, NE content in spleen normalized to tissue weight. Right, NE content in ipsilateral (left) kidney normalized to tissue weight. Comparisons between sham and Dnx by Mann-Whitney U test.
hydroxylase (TH; the rate-limiting enzyme of catecholamine synthesis) in splenic lysate compared to sham animals (Figure 20B-C). Direct measurement of NE content within the spleen showed a 79% reduction in splenic NE as compared to sham-operation (Figure 20D), with no effect on ipsilateral kidney NE content (Figure 20D). To ensure splenic blood flow was not affected by the Dnx procedure, pulsed-wave echo doppler ultrasound demonstrated no significant differences in peak velocity or velocity time integral between sham or Dnx in blood flow of the splenic artery (Figure 21).

After seven days of recovery, sham and Dnx animals were assigned to control or RSDS paradigms. Splenic NE was elevated RSDS mice compared to controls in the sham

![Image of doppler ultrasound and statistical analysis](image)

**Figure 21.** Dnx does not alter blood flow to the spleen.

C57BL/6J wild-type mice were sham-operated or denervated, then assessed for blood flow by B-mode and color mode Doppler imaging under isoflurane anesthetic. A) Representative doppler ultrasound of spleen following sham or Dnx. B) Left, Peak velocity calculated by pulsed wave doppler, with gate placed at point of maximal velocity. Right, velocity time integral by pulsed wave doppler. Statistical analyses conducted by parametric t-test as appropriate.
group, though the difference did not reach statistical significance (Figure 22A). In contrast, Dnx significantly attenuated splenic NE in both control and RSDS compared to respective sham groups (p=0.0197 and p<0.0001, respectively; Figure 22A). Within the plasma, there were no significant differences in NE concentration across all groups (Figure 22B).

Figure 22. Dnx abrogates RSDS alterations in splenic NE, with no effect on plasma NE concentration.

Mice were randomized to ±Dnx and ±RSDS cohorts, and NE was assessed in tissue or plasma by ELISA at the completion of the stress paradigm. A) NE content in spleen normalized to tissue weight (2-way ANOVA group results; Stress p=0.0010, Dnx p<0.0001, Interaction p=0.0055). B) Plasma NE concentration (2-way ANOVA group results; Stress p=0.981, Dnx p=0.3592, Interaction p=0.5241). Statistical analyses by 2-way ANOVA with Tukey post-hoc correction; of interest post-hoc p-values listed on each figure if respective group effects were found significant.
Together, these data demonstrate that Dnx is a feasible, robust, and specific method of attenuating RSDS-induced sympathetic tone.

**Dnx does not alter social or anxiety-like behavior seen in RSDS**

As we and others have previously reported, RSDS resulted in significantly decreased sociability by social interaction test in sham-operated mice (Figure 23A). Interestingly, Dnx mice exposed to RSDS also showed decreased sociability, but the

![Figure 23. Dnx does not affect RSDS-induced antisocial or anxiety-like behavior.](image)

Mice were tested for pro-social and anxiety-like behavior after exposure to ±Dnx and ±RSDS. **A)** *Left*, representation of social interaction test for social behavior. *Middle*, social interaction zone ratio (2-way ANOVA group results; Stress p<0.0001, Dnx p=0.6714, Interaction p=0.0059). *Right*, corner zone ratio (2-way ANOVA group results; Stress p=0.0304, Dnx p=0.9669, Interaction p=0.9218). **B)** *Left*, representation of elevated zero maze for anxiety-like behavior. *Middle*, time spent in open arm of maze (2-way ANOVA group results; Stress p<0.0001, Dnx p=0.1113, Interaction p=0.6793). *Right*, total distance moved (2-way ANOVA group results; Stress p<0.0001, Dnx p=0.5647, Interaction p=0.5719). Statistical analyses by 2-way ANOVA with Tukey post-hoc correction; of interest post-hoc p-values listed on each figure if respective group effects were found significant.
variability precluded corrected statistical significance as compared to sham RSDS (p=0.0777; Figure 23A). Similar to what we have seen and reported previously 243, corner zone ratio did not reliably display significant differences between any groups (Figure 23A).

In order to assess anxiety-like behavior, we performed elevated zero maze tests. Time spent in the open arms of the maze was significantly decreased in RSDS animals compared to controls in both sham-operated and Dnx groups (Figure 23B). Additionally, distance moved during the elevated zero maze test showed similar decreases in RSDS animals with no statistical differences between RSDS-exposed sham or Dnx groups (Figure 23B). Overall, these data suggest splenic Dnx does not greatly affect anti-social or anxiety-like behavior induced by RSDS.

Circulating inflammation due to RSDS is partially ameliorated by splenic Dnx

We recently reported several cytokines are significantly elevated in circulation after RSDS 243, including interleukin 2 (IL-2), 6 (IL-6), 10 (IL-10), 17A (IL-17A), 22 (IL-22), and tumor necrosis factor alpha (TNFα). As expected, RSDS increased circulating levels of these cytokines in sham-operated animals (Figure 24), with IL-10 and TNFα not reaching statistical significance in this cohort (This current study has significantly fewer animals than our previous report). Dnx was able to significantly attenuate RSDS-induced increases in circulating IL-2, IL-17A, and IL-22, whereas IL-6, IL-10, and TNFα were largely unaffected by Dnx (Figure 24). Importantly, IL-2, IL-17A, and IL-22 are produced almost exclusively by T-lymphocytes, whereas the others are produced by a variety of cell types. These data demonstrate that denervation is able to attenuate RSDS-induced increases in specific circulating cytokines, which may be linked to T-lymphocyte subtypes.
Dnx attenuates RSDS-induced splenic T-lymphocyte mitochondrial superoxide

We have previously demonstrated both exogenous NE and RSDS result in an increase in mitochondrial superoxide within T-lymphocytes that is linked to a pro-inflammatory phenotype. As before, RSDS induced an increase in T-lymphocyte mitochondrial superoxide in sham animals compared to controls (Figure 25). Strikingly, Dnx completely attenuated this RSDS-induced increase in mitochondrial superoxide (Figure 25). Additionally, natural killer cells in the spleen also routinely demonstrate increases in
mitochondrial superoxide after RSDS (Figure 26). However, natural killer cell mitochondrial superoxide was not significantly attenuated by Dnx (when comparing to the respective control animals; Figure 26). In examining the effects on immune cell populations, RSDS and Dnx did not show clear effects on T-lymphocytes (CD3; Figure 27), despite previous literature demonstrating small but significant decreases in the number of T-lymphocytes in stress paradigms. Moreover, no other cell type in the spleen demonstrated any population alterations with Dnx (Figure 26). Overall, these data suggest splenic innervation and RSDS have significant and robust effects on mitochondrial superoxide within T-lymphocytes, with minimal impact on cell population distribution.

**Dnx reduces RSDS-induced splenic Th17 lymphocytes and pro-inflammatory gene expression**

Utilizing a hierarchical gating strategy, we found CD4+ helper and CD8+ cytotoxic T-lymphocyte populations were not significantly altered by RSDS or Dnx (Figure 28). Within CD4+ subtypes, Th1 and Th2 were unchanged, whereas Th17 and Treg populations were
increased by RSDS in sham mice (Figure 28). Importantly, Dnx tempered this RSDS effect in both T\(_{H}17\) and T\(_{REG}\) subtypes (Figure 28). We further investigated inflammatory differences by examining overall T-lymphocyte gene expression. Within purified splenic T-lymphocytes of sham mice, RSDS produced increased expression of IL-2, IL-6, IL-10, IL-17A, IL-22, and TNF\(\alpha\) (Figure 29). Within the Dnx group, RSDS attenuated these 6 cytokines within splenic T-lymphocytes (Figure 29), whereas cytokines such as interferon \(\gamma\) and IL-4 showed no differences across any groups. These data provide further evidence for the role of splenic innervation in RSDS-induced T-lymphocyte regulation.
Discussion

In our previous work\(^{238}\), we demonstrated an association between RSDS-induced splenic catecholamines, T-lymphocyte inflammatory gene signatures, and T-lymphocyte mitochondrial superoxide. Herein, we have demonstrated a causal role for neural-derived NE in the regulation of distinct facets of T-lymphocyte-driven inflammation during RSDS. By utilizing a method of splenic denervation which effectively reduced splenic TH and NE, we deduced the role of this neuroimmune connection \textit{in vivo} on the behavioral, inflammatory, and redox phenotypes of RSDS. Dnx did not affect RSDS-induced anti-social or anxiety-like behavior, but did attenuate RSDS-induced increases in circulating cytokines, T-lymphocyte mitochondrial superoxide, T\(_{17}\) and T\(_{reg}\) populations, and T-lymphocyte gene signatures. These data are summarized graphically in Figure 30. Together, these data provide valuable insight into splenic neuroimmune interactions that are responsible for RSDS changes in T-lymphocyte-driven inflammation.

PTSD is a complex behavioral disorder with important physiological manifestations. In order to perform mechanistic investigations, current studies rely on one of many preclinical

![Figure 27. Dnx or RSDS do not alter Splenic T-lymphocytes.](image-url)
mouse models\textsuperscript{237,249}. Among the various PTSD models that have arisen, RSDS has been lauded for its ability to recapitulate inflammatory characteristics of PTSD\textsuperscript{237,249}. Additionally, many works employing RSDS have utilized the social interaction test to divide mice into susceptible and resilient groups based on the social interaction ratio\textsuperscript{239,250}. However, we recently demonstrated this divide does not associate with another commonly utilized behavioral test of anxiety, the elevated zero maze, nor with circulating inflammatory proteins that increase during RSDS\textsuperscript{243}. In the investigations herein, splenic Dnx did not have a meaningful effect on the behavioral parameters measured, but did

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**Figure 28.** Dnx reverses RSDS increases in T-lymphocyte subtypes $T_{H17}$ and $T_{REG}$ while other subtypes remain unaffected.

Mice were assigned to $\pm$Dnx and $\pm$RSDS cohorts followed by immunophenotyping of splenic T-lymphocyte populations by flow cytometry. All values represent frequency of live cells by fixable viability stain, with marker positivity listed below each subtype assay. 2-way ANOVA group results: $CD4$ Stress p=0.9659, Dnx p=0.1315, Interaction p=0.6076; $CD8$ Stress p=0.0195, Dnx p=0.0256, Interaction p=0.5089; $T_H1$ Stress p=0.4305, Dnx p=0.2904, Interaction p=0.2441; $T_H2$ Stress p=0.6024, Dnx p=0.0953, Interaction p=0.9874; $T_{H17}$ Stress p=0.0039, Dnx p=0.0051, Interaction p=0.0036; $T_{REG}$ Stress p=0.0258, Dnx p=0.0069, Interaction p=0.0836. Statistical analyses by 2-way ANOVA with Tukey post-hoc correction; of interest post-hoc p-values listed on each figure if respective group effects were found significant.
significantly impact T-lymphocyte-derived inflammation. Importantly, Dnx did not robustly affect other aspects of inflammation, such as circulating IL-6 and other splenocyte populations. Additionally, the changes to circulating inflammation demonstrated an attenuation of the RSDS-induced increases, not a complete ablation, which belies the known existence of neuroimmune connections within other lymphoid organs. Moreover, splenic denervation likely has no effect on central noradrenergic tone, which may be responsible for the resultant changes in behavior. Overall, the complex relationship between behavior and inflammation is likely systemic, involves numerous immune cell types, and has both neural and hormonal contributions. The connections between

![Figure 29. Dnx alters RSDS-induced changes in gene expression within purified splenic T-lymphocytes.](image)

Splenic T-lymphocytes were purified by negative selection, followed by RNA extraction and cDNA conversion for quantitative real-time RT-PCR. Data shown are fold change normalized to sham-operated control-housed animals, with all ΔCT values normalized to 18s rRNA loading control. 2-way ANOVA group results: **IL-2** Stress p=0.0067, Dnx p=0.0097, Interaction p=0.0028; **IL-4** Stress p=0.4622, Dnx p=0.5022, Interaction p=0.0892; **IL-6** Stress p=0.0038, Dnx p=0.0047, Interaction p=0.0021; **IL-10** Stress p=0.0309, Dnx p=0.0037, Interaction p=0.1969; **IL-17A** Stress p=0.0045, Dnx p=0.0093, Interaction p=0.0333; **IL-22** Stress p=0.0007, Dnx p=0.0014, Interaction p=0.0057; **TNFα** Stress p=0.0032, Dnx p=0.0014, Interaction p=0.0013; **IFNγ** Stress p=0.8341, Dnx p=0.4799, Interaction p=0.6654. Statistical analyses by 2-way ANOVA with Tukey post-hoc correction; of interest post-hoc p-values listed on each figure if respective group effects were found significant.
behavior and inflammation remains a significant area of interest which requires more nuanced approaches that must rely on non-binary outputs of behavior or peripheral inflammation \cite{203,313}. Additionally, future studies examining RSDS and peripheral inflammation would benefit from the inclusion of other behavioral paradigms outside of the social interaction test and elevated zero maze, such as startle responses, hypervigilance, et cetera.

Herein, we similarly examined alterations to peripheral inflammation. We demonstrated that Dnx ameliorates characteristic increases in some circulating cytokines after RSDS (likely not completely due to the targeting of only one secondary lymphoid organ), with a partiality for cytokines more canonically associated with T-lymphocytes (IL-2, IL-17A, IL-22), while other cytokines (IL-6, TNF\textsubscript{\alpha}, and IL-10) were grossly unaffected. Works by pioneers of RSDS like John Sheridan \textit{et al.} have previously examined the effects of RSDS on splenocyte populations, primarily monocytes \cite{242}. They have described that RSDS induces the amount of glucocorticoid-insensitive monocytes in the spleen and circulation, which contribute to the RSDS-induced increase in circulating cytokines such as IL-6, CCL2, and TNF\textsubscript{\alpha} (summarized nicely by Reader \textit{et al.}) \cite{241}. Our data confirm and extend these findings by showing that splenic innervation appears to primarily affect T-lymphocyte populations, while the innate immune system response to RSDS is preserved even in the absence of splenic innervation. Combined with this current work, these data provide further evidence for the specificity—both anatomical, cellular, and hormonal—of immune responses to RSDS.

At the anatomical level, this work is built upon an important premise that the spleen receives exclusively sympathetic innervation, and thus splenic denervation allows for effective study of this single variable. This topic has been reviewed exhaustively elsewhere \cite{161}, with a breadth of data demonstrating a lack of parasympathetic innervation
to the spleen. Conversely, classic and recent functional studies have elucidated splenic sympathetic efferent nerves’ role in various immune responses. In recent works examining the anti-inflammatory reflex, NE released from the splenic nerve has been shown to signal to choline acetyltransferase positive (CHAT+) T-lymphocytes through their β2-adrenergic receptors. Subsequently, these CHAT+ T-lymphocytes produce acetylcholine which signals through the alpha-7 nicotinic receptor (α7-nAchR) on macrophages to suppress the release of inflammatory cytokines into circulation. However, the explicit interactions upstream from the splenic nerve in this pathway is a topic wont with controversy. Work by Tracey et al. has provided evidence this is mediated upstream by the vagal efferent which synapse at the celiac ganglion to modulate the splenic nerve, while McAllen et al. have shown these splenic nerve fibers originate from sympathetic splanchnic nerves. Overall, the data presented herein only provides further evidence for the influence of splenic NE on T-lymphocytes (primarily pro-

**Figure 30. Summative representation of splenic nerve-mediated effect of RSDS on T-lymphocytes.**

Through sympathetic splenic nerves, RSDS induces mitochondrial superoxide increase within T-lymphocytes, which results in altered circulating cytokine secretion, pro-inflammatory gene expression, and T-helper subtype polarization.
inflammatory) in the important context of psychological trauma. The relationship between our findings and various facets of the anti-inflammatory reflex remains unresolved, but are a potential area for future investigations.

At the cellular and molecular level, this investigation provides new insights into how psychological trauma affects the complex ability of the sympathetic nervous system to modulate splenic T-lymphocyte-driven inflammation. It has been well-established that splenic T-lymphocytes are exposed to high concentrations of NE released by sympathetic nerve terminals. However, the response of T-lymphocytes to this NE and other catecholamines has been shown to be largely dependent on experimental details, such as type of immune challenge, timing of sympathoexcitation, activation state of T-lymphocytes, and murine strain. While a significant body of work has pointed to the β2-adrenergic receptor playing a primary role of NE effects in T-lymphocytes, other work (including our own) has also demonstrated α-adrenergic receptors in the redox and inflammatory response of T-lymphocytes. Like many systems, it is highly probably that adrenergic receptors (and other neurotransmitter receptors) on T-lymphocytes do not work autonomously, but rather together in a system-dependent fashion to respond to the neurotransmitter milieu. Identification of these signaling cascades is indeed important to understand regulation of inflammation via autonomic signaling, but given the numerous intercommunicating cell types, dozens of neurotransmitters, and multitudes of receptors involved, this a highly complex and challenging aspect of neuroimmune communication investigations.

Importantly, our previous work has strongly demonstrated that NE-induced increases in specific cytokines, such as IL-6 and IL-17A, is partially mediated by increases in mitochondrial superoxide, as evidenced by an attenuation of these cytokines after treatment with MitoTEMPOL, a selective mitochondrial superoxide scavenger.
Additionally, in a genetic manganese superoxide dismutase (MnSOD) knockout model with increased mitochondrial superoxide, \textit{ex vivo} IL-17A production was distinctly increased \textsuperscript{324}. Importantly, these changes in the mitochondrial redox environment cannot be divorced from the overall metabolic status of these T-lymphocytes \textsuperscript{325}. It is possible that the increase in mitochondrial superoxide we have observed in T-lymphocytes after RSDS may be due to alterations in cellular metabolism, or vice versa. Metabolism has been recently shown to be a primary driver of T-lymphocyte activation and differentiation \textsuperscript{326, 327}, but has not been fully explored in the context of psychological stress disorders. Untangling the cellular mechanism by which altered mitochondrial redox and metabolism are able to ultimately effect T-lymphocyte function during psychological trauma is an important future direction for this research. In furthering this work, we hope to find new therapeutic targets to ameliorate the deleterious pro-inflammatory shifts that are linked to PTSD, with a keen interest in understanding the directionality of PTSD and inflammation.

This study has important implications but is not without limitations. An important concept of note is the ability of immune cells to produce catecholamines, which could function in an autocrine or paracrine fashion to exert similar effects \textsuperscript{2, 102, 279-281}. This has been repeatedly demonstrated, and requires further investigation to understand the potential role for these immune cell-derived neurotransmitters in the context of health and disease. Additionally, RSDS is a preclinical model of PTSD that only recapitulates certain aspects of the human condition. Our interest in the autonomic and inflammatory relationship prompted our usage of this model, but the findings herein could be further validated by utilizing other psychological trauma animal models to probe the role of this neuroimmune connection. Notably, RSDS as a model of PTSD has limitations. Physical interaction allows for wounding which proves a potential confounding factor when investigating inflammation and immune cell populations, although other studies have
demonstrated similar immune changes in witnessed social defeat paradigms 240. Additionally, RSDS does not display certain characteristic features of PTSD, such as decreases in fear extinction or altered hypothalamic-pituitary-adrenal feedback 237, 249. Last, the standardized model of RSDS precludes examination of these effects in females, which is important given their higher risk for PTSD. New adaptations of RSDS that include females do exist, but often rely on differential action of females versus males which prevents direct comparison between the sexes. This further supports the warranting of examination of these findings in additional preclinical models of PTSD as well as in human subjects.

Overall, T-lymphocyte-driven inflammation—specifically signatures of T\textsubscript{H}17-driven inflammation—has been implicated in a variety of inflammation-driven and autoimmune disorders 303, 328, 329, as well as have been shown to be increased in patients with PTSD 230, 233, 274. In the current study, we have presented a clear role for increased sympathetic tone—a known hallmark of PTSD—in being responsible for this T\textsubscript{H}17 phenotype shift. Splenic denervation as a therapeutic technique has already been translated to larger animals, which enhances the clinical relevance of our preclinical PTSD findings described herein. By furthering our mechanistic understanding of how splenic neuroimmune connections in lymphoid organs could be responsible for the increased morbidity and mortality from trauma-induced inflammatory diseases, we may eventually provide novel treatment strategies for patients with PTSD and other trauma-related disorders.
CHAPTER 5: T-lymphocyte Tyrosine Hydroxylase Regulates TH17 T-lymphocytes during Repeated Social Defeat Stress

Abstract

Posttraumatic stress disorder (PTSD) is a debilitating psychiatric disorder which results in deleterious changes to psychological and physical health. Patients with PTSD are especially susceptible to co-morbid inflammation-driven pathologies, such as autoimmunity, while also demonstrating increased T-helper 17 (TH17) lymphocyte-driven inflammation. While the exact mechanism of this increased inflammation is unknown, overactivity of the sympathetic nervous system is a hallmark of PTSD. Neurotransmitters of the sympathetic nervous system (i.e., catecholamines) can alter T-lymphocyte function, which we have previously demonstrated to be partially mitochondrial redox-mediated. Furthermore, we have previously elucidated that T-lymphocytes generate their own catecholamines, and strong associations exist between tyrosine hydroxylase (TH; the rate-limiting enzyme in the synthesis of catecholamines) and pro-inflammatory interleukin 17A (IL-17A) expression within purified T-lymphocytes in a preclinical rodent model of PTSD. Therefore, we hypothesized that T-lymphocyte-generated catecholamines drive TH17 T-lymphocyte polarization through a mitochondrial superoxide-dependent mechanism during psychological trauma. To test this, T-lymphocyte-specific TH knockout mice (TH\textsuperscript{T-KO}) were subjected to repeated social defeat stress (RSDS). RSDS characteristically increased tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)), IL-6, IL-17A, and IL-22, however, IL-17A and IL-22 (TH17 produced cytokines) were selectively attenuated in circulation and in T-lymphocytes of TH\textsuperscript{T-KO} animals. When activated \textit{ex vivo}, secretion of IL-17A and IL-22 by TH\textsuperscript{T-KO} T-lymphocytes was also found to be reduced but could be partially rescued with supplementation of norepinephrine specifically. Interestingly, TH\textsuperscript{T-KO}
T-lymphocytes were still able to polarize to T\(_h\)17 under exogenous polarizing conditions. Last, contrary to our hypothesis, we found RSDS-exposed TH\(^{T-KO}\) T-lymphocytes still displayed elevated mitochondrial superoxide, suggesting increased mitochondrial superoxide is upstream of T-lymphocyte TH induction, activity, and T\(_h\)17 regulation. Overall, these data demonstrate TH in T-lymphocytes plays a critical role in RSDS-induced T\(_h\)17 T-lymphocytes and offer a previously undescribed regulator of inflammation in RSDS.
Introduction

Posttraumatic stress disorder (PTSD) is a debilitating, stress-related disorder which has been described by various names since antiquity. PTSD is characterized by exposure to a traumatic event in conjunction with the persistence of a constellation of symptom clusters meeting specific diagnostic and statistical manual of mental disorders (DSM) criteria, such as affective changes, avoidance behavior, dissociative symptoms, and hyperarousal. PTSD as a mental health disorder presents a tremendous cost to the individual and society, with 40% of patients suffering from PTSD even 10 years after their initial diagnosis.

Importantly, there is a breadth of research demonstrating a connection between PTSD and deleterious changes to physical health. Patients with PTSD are at an increased risk for a variety of inflammation-driven diseases, ranging from rheumatoid arthritis to cardiovascular disease, which ultimately contribute to the decreased quality of life and lifespan of these patients. Intimately related to this risk for co-morbid diseases, patients with PTSD display distinctive physiological changes, specifically within the nervous and immune systems. Patients with PTSD demonstrate increased sympathetic tone by measures such as urinary norepinephrine (NE) and baroreflex sensitivity, in addition to heightened T-lymphocyte-driven inflammatory markers. There is a breadth of work demonstrating the effects of NE on T-lymphocyte functional status both in vitro and in vivo. For example, our in vitro work demonstrated NE supplementation to activated T-lymphocytes resulted in increased pro-inflammatory cytokine profiles, such as IL-6 and IL-17A, partially mediated by a mitochondrial redox mechanism. Thus, the link between PTSD and inflammatory diseases may lie in connection of catecholamines and T-lymphocytes.
Critically, it has been shown in numerous investigations that T-lymphocytes themselves generate, release, and respond catecholamines (dopamine, norepinephrine, and epinephrine).\textsuperscript{102, 155} This has been demonstrated at the single cell level in both isolated and immortalized lymphocytes, while \textit{in vitro} studies utilizing pharmacological inhibition of catecholamine synthesis demonstrated these catecholamines influence the functionality and activation state of T-lymphocytes.\textsuperscript{102} In our own work, we investigated how increased sympathoexcitation in the murine model of PTSD known as repeated social defeat stress (RSDS) could affect T-lymphocyte inflammatory signatures.\textsuperscript{238} We discovered that T-lymphocytes from RSDS animals had increased in the gene expression of tyrosine hydroxylase (TH; the rate-limiting step in catecholamine synthesis), which strongly correlated with expression of pro-inflammatory IL-17A in purified T-lymphocytes. Importantly, IL-17 and T\textsubscript{H}17 cells have been heavily implicated in several inflammatory and autoimmune diseases.\textsuperscript{67, 329}

To this end, we sought to examine the role of T-lymphocyte-generated catecholamines in T-lymphocyte-driven inflammation seen during RSDS. By generating a conditional T-lymphocyte TH knockout mouse (TH\textsuperscript{T-KO}), we were able to selectively investigate this mechanism and further define the role for catecholamines in T\textsubscript{H}17-mediated inflammation.

\textbf{Materials and Methods}

\textit{Mice}

In order to specifically investigate the role of TH in T-lymphocytes, a cell-type specific (conditional) TH knockout mouse was created and utilized herein. First, mice possessing loxP elements flanking exon 1 of the TH gene locus (\textit{i.e.}, B6.Cg-Th\textsuperscript{im4.1-Rpa}; TH\textsuperscript{lox/lox}) were graciously obtained from Michael Iuvone at Emory University.\textsuperscript{332} TH\textsuperscript{lox/lox} mice were then crossed to mice expressing codon-improved cre recombinase under the control of the
distal promoter of T-lymphocyte-specific tyrosine kinase (Lck) (*i.e.*, B6.Cg-Tg(Lck-
cre)^3779Nik/J), originally generated by Nigel Killeen. This distal Lck promoter is active at
or after T-cell receptor (TCR) upregulation during positive selection of thymocytes and has
shown activity in both αβ and γδ T-lymphocyte subsets. Mice were crossed to the F3
generation, allowing for 100% progeny possessing homozygous TH^lox/lox^ alleles, 50%
bearing Lck cre recombinase (*Figure 31*; T-lymphocyte specific TH knockouts; referred to
as TH^{T-KO}), and 50% cre negative (*Figure 31*; TH^lox/lox^ cre recombinase negative controls;
referred to as TH^{Con}).

For further detail on how mice were housed, dissected, and euthanized, refer to the
*M*aterials and Methods section of Chapter 2. When possible, experimenters were blinded
to the genotype and stress-exposure of mice until the completion of the study. All
procedures were reviewed and approved by both the University of Nebraska Medical
Center and Texas A&M University Institutional Animal Care and Use Committees.

**Genotyping TH KO**

Rationally designed primers and polymerase chain reaction (PCR) were utilized to
assess recombination of the TH gene. DNA was first isolated through use of GeneJET
Genomic DNA Purification Kit (#K0722, Thermo scientific, Waltham, MA). Oligonucleotide
primers (Forward: 5'-GAAGACCCTAGGGAGATGCCAAA-3'; Reverse 5'
TTTCCCTTACTTCA-CAAAATAGGACCCACGAA) were specifically designed which
could distinguish the recombined KO TH allele from the unperturbed TH^lox/lox^ genotype by
size. PCR products were loaded onto a 1.5% agarose gel with 1 mg/mL of ethidium
bromide for intercalation, separated by electrophoresis at 100 V, and visualized under
ultraviolet light alongside a DNA ladder (GeneRuler 1kb Plus, #SM1331, Thermo
Scientific).
Social defeat stress paradigm

An adapted version of RSDS as described by Golden et al. was utilized for all studies. For further detail on the RSDS paradigm, refer to the Materials and Methods section of Chapter 2, with the paradigm summarized in Figure 11A. At the end of the 10-day period (day 11), all mice were behaviorally tested. After testing, control and experimental mice remained in their former co-housed barrier cage until the following day (day 12) when they were sacrificed for biological analysis.

Behavioral testing

For further detail on behavioral testing, refer to the Materials and Methods section of Chapter 2.

Splenic T-lymphocyte isolation

Pan, CD4+, and CD8+ murine T-lymphocytes were magnetically negatively selected from whole spleens as has been previously described. For further detail on Splenic T-lymphocyte isolation, refer to the Materials and Methods section of Chapter 4.

T-lymphocyte activation, culture, and catecholamine supplementation

T-lymphocytes were cultured as has been previously described. Briefly, after T-lymphocyte isolation, 8.0 x 10⁵ cells/mL were plated with anti-CD3/anti-CD28 magnetic activation beads in a 1:1 ratio with cells (Dynabeads™, #11456D, Thermo Fisher Scientific), and incubated for 72 hours at 37°C prior to harvest and analysis.

For catecholamine treatment conditions, freshly harvested splenic CD4+ T-lymphocytes were isolated and plated in 24 well plates. Wells were supplemented daily with 10 µM of dopamine hydrochloride #AAA1113606, Thermo Scientific Chemicals), L-norepinephrine (#AAL0808703, Thermo Scientific Chemicals), or L-epinephrine (#AAL0491106, Thermo Scientific Chemicals) reconstituted in 1x sterile PBS.
Catecholamine concentrations were based on our previous published work with ex vivo catecholamine dosage curves of T-lymphocytes. T-lymphocytes from a single animal received each treatment condition allowing for paired analyses, with 3 technical replicates for each sample and treatment combination.

T-lymphocyte growth curves were obtained by live cell analyses. CD4+ or CD8+ T-lymphocytes were isolated and plated as stated above in 96-well flat bottom plates and then placed into an Incucyte S3 Live-cell analysis system (Essen Bioscience) housed within an environmental chamber (37°C, 5% CO2) for 72 hours. Live cell images for confluence assessments were taken every 5 minutes for the 72-hour period. Confluence was normalized to starting densities after optical focusing, with >5 technical replicate wells used per sample. Data were analyzed using Incucyte S3 analysis software.

**T-lymphocyte Th17 polarization**

CD4+ splenic T-lymphocytes were polarized to Th17 ex vivo by use of CellXVivo Mouse Th17 Cell Differentiation Kit (#CDK017, R&D Systems). Briefly, CD4+ T-lymphocytes were isolated and cultured as described above with RPMI media additionally supplemented with a cocktail of proprietary polarizing reagent antibodies which promote Th17 polarization and prevent Th1 and Th2 differentiation. Following 5 days of culture and activation, T-lymphocytes were harvested for flow cytometric staining and analysis.

**Flow cytometric cellular staining**

Extracellular and intracellular flow cytometric staining for T-lymphocyte populations was performed as has been previously described. Briefly, cells (were incubated at 37°C for 4 hours in RPMI media supplemented with phorbol 12-myristate-13-acetate (PMA; 10 ng/mL), ionomycin (0.5 mg/mL), and BD GolgiPlug Protein Transport Inhibitor (containing brefeldin A; 1 mg/mL; BD Biosciences). Cells were washed, resuspended in PBS, and amine-reactive viability stained for 30 minutes at 4°C with Live/Dead Fixable
Cell Stain Kit (#L34960, Thermo Fisher Scientific). Next, cells were washed and resuspended in RPMI media supplemented with antibodies targeting the following extracellular markers: CD3ε PE-Cy7 (clone 145-2C11, eBioscience), CD4 Alexa Fluor 488 (clone GK1.5, eBioscience), and CD8α APC (clone 53-6.7, BD Biosciences). In order to stain for intracellular proteins, cells were then fixed and permeabilized utilizing the FOXP3 Fixation and Permeabilization kit (#00-5523-00, eBioscience) per the manufacturer’s instructions. Cells were washed again and resuspended for 30 minutes in permeabilization buffer containing IL-17A PE (clone TC11-18H10, BD Biosciences) and primary rabbit anti-tyrosine hydroxylase antibody (clone EP1533Y, Abcam). Next, cells were washed and resuspended in RPMI media supplemented with secondary goat anti-rabbit QDOT 605 (Q11402MP, Invitrogen). Cells were then washed and resuspended in cold PBS for immediate analysis. Data were acquired using a customized BD LSRII flow cytometer (UNMC, BD Biosciences) or a 4-laser Attune NxT flow cytometer (Thermo Fisher Scientific). All flow cytometry experiments were completed with single-color and fluorescence minus one (FMO) control samples, with indirect intracellular staining also employing a primary antibody only control sample. All analyses were conducted on FlowJo 10 software (BD Bioscience).

**Flow cytometric redox assessment**

Mitochondrial-specific assessment of superoxide was performed as has been previously described\(^\text{24}\). Briefly, cells were stained with cell-type specific fluorescent antibodies [anti-CD3ε PE-Cy7 (clone 145-2C11, eBioscience), CD19 APC-Cy7 (clone 6D5, BioLegend), CD11b SB-436 (clone M1/70, eBioscience), CD11c APC (clone N418, eBioscience), and NK1.1 SB-600 (clone PK136, eBioscience)], om addition to staining with 1 µM of \(\text{O}_2^-\)-sensitive mitochondrial-localized probe, MitoSOX Red (Thermo Fisher Scientific #M36008, Waltham, MA), for 30 minutes at 37°C. Cells were analyzed on a
LSRII flow cytometer at 488/610 nm excitation/emission, respectively, and data analyzed using FlowJo software.

**RNA extraction, cDNA production, and real-time RT-qPCR**

For further detail on the gene expression assessment, refer to the Materials and Methods section of Chapter 3. Generated cDNA was assessed for transcript levels by qPCR using respective gene targeted intron-spanning gene-specific unlabeled primers and dual-labeled fluorescent FAM probes with quencher (PrimePCR™, Bio-Rad, Hercules, CA). Thresholds were set objectively to determine cycle thresholds (CT), with 40S ribosomal protein S18 (RPS18) utilized as a loading control to determine ∆CT. All values were normalized to THCon control samples to determine ∆∆CT values, which were then transformed to generate fold changes by the $2^{-\Delta\Delta CT}$ method.

**Cytokine analyses**

For further detail on circulating cytokine analysis, refer to the Materials and Methods section of Chapter 2. For cytokine assessment in culture media, spent media was harvested after 72 hours of T-lymphocyte activation and culture (described above). Antigenic activation beads were magnetically removed, followed by centrifugation at 500xg for 2 minutes to pellet T-lymphocytes. Supernatant was removed and immediately frozen at -80°C until assay. All experiments were conducted per manufacturer’s instructions and quantified on a Meso Scale Discovery Quickplex SQ 120, with analyses conducted using Mesoscale Discovery Workbench software (Rockville, MD, USA).

**Statistics**

A total of 107 animals (53 THCon, 54 THT-KO) were utilized for the studies described herein. All data are presented as mean ± standard error of the mean (SEM) with sample
numbers displayed as individual markers, same for repeated measures experiments, where n values are included within figure legend. Due to the nature of the experiments herein, not every biological assay was completed on each mouse within the RSDS or control paradigms. At least 3 independent experimental repeats were conducted for each experimental design. For comparisons with two independent groups, Shapiro-Wilk normality was performed followed by statistical testing by Mann-Whitney U or Student t-test as appropriate. In experiments with two levels of categorical variables (such as RSDS/Control, and TH\textsuperscript{Con}/TH\textsuperscript{T-KO}), full model two-way ANOVA was utilized. Two-way ANOVA Šidák multiple comparison tests of interest are listed in figures if respective group or interaction effects were found to be significant, with ANOVA p values listed within figure legend. All statistics were completed in GraphPad Prism (V9, GraphPad).

**Results**

\textit{TH}\textsuperscript{T-KO} mice are viable model to assess TH within T-lymphocytes

In order to investigate the role of TH in T-lymphocytes exclusively, a conditional knockout mouse was generated and validated (Figure 31A). In Lck-driven cre-expressing cells (\textit{i.e.}, T-lymphocytes), exon 1 of the TH gene was successfully removed, resulting in an ~800 bp shifted product by PCR (Figure 31B). Due to the likely imperfect T-lymphocyte purification, faint non-recombined bands can be seen within TH\textsuperscript{T-KO} T-lymphocyte PCR products (Figure 31B). Furthermore, TH\textsuperscript{Con} and TH\textsuperscript{T-KO} flow cytometric assessment demonstrated nearly absent TH within T-lymphocytes [CD3\textsubscript{ε}; p=0.0038, Figure 31C]. Overall, these data demonstrate effective knockout of TH within TH\textsuperscript{T-KO} Pan T-lymphocytes.
Behavioral responses to RSDS are not altered in \( TH^{T\text{-KO}} \) animals

Following exposure to RSDS or control paradigms, \( TH^{\text{Con}} \) and \( TH^{T\text{-KO}} \) were assessed for differences in sociability and anxiety-like behavior by social interaction test and elevated zero maze, respectively. By elevated zero maze, control \( TH^{T\text{-KO}} \) demonstrated
less distance moved after RSDS-exposure, independent of T-lymphocyte TH (p=0.0059, TH\textsuperscript{Con} Con vs. RSDS, and p=0.0067, TH\textsuperscript{T-KO} Con vs. RSDS; **Figure 32A**). Time within the open arm was significantly different by RSDS only (p=0.0040, Two-way ANOVA), with multiple comparison tests nearing significance (p=0.1114, TH\textsuperscript{Con} Con vs. RSDS, and p=0.1114, TH\textsuperscript{T-KO} Con vs. RSDS; **Figure 32A**). From the social interaction ratio, RSDS-exposed TH\textsuperscript{Con} and TH\textsuperscript{T-KO} mice demonstrated significant reductions in sociability (p=0.0063, TH\textsuperscript{Con} Con vs. RSDS, and p=0.0159, TH\textsuperscript{T-KO} Con vs. RSDS; **Figure 32B**), with no effect of genotype (p=0.9233, Two-way ANOVA). These assessments demonstrate established behavioral changes following RSDS exposure, with TH T-lymphocyte having no influence on these RSDS-induced behaviors.

**Figure 32.** T-lymphocyte TH knockout does not affect RSDS-induced anxiety-like or social behavior.

Mice were tested for anxiety-like and pro-social behavior following control or RSDS paradigms. A) Left, Representation of elevated zero maze for anxiety-like behavior. Middle, Total distance moved on elevated zero maze, Two-way ANOVA results: stress p<0.0001, genotype p=0.3042, interaction p=0.8672. Right, Time in open arm of elevated zero maze, Two-way ANOVA results; stress p=0.0040, genotype p=0.0575, interaction p=0.4305. B) Left, Representation of social interaction test for social behavior. Right, Social interaction ratio (defined as time spent with CD-1 present/time spent with empty enclosure), Two-way ANOVA results; stress p=0.0001, genotype p=0.9233, interaction p=0.9101. Two-way ANOVA Šidák multiple comparison tests of interest are listed if respective group or interaction effects were found to be significant.
Circulating Th17 cytokines differ in THT-KO mice after RSDS, reflecting changes in Th17 populations and cytokine expression

We have previously demonstrated RSDS significantly increases circulating pro-inflammatory cytokines in WT mice, such as IL-2, IL-6, IL-10, IL-17A, IL-22, TNFα, CCL2, and CXCL2. Importantly, many of these cytokines are related to adaptive immune function and can be generated by T-lymphocytes. In particular, IL-17A and IL-22 are cytokines released in large quantities by Th17 T-lymphocytes, a subtype of CD4+ T-lymphocytes which have been implicated in autoimmunity. In ThCon animals, RSDS induced a significant increase in many of these cytokines paralleling earlier studies, such as IL-6, TNFα, IL-17A, and IL-22, despite smaller sample sizes across the four groups (p=0.0429, 0.0004, 0.0003, and 0.0054, respectively, THCon Con vs. RSDS; Figure 33A). In THT-KO mice, IL-6 and TNFα in circulation were increased after RSDS (p=0.0571 and <0.0001, respectively, TH T-KO Con vs. RSDS; Figure 33A). However, this increase in RSDS-exposed TH T-KO animals was significantly attenuated in IL-17A and IL-22, specifically (p=0.8930 and 0.9994, respectively, TH T-KO Con vs. RSDS; Figure 33A), with differences in IL-17A and IL-22 by genotype in RSDS-exposed animals (p=0.0058 and 0.0061, respectively, THCon RSDS vs. TH T-KO RSDS; Figure 33A).

To further investigate these cytokine changes, splenic T-lymphocytes were isolated for gene expression analyses by real-time qPCR. We observed statistically significant increases in IL-6 and TNFα gene expression independent of genotype (p=0.0292 and 0.0001, respectively, THCon Con vs. RSDS; p<0.0001 and 0.0002, respectively, THT-KO Con vs. RSDS; Figure 33B). However, we demonstrated a similar attenuation of RSDS-induced increases in expression of IL-17A, with a trend toward significance in IL-22, in
1. THT-KO T-lymphocytes as compared to TH Con (p=0.0001 and 0.0726, respectively, TH Con RSDS vs. THT-KO RSDS; Figure 33B).

We next investigated TH17 populations in our TH T-KO and RSDS paradigms by a multi-parametric flow cytometric panel to identify TH17 T-lymphocytes (CD3ε+CD4+CD8-IL-
17A+ splenocytes; Figure 34). RSDS induced an increase in \( T_H^{17} \) T-lymphocytes in \( TH^{Con} \) mice but had no effect \( T_H^{17} \) populations of \( TH^{T-KO} \) mice (\( p=0.0326, \) \( TH^{Con} \) Con vs. RSDS; \( p>0.9999; \) \( TH^{T-KO} \) Con vs. RSDS; Figure 34), with significant differences in RSDS-exposed animals by genotype (\( p=0.0032, \) \( TH^{Con} \) RSDS vs. \( TH^{T-KO} \) RSDS; Figure 34). Overall, these data demonstrate RSDS induces \( T_H^{17} \) polarization and cytokine production in a TH-dependent fashion within T-lymphocytes.

Overall, these data demonstrate RSDS induces \( T_H^{17} \) polarization and cytokine production in a TH-dependent fashion within T-lymphocytes.

**Figure 34.** \( TH^{T-KO} \) animals have attenuated \( T_H^{17} \) polarization following RSDS.

Following RSDS or control paradigms, splenocytes were harvested for multiparametric flow cytometry for \( T_H^{17} \) T-lymphocytes (CD3ε+CD4+CD8-IL-17A+). Hierarchical gating strategy was employed to assess viability and percentage \( T_H^{17} \) positivity. Two-way ANOVA results, Stress \( p=0.0398, \) genotype \( p=0.0007, \) interaction 0.0328. Two-way ANOVA Šidák multiple comparison tests of interest are listed if respective group or interaction effects were found to be significant.

**\( TH^{T-KO} \) T-lymphocytes demonstrate altered secretion of IL-17A and IL-22 despite similar growth**

To focus our investigation on the role of TH in T-lymphocytes, \( TH^{T-KO} \) CD4+ and CD8+ T-lymphocytes from control (unstressed) animals were isolated for culture and activated by antigen-independent anti-CD3/CD28 stimulatory beads. Utilizing live-cell imaging, we detected no differences in growth in \( TH^{T-KO} \) CD4+ or CD8+ T-lymphocytes (\( p=0.6912 \) and
0.3516, respectively; Figure 35A). After 72 hours of activation, cytokine analyses of spent media of TH\textsuperscript{T-KO} CD4+ T-lymphocytes revealed altered content of critical pro-inflammatory cytokines; IL-6 and TNF\(\alpha\) were unchanged or trended towards statistical significance (\(p=0.8353\) and 0.0606, respectively; Figure 35B) while IL-17A and IL-22 were significantly decreased (\(p=0.0040\) and 0.008, respectively; Figure 35B). These cytokines were found unchanged or undetectable in CD8+ T-lymphocyte culture media (data not shown).

Figure 35. TH\textsuperscript{T-KO} CD4+ T-lymphocytes demonstrate altered Th17 cytokine secretion, but similar growth.

A) CD4+ or CD8+ T-lymphocytes were isolated by negative selection, plated with anti-CD3/CD28 beads, and imaged for confluence 72 hours. N=4 samples, with >5 technical replicates per well. CD4+ Repeated measures Two-way ANOVA results, Genotype \(p=0.6912\), Time \(p<0.0001\), Interaction \(p>0.9999\); CD8+ Repeated measures Two-way ANOVA results, Genotype \(p=0.3516\), Time \(p=0.0003\), Interaction \(p=0.0002\). B) CD4+ T-lymphocytes were isolated and activated as above, with spent culture media collected and assessed for cytokines by Meso Scale Discovery Th17 (Combo 2) assay, with concentrations normalized to final cell counts. Listed \(p\) values were calculated by unpaired t-test or Mann-Whitney U test where appropriate from Shapiro-Wilk normality testing.
Overall, this investigation provides further evidence of differences in TH\textsuperscript{T-KO} T-lymphocytes ability to produce T\textsubscript{H}17 cytokines.

**TH\textsuperscript{T-KO} T-lymphocyte IL-17A and IL-22 cytokine production can be restored with catecholamine supplementation**

To explore the role of specific catecholamines in TH\textsuperscript{Con} and TH\textsuperscript{T-KO} CD4+ T-lymphocyte cytokine production, CD4+ T-lymphocytes were isolated, activated, and cultured *ex vivo* with 10 µM of each dopamine, norepinephrine, or epinephrine for paired analyses after 72 hours. IL-17A and IL-22 production was rescued primarily by NE supplementation (p=0.7989, TH\textsuperscript{Con} Vehicle vs. TH\textsuperscript{T-KO} NE; Figure 36A), with dopamine and epinephrine supplementation demonstrating modest effects. TNF\textsubscript{α} demonstrated no significant changes with catecholamine supplementation or by genotype (Figure 36A). Overall, this further validates a role for T-lymphocyte TH and subsequent catecholamines in T\textsubscript{H}17 salient cytokines.

**T-lymphocyte TH is not necessary for ex vivo T\textsubscript{H}17 polarization and does not function through a mitochondrial superoxide mechanism**

We next investigated necessity versus sufficiency of T-lymphocyte TH in T\textsubscript{H}17 polarization *ex vivo*. CD4+ TH\textsuperscript{Con} and TH\textsuperscript{T-KO} T-lymphocytes were isolated and cultured with T\textsubscript{H}17 exogenous polarizing conditions. T\textsubscript{H}17 polarization was evident by flow cytometric analysis after 5 days, with no observable changes between genotypes (p=0.8012, Figure 36B), suggesting T-lymphocytes can still polarize to T\textsubscript{H}17 in the absence of TH if given a strong enough stimulus. Lastly, as we have previously shown a role for mitochondrial superoxide in T\textsubscript{H}17 polarization,\textsuperscript{24, 324, 334} mitochondrial superoxide was assessed herein. Unexpectedly, mitochondrial superoxide was increased by RSDS in both TH\textsuperscript{Con} and TH\textsuperscript{T-KO} T-lymphocytes (p=0.0020, TH\textsuperscript{Con} Con vs. RSDS; p=0.0067 TH\textsuperscript{T-KO} Con vs. RSDS; p=0.0067 TH\textsuperscript{T-KO} RSDS vs. TH\textsuperscript{Con} RSDS).
KO Con vs. RSDS; Figure 36C), implying an uncoupling of between T-lymphocyte TH mechanisms and the mitochondrial redox environment.

Figure 36. TH17 cytokine production can be rescued with catecholamine supplementation, but T-lymphocyte TH is not necessary for TH17 polarization, nor does it rely on mitochondrial redox.

A) CD4+ T-lymphocytes were isolated and activated with 10 µM of respective catecholamines supplemented. After 72 hours, spent culture media was collected and assessed for cytokines by Meso Scale Discovery TH17 (Combo 2) assay. Cytokine concentrations are normalized to THCon and final cell counts. P-values represent nonparametric, paired measures ANOVA multiple comparisons tests (Friedman) compared to THCon vehicle. B) CD4+ T-lymphocytes were isolated, activated, and cultured under TH17 polarizing conditions for 5 days, then analyzed by flow cytometry. Left, Representative zebra plot. Right, TH17 T-lymphocytes following polarization; p-value by unpaired t-test. C) Following RSDS or control-housing exposure, splenic T-lymphocytes (CD3ε+) were stained with MitoSox Red to assess mitochondrial superoxide levels. Two-way ANOVA results, Stress p<0.0001, Genotype p=0.7096, Interaction p=0.8952.
Discussion

Herein, we successfully generated a murine TH-deficient T-lymphocyte model in order to assess its role after psychological trauma. Firstly, we demonstrated the viability of TH\textsuperscript{T-KO} mice and a successful reduction in TH in TH\textsuperscript{T-KO} T-lymphocytes, while also finding patent RSDS-induced changes to anxiety-like or pro-social behavior in TH\textsuperscript{T-KO} mice. In circulation, RSDS exposure increased canonical pro-inflammatory cytokines in TH\textsuperscript{Con} animals, while TH\textsuperscript{T-KO} mice demonstrated attenuated IL-17A and IL-22. Additionally, we found reduced expression of these respective genes in isolated T-lymphocytes, in addition to reduced TH\textsubscript{17} (CD3\textsubscript{ε}+CD4+CD8-IL-17A+) T-lymphocytes in RSDS-exposed TH\textsuperscript{T-KO} as compared to TH\textsuperscript{Con}. When activated \textit{ex vivo}, TH\textsuperscript{T-KO} CD4+ T-lymphocytes secreted less IL-17A and IL-22 in culture, with no growth disparities, which could be rescued with NE supplementation. Importantly, TH\textsuperscript{T-KO} T-lymphocytes were still able to polarize to TH\textsubscript{17} under exogenous TH\textsubscript{17} polarizing \textit{ex vivo} conditions, and RSDS-induced mitochondrial superoxide shifts were not influenced by TH in T-lymphocytes. Overall, this investigation provides new insight into the role for TH in T-lymphocytes in the context of psychological trauma, while making way for further neuroimmune investigations.

The genesis for this investigation was based on a simple finding from our group\textsuperscript{238}. Across animals in both RSDS and control conditions, the gene expression of IL-17A and TH was strongly, positively correlated (R=0.8722) in purified T-lymphocytes. Furthermore, RSDS increased expression of TH within T-lymphocytes. To test the potential causal relationship of these findings, we generated a conditional T-lymphocyte-specific knockout model of TH. While sparse, previous reports have identified catecholamine generation from T-lymphocytes, but have primarily focused on \textit{in vitro}, pharmacological approaches. More recently, Yang \textit{et al.} created a TH-deficient T-lymphocyte model using a ROR-\gamma\textsubscript{t} promoter-driven cre recombinase after demonstrating a selective increase in the
epinephrine and phenylethanolamine N-methyltransferase (PNMT; the synthetic enzyme preceding its production) in Th17 polarized cells. After deleting TH in T-lymphocytes, they examined the phenotype of experimental autoimmune encephalitis (EAE), but interestingly did not observe any difference in clinical scores or lymphocyte infiltration into the CNS. Critically, EAE is an antigen-dependent response that develops after immunization by myelin basic protein, whereas RSDS-induced pro-inflammation and IL-17A is not known to be mediated through a single specific antigen, and may be a much weaker immune stimulus compared to an autoantigen. Additionally, much of the work by Yang et al. assessed the role of TH in T-lymphocytes through use of in vitro assays where Th17 polarization was accomplished by exogenous administration of polarizing cytokines (i.e., anti-IFN-γ, anti-IL-4, IL-6, TGFβ). In the work herein, we also demonstrate that T-lymphocyte TH is not necessary for exogenous, ex vivo Th17 polarization. Together, our works provide valuable insights into the critical relationship between T-lymphocyte-derived catecholamines and Th17 T-lymphocytes, with two differing disease models and approaches showing differential effects of TH loss.

Importantly, the exact mechanism that connects T-lymphocyte-generated catecholamines and Th17 cells is still unknown. In our previous work, we have demonstrated the role for neuronally-derived NE in driving Th17 profiles through a mitochondrial superoxide mechanism. However, the data presented herein indicates that T-lymphocyte mitochondrial superoxide operates upstream or in parallel of T-lymphocyte TH in inducing Th17, since it was increased after RSDS independent of genotype. The loss of TH within T-lymphocytes would result in deficient production of all catecholamines, which canonically bind adrenergic receptors to influence T-lymphocyte functionality. There is a breadth of literature which has focused on the intracellular cascade that follows adrenergic receptor (AR) binding, and there are several potential
pathways which could explain this relationship. In our work herein, we were able to rescue the attenuated ex vivo production of IL-17A and IL-22 by TH\textsuperscript{T-KO} T-lymphocytes through primarily norepinephrine supplementation, thereby further validating their role in this overall mechanism. While outside of the scope of this work, future work could serve to utilize various adrenergic α and β agonists and antagonists to further refine this pathway. Additionally, delineating the reception of T-lymphocyte-generated catecholamines specifically is an important future direction, especially giving special attention to non-canonical reception of catecholamines and intracellular signaling, as reviewed thoughtfully by Bellinger et al\textsuperscript{111}. It should be noted that adrenergic pharmacological activation and inhibition of T-lymphocytes presents a number of technical challenges, further discussed in our previous work\textsuperscript{2}.

This work is not without limitations. RSDS is murine model of PTSD which induces a robust T-lymphocyte inflammatory response\textsuperscript{238, 243, 334}. However, RSDS utilizes retired male breeder CD1 mice to induce psychological distress, and thus limited the inclusion of female mice herein. While models of RSDS have been developed which utilize females, many of these often utilize differential stress induction for the female mice, which introduces further variability and precludes direct comparison between male and female mice. As new PTSD models adjacent to RSDS are further developed and refined\textsuperscript{237}, examining the phenotype in TH\textsuperscript{T-KO} is an important extension of these investigations. Additionally, cre recombinase in our experimentation was driven by the distal promoter of Lck in our model. This cre activation at the double positive (CD4+CD8+) thymocyte stage results in cre expression in all αβ T-lymphocytes\textsuperscript{333}. Since this is relatively early in T-lymphocyte development, TH knockout at this stage (and subsequent T\textsubscript{H}17 dysregulation) could be the result of a developmental defect. Additionally, IL-17 and IL-22 are also produced by γδ T-lymphocytes, which additionally can utilize the distal promoter of Lck\textsuperscript{336},
thus indicating their possible contribution to the phenotype seen. Future work could further describe the involvement of γδ T-lymphocytes in pro-inflammatory cytokine production after psychological trauma exposure.

Overall, this work provides new insights into the role for T-lymphocyte TH, specifically during psychological trauma. By utilizing an in vivo model, we were able to effectively demonstrate how T-lymphocyte-generated catecholamines are necessary for the T_{H17}-skewed inflammation seen during RSDS. Continued work investigating the mechanism of T-lymphocyte generated catecholamines, as well as their potential clinical relevance is an important future direction. By fully elucidating the nuance of these neuroimmune connections, we can further our understanding of fundamental T-lymphocyte biology and inflammation associated with PTSD.
CHAPTER 6: Discussion

Summary of Findings

The findings described herein contribute significantly to the literature to further our understanding of the intimate relationship between psychological trauma and T-lymphocyte inflammation, especially as mediated by sympathetic and redox mechanisms. Firstly, we demonstrated that our adapted murine RSDS paradigm reliably alters anxiety-like behavior, social behavior, and circulating inflammation, as evidenced by well-powered cohorts. These robust inflammatory changes (IL-2, IL-6, IL-10, IL-17A, IL-22, CCL2, CXCL2, TNFα) are generally related to T-lymphocyte-driven inflammation, although not exclusively. Additionally, we found that distinctions of susceptible and resilient by social interaction tests did not divide mice adequately by inflammation, whereas anxiety-like behavior (by elevated zero maze) was predicted well by a panel of circulating inflammatory markers.

Based on these findings, we focused on the potential role for T-lymphocytes, with a particular emphasis on the interactions of autonomic gene signatures and the redox environment. We demonstrated RSDS induced significant changes in sympathetic outflow to the spleen at the protein and molecular level (TH and NE, respectively). Moreover, we found that RSDS increased mitochondrial superoxide specifically within T-lymphocytes. Importantly, we previously investigated the interaction between exogenous NE and T-lymphocytes, during both ex vivo administration and in vivo infusion24, 25. In these works, Case et al. demonstrated that NE induced increased secretion of pro-inflammatory cytokines, such as IL-6 and IL-17A. Furthermore, this was shown to be partially reversible when the mitochondrially-targeted superoxide scavenger, mitoTEMPOL, was concurrently administered24. Taking this work further, we showed RSDS resulted in increased
circulating IL-6 and IL-17A, as well as their increased expression in purified T-lymphocytes. Furthermore, we demonstrated strong, positive correlations in local sympathoexcitation and inflammatory gene signatures (TH and IL-17A, respectively).

From these exploratory studies, we sought to specifically deduce the contribution of sympathoexcitation to the inflammatory signatures seen during RSDS. After designing and validating a method to ablate the splenic nerve, we found that RSDS-exposed mice had a reversal of T-lymphocyte-associated pro-inflammation (IL-2, IL-17A, IL-22; demonstrated at the circulating, cellular, and gene expression level), yet other more global markers of RSDS inflammation, such as IL-6, remained unchanged. Moreover, while RSDS-exposed, sham-operated mice had increased T-lymphocyte mitochondrial superoxide, splenic denervation completely attenuated this redox signal, even in RSDS-exposed mice.

After determining the contribution of sympathetic nerves to the T-lymphocyte inflammation, we then focused on T-lymphocyte-generated catecholamines in RSDS. We generated a T-lymphocyte-specific TH genetic knockout mouse, which demonstrated an even further selective ablation of IL-17A production and expression, with modest changes to IL-22. This had no effect on mitochondrial superoxide, pointing to a potentially different mechanism altogether. Taken together, this work can be contextualized within the literature, since it spans many different research areas outside of PTSD specifically. This discussion will loosely focus on the preceding chapters thematically by discussing 1) behavioral paradigms, 2) bidirectionality of psychoneuroimmunology, and 3) regulation of T-lymphocyte inflammation. While each individual chapter previously discussed limitations of each study, these will be further discussed herein. Additionally, like any good research investigation, these studies have tended to generate just as many questions as they have answered. In the following section, we will also discuss future directions for these research projects.
Implications, Limitations, and Future Directions

**Behavioral paradigms: blunt tools for dissection**

In Chapter 2, we sought to investigate how inflammation and behavior are interrelated in the RSDS paradigm. These circulating immune changes corroborate and greatly expand upon much more limited studies which investigated RSDS and inflammation\(^{239, 241, 242, 337}\). In order to contextualize these findings, a broader discussion of inflammation and behavior is warranted.

Behavioral research is inherently difficult, both in human and preclinical models. Each behavioral test is primarily developed to assess a facet of cognition and/or emotion expressed as a measurable behavioral manifestation (e.g., anxiety-like behavior by avoidance an open area in a novel environment), but it is not intrinsically able to assess a specific psychological disorder (e.g., major depression, PTSD)\(^{338}\). Further complicating this, there are a vast number behavioral tests meant to assess each facet. For example, anxiety-like behavior can be assessed by elevated zero maze, elevated plus maze, light and dark box tests, novelty suppressed feeding, among others\(^{338}\). Each of these tests may have adequate face, construct, and content validity, but their interchangeable use in the literature can convolute findings, especially when the same test is conducted differently despite being eponymous. Furthermore, external factors such as housing conditions\(^ {339, 340}\) and individual factors of investigators handing rodents\(^ {341}\), have been shown to greatly affect behavior. Therefore, it is of great importance that scientists continue to explore new and improved methods to assess and describe behavior. As discussed within Chapter 2, distinctions in PTSD preclinical research of “susceptible” and “resilient” by a single parameter from a single behavioral test (i.e., social interaction) is modestly reductionistic
when more nuanced approaches are undoubtedly necessary. By combining multiple parameters from multiple behavioral paradigms, we can better characterize animals along axes of anxiety-like behavior, sociability, et cetera. While we only conducted two behavioral tests herein, we attempted to utilize multiple parameters, and especially those that we found most aligned with inflammation in our model (i.e., anxiety-like behavior by elevated zero maze). This type of work is difficult, but will facilitate a more externally valid, systems approach to behavioral research. Systems such as home-cage monitoring are a promising future for behavioral assessment but remain limited and cost-prohibitive for many investigators. Overall, there is a continued need for new and improved methods for assessing behavior in preclinical models in order to further our understanding of various psychopathologies.

In addition to refining the behavioral tests and outputs we and others have utilized, similar investigations into inflammation by use of different PTSD models is warranted. An important caveat to all the investigations presented herein is the exclusion of female mice. This is a tremendous limitation, especially when epidemiological human data demonstrates that women are more likely to develop PTSD. As discussed within the chapters, there has been a burgeoning interest in this problem which has led to novel methods for inducing psychological trauma in female mice\textsuperscript{254, 278}. However, many of these models are inherently different than RSDS but still warrant investigation to demonstrate corroboration of the inflammatory changes described herein. Additionally, PTSD models which do not allow for physical trauma, such as single prolonged stress, would be excellent complements to the current studies of inflammation.

**Psychoneuroimmunology: a two-way street**

In order to continue to refine our understanding of the interaction between the nervous and immune systems, studies such as those described herein are essential in how we
conceptualize this intricate relationship in order to better harness to prevent, diagnose, and treat disease. In this bidirectional communication in neuroimmunology, it is crucial to discuss approaches that utilize one to target the other (neuromodulation of immunity) and vice versa (immunotherapy of psychiatric diseases).

Neuromodulation of immunity

The term “neuromodulation” loosely encompasses any technical approach which directly acts upon the nerve fibers, through electromagnetic, chemical, or sonic devices to alter nervous systemic function. Neuromodulation has been implemented clinically for decades, with techniques ranging from electro-conclusive therapy in refractory depression to more recent approaches, such as deep-brain stimulation for movement disorders. Critically, these approaches rely on modulating the nervous system in order to affect psychological and neurological function. What remains to be fully investigation is the utility of neuromodulation in treating inflammatory diseases, especially related to psychiatric disorders.

Sympathetic modulation

Herein, we focused on the role of splenic sympathetic nerves to T-lymphocyte inflammation in the context of psychological trauma. Our study utilized a specific surgical approach to ablate sympathetic effector signals (i.e., NE) from reaching immune cells, while avoiding the confounds of systemic treatment. By using RSDS, we utilized system that increases NE within the spleen. We solely focused on T-lymphocyte-driven inflammation based on previous findings, literature, and the cytokine signatures seen, but it is likely other immune cell subtypes receive catecholamines and could be ostensibly changed in ways we did not assess. While we investigated T-lymphocyte-driven inflammation in RSDS, a recent publication from Zhang et al. focused on how the splenic nerve (with signals originating in CRH neurons in the PVN) controls T-lymphocyte-
dependent B-lymphocyte responses to a classical immunization paradigm, which could be stimulated through a mild, novel stressor\textsuperscript{342}. This study provides interesting data on the dose-dependent nature of psychological stress, while also investigating B-lymphocytes, which make up a majority of murine splenocytes. Undoubtedly, there are differences in splenic anatomy between rodent and human, and two recent publications focused on porcine models of splenic denervation and demonstrated targeting of the splenic nerve could effectively modulate immune responses\textsuperscript{312, 343}. Importantly, these researchers additionally completed sympathetic nerve recordings while we focused solely on ablation, which provides valuable information about the nature of the nervous signals being sent, which themselves could contain important physiological insights\textsuperscript{344}. These comparative investigations are foundational in delineating which approaches might be clinically practical and useful for human diseases.

While neuromodulation of the sympathetic efferent nerves to affect inflammation is a nascent field, sympathetic denervation has been implemented within the kidney for treatment-resistant hypertension\textsuperscript{310, 311, 345}. Although marred by mixed trial results due to study design and technical/user failures\textsuperscript{308}, renal denervation is an elegant example of preclinical work finding a safe and effective use in a niche clinical setting.

\textit{Parasympathetic modulation}

Importantly, adjacent approaches to target autonomic function through the parasympathetic nervous system, are another keen area of interest outside of the scope of our work. Not long the original psychoneuroimmunology experiments by Ader and Cohen, and in the tradition of scientific serendipity, Kevin Tracey and colleagues stumbled upon the details of vagus nerve in the anti-inflammatory reflex (discussed within introduction). Due to our ever-expanding understanding of this pathway, the vagus nerve is now a prime target for neuromodulation. Specifically, electrical vagal nerve stimulation
(VNS) is an FDA-approved treatment for refractory epilepsy and depression, and more recently has shown promising results for helping to control autoimmune diseases such as rheumatoid arthritis\textsuperscript{346} and Crohn’s disease\textsuperscript{347}. More saliently, a recent randomized sham trial demonstrated VNS was able to attenuate in stress-induced activation of IL-6 and IFN-γ in patients with PTSD \textsuperscript{348}. Additionally, there is an ongoing clinical trial utilizing VNS in patients with PTSD which will examine psychiatric and inflammatory outputs (Clinical Trials ID: NCT03858985). The data from this trial will provide insight into the viability of a clinically useful method of neuromodulation as a treatment for the inflammatory aspect of PTSD.

Future directions

In seeking to extend our work, studies paralleling those mentioned above would contribute significantly to the literature. While we demonstrated increased sympathetic tone locally within the spleen, we did not examine CNS control centers of sympathetic tone. Much of the current research involving RSDS has focused on inflammation within the CNS, and demonstrated increase pro-inflammatory cytokines (\textit{e.g.}, IL-1β, IL-6, TNFα) and increased microglial activation\textsuperscript{241}. Since our interest lies in how sympathetic nerves control T-lymphocyte inflammation in RSDS, future directions could focus on how this process is regulated more rostrally. Through modern optogenetic and chemogenetic techniques, such as Designer Receptors Exclusively Activated by Designer Drugs (DREADD), we could temporally control sympathoexcitation to further validate its role in RSDS-induced inflammation. Indeed, we have demonstrated effective insertion of mCherry-labelled Gq-DREADD or Gi-DREADD within the RVLM of WT mice in order to activate or inhibit sympathoexcitation, respectively (data not shown). Additionally, this approach could be combined or validated with aforementioned more technical measures of sympathetic tone, such as nerve recordings.
In future works, we can additionally focus on the converse approach by targeting parasympathetic control of immune function. While we measured expression of ACHE and CHAT within T-lymphocytes after RSDS, we did not further investigate parasympathetic signals for a variety of reasons. First, acetylcholine is extremely labile and degraded quickly by endogenous cholinesterases, while additionally being difficult to measure due to choline supplementation within critical cell media supplements, such as fetal bovine serum (FBS). Additionally, as discussed thoroughly in the introduction, there is little evidence for parasympathetic nerve fibers reaching the splenic parenchyma, with more research focused on T-lymphocyte produced Ach. Interestingly, we demonstrated the catabolic enzyme ACHE was greater in RSDS-exposed T-lymphocytes, while CHAT was unaffected. In future experiments, similar chemogenetic systems could be used to modulate vagal efferent outputs in order to understand their role more fully in RSDS inflammation. This area of research is already at a more clinically relevant stage, given the aforementioned work utilizing VNS in PTSD inflammation. Yet, bedside to bench approaches have utility in mechanistic understanding which could further refine our ability to harness vagal nerve signaling clinically.

Overall, these future directions will greatly expand on this current body of work. By continuing to elucidate the minutia of specific neuroimmune pathways (and their role in psychiatric disorders), we might further incorporate new and old approaches into clinical practice through pharmacological or neuromodulatory therapies.

Importantly, this discussion has largely focused on how direct neuromodulation might alter inflammation, but undoubtedly interventions which act through cognition and behavior are an absolutely foundational pillar to the successful treatment of psychiatric disorders. Psychosocial methods like cognitive behavioral therapy, meditation, lifestyle interventions, and others have demonstrated profound effects of psychological and
physical health in PTSD\textsuperscript{349}. A recent meta-analysis demonstrated robust overall improvements in a variety of immune outcomes after psychosocial interventions\textsuperscript{350}. As discussed within the introduction, the current literature remains conflicted in understanding causality in psychological trauma and immune dysfunction\textsuperscript{236}. However, there is a great deal of literature and interest in how we can harness the nervous system to better modulate immune function.

\textit{Immune targeting in PTSD}

For too long, the brain (and thus psychology) was erroneously deemed to be immune privileged and impervious to altered inflammation outside of the CNS\textsuperscript{7}. More recently, multiple groups re-demonstrated the existence and intricacy of the brain lymphatic system\textsuperscript{351, 352}, wherein immune cells from the periphery actively circulate through and reside within the meninges and choroid plexus\textsuperscript{9}. These anatomical findings plainly demonstrated an obvious site where immune function could directly influence nervous system. Moreover, these meningeal immune cells, specifically T-lymphocytes, and their respective cytokines directly affect behavior, in behavioral measures such as sociability and spatial learning\textsuperscript{289, 290}. Additionally, a series of studies by Tonelli and colleagues\textsuperscript{353, 354} demonstrated that adoptively transferring GFP-labelled T-lymphocytes (administered peripherally) into RAG2\textsuperscript{-/-} animals resulted in CNS T-lymphocyte expansion and rescued social behavior in neonates\textsuperscript{355}. While they begin from an extreme lymphocyte-deficient phenotype, these investigations demonstrate how changes to the peripheral inflammatory milieu can be relayed to result in behavioral change. Since our work focusing on behavior and circulating inflammation in RSDS makes no assertions about mechanism, investigations such as these are critical in order to develop a conceptual understanding of pathways in which inflammation might precede behavioral change.
Building on their early work in meningeal T-lymphocytes, Kipnis et al. have continued these investigations to demonstrate specifically how meningeal γδ T-lymphocytes produce IL-17 to directly influence neuronal signaling, which thus alters behavior.356 Additionally, other recently works have demonstrated the role of IL-17 in maternal immune activation models357 and regulation of adipose thermogenesis358. Clearly, IL-17 is an important regulatory cytokine in both health and disease, especially within the context of the central nervous system. In light of these recent exciting findings, our work provides a conversely critical perspective for the role of psychological regulation by IL-17. Importantly, while these studies provide novel insights into T-lymphocyte (and IL-17 specifically) inflammation and behavior, they ultimately focus on T-lymphocytes native to the central nervous system and outside the context of psychological trauma. A recent publication from Kim et al. utilized a novel “cumulative mild stress” paradigm and after demonstrating increased anxiety-like behavior and CNS Th17, was able to reverse this phenotype through peripheral administration of an IL-17 blocking antibody.359 This simple study provides a piece of evidence for how peripheral blockade of IL-17 could influence behavior in a novel model of PTSD that involves prenatal and maternal stress paradigms.

In our work, we demonstrated robust increases in circulating IL-17A after RSDS, which have multiple peripheral T-lymphocyte subtype sources, including γδ T-lymphocytes. In pilot studies, we examined IL-17A+ γδ T-lymphocytes within both the spleen and circulation. We found that RSDS significantly increased circulating IL-17A+ γδ T-lymphocytes, while only trended towards significance within the spleen (Figure 37). Further investigation is needed to confirm this finding, as well as validate the role of γδ T-lymphocytes in psychological trauma and sympathetic pathways generally. In an elegantly simple study in humans, Anane et al. showed high-intensity exercise and β-agonist infusion resulted in increased γδ T-lymphocytes, whereas an acute stressor speech task
did not. This experiment provides some insights, but more work is needed to understand the role of γδ T-lymphocytes (both peripheral/meningeal, and IL-17+ and not) in the context of RSDS and PTSD. A more general discussion of regulation of T-lymphocytes in RSDS will follow.

Connecting peripheral inflammation to CNS function is practically and translationally relevant, especially within the context of psychiatric diseases. This interconnection has already been arrived at serendipitously, wherein patients treated with interferon for viral diseases often demonstrated psychiatric side effects. Alternatively, large trials testing anti-inflammatory drugs for efficacy against autoimmune disorders found improved depressive symptoms in a subgroup of patients, which is thoroughly summarized in a meta-analysis by Wittenberg et al. They demonstrated subgroups of patients with highest burden of major depression showed modest improvements in core measures, with drugs targeting IL-6 and IL-23 having the largest effects. Relevant to our studies herein, IL-17 blockers have demonstrated positive effects for autoimmune diseases such as
psoriasis and ankylosing spondylitis\textsuperscript{362}, but also resulted in an increased risk of non-severe infections\textsuperscript{363}, making them less attractive targets for psychiatric disorders. A recent trial which repurposed the antihypertensive drug losartan for the treatment of PTSD saw profoundly negative results\textsuperscript{364}. Within the work described herein, no clear target for T-lymphocyte-driven inflammation in RSDS emerged. However, ongoing investigations within our lab are investigating novel targets which would have less off-target effects than currently available immunosuppressants. Critically, immunotherapies which can target disorders are especially enticing if they are able to avoid the canonical adverse effects seen with current psychiatric therapeutics, which range from sexual dysfunction to severe neutropenia. These continued efforts to address the subset of patients with inflammation-driven psychiatric disease is an important future direction for preclinical researchers.

\textbf{Immune cell-generated catecholamines: still misunderstood}

Neurotransmitters are an essential means of communication within the nervous system, are yet understood to be ubiquitous signaling molecules between the nervous and immune systems. T-lymphocytes are able to synthesize and receive a variety of neurotransmitters, with an amazing amount of nuance and complexity. Catecholamines specifically can result in massive changes in the main measures of T-lymphocyte function such as proliferation, polarization, cytokine production, and activation. However, far too often, the results of these studies seemed contradictory due to experimental and technical differences (See introduction). In our early studies, we sought to understand (within our own experimental setup) how T-lymphocytes responded adrenergic receptor agonism as compared to NE. We found that 1 \textmu M of NE resulted in unique effects on cytokine secretion which was not recapitulated by agonism of any one adrenergic receptor, namely in the pro-inflammatory cytokine of interest, IL-17A (Figure 38). Furthermore, we sought to assess the role of \( \beta \)-ARs in RSDS by use of a genetic \( \beta \textsubscript{1} \) and \( \beta \textsubscript{2} \) knockout model.
(Adrb1tm1Bkk, Adrb2tm1Bkk/J), but found that it was embryonically lethal on a C57BL/6J background (data not shown). Without this genetic model, and due to the infidelity and toxicity of both adrenergic agonists and antagonists, we were unable to fully deduce the involvement of each specific adrenergic receptor within RSDS, Dnx, or TH^{T-KO} experiments. This is a limitation to the studies included herein which would necessitate the generation of multiple adrenergic receptor knockouts, which is certainly outside of the current scope. As a future direction, investigations could utilize conditional and/or inducible T-lymphocyte-specific knockouts to target a specific AR and deduce its role within RSDS inflammation.

An important area of research which remains completely unexplored is how immune cell-generated neurotransmitters might affect neuronal function. There is a great deal of preclinical work which has definitively shown that T-lymphocytes synthesize catecholamines and differentially regulate their production (discussed in introduction). To our knowledge, there is no current literature which demonstrates that immune cell-generated neurotransmitters alter nervous system function and behavior. Knowing that immune cell-generated cytokines and chemokines are essential mediators of many behavioral characteristics, neurotransmitters might be another signal by which immune cells can influence the nervous system. While these molecules are inherently more labile, the meningeal lymphatics provide an obvious nidus whereby this interaction could

![Figure 35. CD4+ T-lymphocyte IL-17A production after NE treatment is not recapitulated by other adrenergic agonists.](image)

CD4+ T-lymphocytes from WT mice were activated (anti CD3/28) and treated for 72 hours. Spent media was assessed by mesoscale multiplex assay. Concentrations normalized followed by statistical analyses by one-way ANOVA versus vehicle control (**=p<0.001, *=p<0.05).
potentially occur. Importantly, our work in chapter 5 examining behavior (Figure 32) showed no significant change in two standard measures of anxiety-like and social behavior when T-lymphocytes were catecholamine-deficient (TH^{T-KO}). However, additional investigations are warranted in order to determine how immune-cell neurotransmitters might affect behavior. These approaches could vary to improve their impact by investigating other behavioral outputs, neurotransmitters (e.g., serotonin), or immune cells which might generate these changes.

**Antigen-presenting cell catecholamines**

From these aforementioned approaches, expanding our focus to the generation of catecholamines by immune cells outside of solely T-lymphocytes is an important future direction for this work. Most critically, antigen-presenting cells (APCs) represent an immune cell which are definitionally in close proximity to T-lymphocytes, due to their need to present processed antigens directly to the TCR. Our results have demonstrated T-lymphocyte-driven inflammation, but this inflammation could be easily controlled upstream by APCs. Work from the lab of Rodrigo Pacheco has focused on how APCs, such as dendritic cells (DCs), produce dopamine to regulate the function of T-lymphocytes to promote subtype polarization, while also demonstrating that DCs are unable to generate any catecholamines downstream of dopamine, due a lack of Dopamine β-hydroxylase113,117,365. Additionally, they demonstrated this dopamine was generated by DCs themselves and not transported from the extracellular milieu. In a continuation of this work, Prado et al. demonstrated DC-generated dopamine is an autocrine signal which allows for stimulated DCs to produce IL-23, thereby polarizing T-lymphocytes to T_{H17}117. In our lab, we have crossed a CD11c-driven Cre mouse [B6.Cg-Tg(Itgax-cre)^{1-1Reiz/J}] to the aforementioned TH^{lox/lox} mouse, thus generating a conditional, DC TH knockout mouse.
This will be a novel and precise system to assess the influence of DC-generated dopamine on the inflammatory and behavioral phenotype in RSDS.

Overall, the physiologic role of immune cell generated catecholamines is complex and not as of yet well elucidated, especially in regard to their functional effects in vivo. This gap in the current literature is critical as new studies emerge which implicate T-lymphocytes and other immune cells in the pathogenesis and deleterious health effects of psychiatric diseases, like PTSD.

**Regulation of T-lymphocyte inflammation in RSDS**

The regulation of T-lymphocyte inflammation is an area of intense inquiry amongst researchers, with a recent interest focusing on their role in psychiatric disease. While it has been well documented that patients with PTSD demonstrate increased inflammation, with a specific skew that is T-lymphocyte-driven, the overall mechanism and potential antigen that drives this connection is still unknown in clinical and preclinical PTSD. However, the regulation of specific T-lymphocyte subset inflammation is a crucial area of discussion to contextualize the work described herein, especially given this and previous work demonstrating an important role for reactive oxygen species (ROS) and metabolism.

**Reactive oxygen species and metabolism**

These investigations contribute to the breadth of literature which demonstrate that reactive oxygen species (ROS) are not merely an aberrance of cellular metabolism, but concerted signals utilized by cells to regulate their function. Our data described herein demonstrate how sympathetic efferent nerve signals result in increased T-lymphocyte mitochondrial superoxide, which we have previously shown to be critical T-lymphocyte cytokine production. The actual mechanism by which a transient,
mitochondrially-localized signal could result in alterations of the transcription, translation, and secretion of specific cytokines remains incomplete. There are a number of mechanisms by which this could take place, ranging from mitochondrially-controlled metabolic signals to redox-sensitive transcription factors, which have been discussed exhaustively elsewhere. This is an important future direction within the field generally, and an active area of research within our own work. Previously, there has been significant work which has demonstrated that oxidative phosphorylation is critical to proper T-lymphocyte activation, which acts through ROS signals. Moreover, Moshfegh et al. showed that increased T-lymphocyte mitochondrial superoxide levels in T-lymphocytes resulted in increased cytokine secretion, which was linked to single-carbon metabolic shifts which control epigenetic mechanisms. More specific to the investigations reported herein, there are a breadth of investigations which have specifically looked at the role of metabolism in TH17 polarization and control. Kaufman et al. demonstrated that pathogenic TH17 cell function is first initiated by calcium influx (through STIM1), which controls oxidative phosphorylation and mitochondrial ROS, which they further showed are both necessary for TH17-driven autoimmunity. Additionally, work from Xu et al. described how glutamine and TCA catabolite 2-hydroxyglutarate (2-HG) resulted in hypermethylation of FOXP3, resulting in an imbalance of the TH17/TREG axis. These recently discovered pathways could represent a method by which neuronally or T-lymphocyte derived catecholamines could alter TH17 expression and function, and we could further target TH17-driven disorders. Both intracellular calcium flux and TCA metabolism have been demonstrated to be directly altered by adrenergic reception, thus serving as potential pathways through which T-lymphocyte-generated catecholamines could affect TH17. Overall, further work is needed to examine these metabolic, epigenetic, and redox pathways in our TH17KO model.
Taken together, it is extremely evident that inflammation, redox, and metabolism are linked. Yet, there is a paucity of preclinical investigations focused on the intersection of these areas, especially in the context of psychiatric disorders. Just as with neuromodulation, preclinical work is imperative in its ability to illuminate potential targets and pathways for clinical application. Currently, redox biology remains in a dark age in its clinical application, marred by the failures of large studies which utilized non-specific antioxidants (e.g., Vitamin E) and led to small/no effect or increased morbidity or mortality\textsuperscript{372-377}. By developing a more nuanced understanding of redox biology within specific systems, such as T-lymphocytes, we can better design therapeutics which could target these pathways. These basic science investigations of metabolic and redox control of immunity have been repeatedly identified as crucial gaps in our foundational knowledge of immune function and regulation and remain critical areas for research institutes like the National Institute of General Medical Sciences (NIGMS).

Other potential mediators of RSDS inflammation

\textit{Calprotectin}

While the work herein has focused on catecholaminergic regulation and interaction with T-lymphocyte inflammation, there is likely a number of other critical mediators involved. After establishing the robust T-lymphocyte inflammation seen in RSDS, we conducted a single-cell RNA sequencing on splenocytes of RSDS and control animals\textsuperscript{238}. From this, firstly we found significant increases compared to controls of transcripts for the inflammatory protein calprotectin, a heterodimeric calcium-binding protein which is clinically associated with the inflammatory response seen in inflammatory bowel diseases\textsuperscript{378}. Furthermore, after RSDS we found significant and consistent increases in the expression of calprotectin and IL-6 within T-lymphocytes, which were correlated. From this, we obtained a genetic calprotectin KO mouse model and preliminary evidence is
contrary to our hypothesis; loss of calprotectin worsened anxiety-like behavior, increased RSDS-induced increases in circulating IL-6 and IL-17A, and resulted in increased T-lymphocyte mitochondrial superoxide\(^{379}\). Together, these data suggest that calprotectin may in fact play a protective role in attenuating RSDS inflammation, and future work will continue along these investigations.

**Hemoglobin**

Secondly, within the single-cell RNA sequencing data, we found that T-lymphocytes from RSDS-exposed animals displayed significantly increased transcripts for hemoglobin. We further validated the differential expression of hemoglobin in RSDS paradigms, as well as its basal expression purified and immortalized T-lymphocytes (data not shown). Critically, there have been multiple publications which have demonstrated a critical role for hemoglobin outside of oxygen transport within erythrocytes, such as work

![Figure 36. T-lymphocyte Hba KO mice (Hba\(^{\text{AlcK}}\)) have altered redox flux.](image)

Compared to flanked Hba loxP control (Hba\(^{\text{Cre}}\)) animals, T-lymphocytes preliminarily have altered redox at baseline. Statistical analyses not conducted due to n<4 in some groups.
demonstrating hemoglobin could act as a regulator of nitric oxide within endothelial and vascular smooth muscle junction\textsuperscript{380}. In order to more elegantly test the role of hemoglobin in T-lymphocytes within the context of psychological trauma, we graciously obtained from Dr. Adam Straub at the University of Pittsburgh a mouse with hemoglobin alpha 1 flanked by loxP which we have since crossed with the aforementioned Lck Cre recombinase mouse (HBα\textsubscript{ΔLck}). This mouse model is viable and in pilot studies demonstrated significantly dysregulated mitochondrial redox flux (\textbf{Figure 39}), and is an important future direction for our research, both in RSDS and examining a fundamental T-lymphocyte pathway.
Epilogue

"...There's a condition in combat that occurs when a soldier is completely stressed out and is on the verge of a nervous collapse. In World War I it was called 'shell shock.' Simple, honest, direct language. Two syllables. Shell shock. It almost sounds like the guns themselves. That was more than eighty years ago. Then a generation passed, and in World War II the same combat condition was called 'battle fatigue.' Four syllables now; takes a little longer to say. Doesn't seem to hurt as much. 'Fatigue' is a nicer word than 'shock.' Shell shock! Battle fatigue. By the early 1950s, the Korean War had come along, and the very same condition was being called 'operational exhaustion.' The phrase was up to eight syllables now, and any last traces of humanity had been completely squeezed out of it. It was absolutely sterile: operational exhaustion. Like something that might happen to your car. Then, barely fifteen years later, we got into Vietnam, and, thanks to the deceptions surrounding that war, it's no surprise that the very same condition was referred to as 'post-traumatic stress disorder.' Still eight syllables, but we've added a hyphen, and the pain is completely buried under jargon: post-traumatic stress disorder (sic). I'll bet if they had still been calling it 'shell shock,' some of those Vietnam veterans might have received the attention they needed."

As alluded to by the infamous George Carlin above, our definitions (and euphemisms) of PTSD continue to change, but the reality of the situation for those who suffer the most has not. In the last three decades, there have been little to no new advances specific for the treatment of PTSD. Moreover, very few treatments and medical approaches have sought to address the robust changes to inflammation that these patients clearly face. Yet, there is promising research ongoing in PTSD. Psychiatrists have sought to repurpose old therapeutics, ranging from antihypertensives to psychedelics, in the diligent pursuit of finding ways to help patients here and now. Basic scientists continue to delineate new
pathways and search for yet unknown mediators. These two systems must work in concert in order to facilitate safe and effective therapies for psychiatric disorders.

While difficult to understand or define in their time, the findings of basic science investigations will ideally prompt and promote our overall betterment, scientifically and beyond. I hope to straddle the line between basic and clinical science, taking the skills and training I have learned to help translate findings to ultimately help patients across a diversity of clinical fields. I am forever grateful for the opportunity to peel away uncertainty and uncover truths about the natural world, and optimistically look forward to my small contributions to the scientific literature someday, in some way, facilitating the improvement of the lives of those around me.
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APPENDIX A: Role of T-lymphocytes in Social Defeat Stress Hypertension Sensitization

Introduction

The breadth of literature discussed herein paints a complex, yet influential picture of how catecholamines of both neuronal and immune origin might result in functional changes to T-lymphocytes in the context of RSDS. In taking this work further, we sought to examine the role of T-lymphocyte-driven inflammation in the role of PTSD and another co-morbid inflammatory disease. By understanding a specific disease state which ultimately contributes to the mortality of these patients, we might further understand our previous findings in a more translational context.

Metabolic syndrome is one of the most important risk factors to the development of cardiovascular disease. This syndrome represents a number of separate but interlinked pathophysiological states (e.g. obesity, insulin resistance, hyperlipidemia, hypertension, and others) that converge to create a phenotype with a high risk for cardiovascular events. Furthermore, autonomic dysregulation as well as chronic low-grade inflammation associated with metabolic syndrome and cardiovascular diseases are both widely accepted. This low-grade inflammation in cardiovascular disease begets an etiological question: Is the pro-inflammatory phenotype a response to deranged physiology, or does the pro-inflammation precede the changes in cardiovascular physiology? Moreover, how does the interaction between neurotransmitters and T-lymphocytes alter this relationship?
T-Lymphocytes and hypertension

Enhanced sympathetic outflow is a hallmark of many forms of hypertension. NE activates ARs in the kidney, which results in increased retention of Na\(^+\) as well as renin release. This activation of the classical renin-angiotensin-aldosterone system (RAAS) contributes to the maintenance of elevated mean arterial pressure in hypertension (reviewed extensively by Nishi et al.). Angiotensin II (Ang II), the main effector peptide of the RAAS system, is a major component and current therapeutic target of hypertension. Intriguingly, Ganta et al. demonstrated that application of Ang II centrally increased splenic pro-inflammatory cytokine production (e.g. IL-1\(\beta\) and IL-6), and was diminished by ablation of the splenic nerve. This phenomenon is suggestive of a neural-immune reflex, but further indicates significant harmony between the cardiovascular, neural, renal, and immune systems.

To this end, we must first discuss that it has already been well established that T-lymphocytes are necessary for the development of hypertension, with investigations starting in the 1960’s, followed by clinical work with the lymphocyte-depleting drug mycophenolate mofetil resulting in a reversal of hypertension in patients with autoimmune disorders. This has been further validated as a causal link by multiple groups showing that mice lacking lymphocytes are resistant to hypertension of various etiologies with adoptive transfer studies of T-lymphocytes restoring this response. This intimate connection is mediated through direct interactions of the nervous and immune components, with local inflammation evident in the kidneys, heart, brain, and vasculature. It follows that interruption of these sympathetic signals should result in an attenuated inflammatory and hypertensive phenotype.

The central nervous system mediates changes in blood pressure most prominently by altering sympathetic tone and humoral factors. The anteroventral third ventricle
(AV3V) region includes various circumventricular regions that, when ablated, results in loss of many Ang II-mediated effects, such as vasopressin release and sympathetic tone\textsuperscript{406}. Marvar and colleagues found that this ablation of this region in Ang II-treated mice resulted in decreased activation of T-lymphocytes and vascular infiltration\textsuperscript{407}. This inflammation was found to be mediated by pressure alone, since NE-induced pressure increases bypassed the AV3V lesion and led to inflammation as well as the administration of the anti-hypertensive hydralazine (with no known Ang II effects) resulted in a decrease of the same inflammatory markers. These investigations provide some insight to the question posited in the introduction to this section. It is likely the combined, feed-forward effect of centrally-mediated changes give rise to inflammatory perturbations, which result in frank hypertension. This communication is facilitated at the cellular level by interactions between sympathetic outflow and T-lymphocytes.

One area this neuroimmune interaction may take place is the kidneys, where sympathetic tone and neurohumoral factors can sway the titrated control of intravascular volume and thus, pressure. However, it has become clearer that not all hypertension models are created equal, especially in regards to their effects on the kidneys. Work by Xiao et al. found that renal denervation prevented T-lymphocyte infiltration and damage to the kidneys, while also reducing blood pressure in Ang II-treated mice\textsuperscript{306}. Additionally, adoptive transfer of splenic DCs from Ang II-treated mice resulted in T-lymphocyte activation and a hypertensive phenotype in untreated mice, an effect that could be ameliorated by bilateral renal denervation. Dextroxcortiosterone-acetate salt (DOCA-salt) is another commonly used hypertensive stimulus that has similarly been shown reversible through renal denervation. Interestingly, in this model, targeted chemical or physical afferent renal nerve ablation shows a similar decrease in pressure as compared to complete (i.e. afferent and efferent sympathetic nerves) denervation\textsuperscript{408, 409}. In addition to the antihypertensive effect of total renal denervation, markers of renal inflammation such
as T-lymphocyte infiltration and cytokine content also were reduced. Interestingly, a follow-up study by the same group found that afferent denervation alone did not reduce renal T-lymphocyte or macrophage infiltration caused by DOCA-salt treatment\textsuperscript{410}. Osborn \textit{et al.} investigated this work in another model, wherein mice were exposed to either traditional chow or a high-fat diet (45\% of caloric content), with the latter group developing the characteristic metabolic syndrome phenotype of increased arterial pressure, hyperglycemia, and increased fat mass. Both groups then underwent complete renal denervation, but only the high fat group displayed a reduction in systemic blood pressure\textsuperscript{307}. However, renal denervation did not result in any changes in glucose metabolism or markers of renal inflammation, again showing the complexity of hypertension, renal denervation, and renal inflammation dependent on the etiology and possibly extent of the hypertension.

These works provide evidence of a crucial neuroimmune interaction that results in the development of hypertension. It also serves as confirmation that the etiology of the hypertension plays a role in its pathophysiology, with different models responding differently to renal denervation. The ability to treat resistant hypertension with renal nerve ablation is not a new idea clinically, with multiple international clinical trials testing catheter-based ablation methods. Interpretation of these trials is convoluted and nuanced (reviewed exceptionally by Osborn \textit{et al.} \textsuperscript{308}), but it stands to reason that modulation of sympathetic signals to an organ heavily involved in the maintenance of hypertension are yet still a potential therapeutic target for hypertension.

In addition to the renal nerves involvement, the splenic nerve provides a potential location for the priming of T-lymphocytes by sympathetic tone (discussed thoroughly by Lori \textit{et al.}\textsuperscript{411}). T-lymphocytes migrating to the kidneys and vasculature during Ang II hypertension have been shown to originate in the spleen\textsuperscript{412}. This was shown elegantly by adoptive transfer of donor mouse splenocytes bearing the leukocyte antigen CD45.1 into
a CD45.2 splenectomized recipient mouse. The input these T-lymphocytes receive from the splenic nerve can severely alter their phenotype, as seen from the prior discussions of local NE concentrations in the spleen and its complex effects on T-lymphocytes in vitro. Osborn and colleagues found that celiac ganglionectomized Dahl rats, which upon high salt exposure develop a well-established form of neurogenic hypertension, had decreased arterial pressure when compared to sham controls. This reduction in pressure was shown to have a different mechanism than that of renal denervation, since rats who underwent both ablations had even greater decreases in pressure. This data, in conjunction with evidence of the cholinergic anti-inflammatory reflex in the spleen, represents yet a major anatomical location in which sympathoexcitation can alter T-lymphocytes in a way that can promote proinflammatory states related to hypertension and other cardiovascular diseases.

Our group has also examined the role of sympathoexcitation-driven T-lymphocyte activation during hypertension. In a model of increased sympathetic tone through NE-infusion, we found that splenic T-lymphocytes demonstrate an altered phenotype once activated. The phenotype of these T-lymphocytes was overall suppression, with decreases in pro-mitotic cyclins and proinflammatory cytokines IFN-γ and TNF-α, which was suggestive of canonical adrenergic stimulation of cAMP leading to diminished activation. However, this inhibition of T-lymphocytes during NE-induced hypertension was at least partially caused by increases in superoxide, as evidenced by antioxidant supplementation modestly restoring the growth and cytokine profile of these T-lymphocytes. This phenotype of T-lymphocytes during NE-infusion is in contrast to previously discussed findings, in which NE infusion resulted in hypertension and activated T-lymphocyte infiltration into the vasculature despite AV3V lesions. The differential activation of T-lymphocytes during NE-infusion and hypertension is thus highly dependent on the location of these T-lymphocytes. The dual nature of NE signaling on T-
lymphocytes is thus evident, with splenic T-lymphocytes responding to NE in a different manner, likely due to their differing state of activation. This work demonstrates a novel role of redox communication as a downstream mediator in neurotransmission in T-lymphocytes, which is highly relevant to the cardiovascular field where neurotransmission and redox environments are reported to be significantly disrupted.

Another burgeoning area of interest is the role progenitors of T-lymphocytes and other immune cells play in hypertension. The bone marrow serves a primary lymphoid organ, where bone marrow (BM) cells respond to sympathetic input to differentiate and enter the circulation. These BM cells express all β-ARs, and their stimulation modulates the release of immune cells into circulation in a diurnal pattern corresponding to increased activity or risk of infection. By adoptively transferring β-AR-deficient BM cells into near lethally irradiated mice, Zubcevic and colleagues found that there was an association between the lack of β-ARs on immune cells and the observed decreases in blood pressure. In addition, transcriptomic analysis of these BM cells showed altered expression of transcripts relevant to many immune processes, which pathway analysis revealed were shown to be critical in T-lymphocyte activation, proliferation, adhesion, and chemotaxis. This research highlights how adrenergic signaling in immune cells is involved upstream from many of the inflammatory changes seen in hypertension. That is, how early changes in sympathoexcitation could potentiate deleterious proinflammatory changes in T-lymphocytes and other immune cells, as mediated by ARs specifically. Overall, T-lymphocytes have proven to be necessary for the development of hypertension, while also having been shown to be extremely responsive to signals mediated by neurotransmitters.

**T-lymphocytes in a preclinical model of PTSD and hypertension**

In conjunction with the preceding literature, this introduction clearly presents that common to PTSD and hypertension is frank derangement in T-lymphocyte-driven
inflammation. In order to further investigate the nature of this relationship, herein we examined pressor responses to RSDS by radiotelemetry. First, we demonstrate that after RSDS, infusion of a subpressor dose of the hypertensive stimulus Angiotensin II (AngII) resulted in differential responses in RSDS-exposed mice as compared to controls. By utilizing RAG2−/− which lack T and B lymphocytes, we repeated this paradigm and demonstrated this RSDS-induced sensitization to AngII is lymphocyte-dependent.

Materials and Methods

Mice

For further detail on how mice were housed, dissected, and euthanized, refer to the Materials and Methods section of Chapter 2. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

RSDS paradigm

For further detail on the RSDS paradigm, refer to Materials and Methods section of Chapter 2. Following the 10-day RSDS or control paradigms, mice recovered for 10 days, then were implanted with osmotic mini pumps (above). Throughout, all mice were assessed for visual wounding (>1 cm) or lameness and dutifully excluded.

Hemodynamic measurement

Hemodynamics, such as mean arterial pressure (MAP) and heart rate were measured by radiotelemetry (Data Sciences International #PA-C10, Minneapolis, MN). This method allows for conscious, unrestrained hemodynamic assessment within their home or RSDS cage, which is essential in stress-response studies. Radiotelemeter implantation was accomplished as has been previously described277. Briefly, mice were anesthetized in
order to insert an arterial catheter within the left carotid artery, which was then sutured securely in place. Mice recovered for 7 days following telemeter implantation prior to start of RSDS. Hemodynamic measurements were recorded for 20 seconds every minute for 2 hours daily. Average mean arterial pressure was calculated during periods with the lowest activity levels, as reported from telemetry accelerometers.

**AngII Infusion**

Following a 10-day recovery after RSDS, mice were implanted subcutaneously with osmotic mini-pumps (Alzet #1002, Cupertino, CA) which delivered normal saline (0.9%; vehicle) or Angiotensin II (AngII; 200 ng/kg/min). Implantation and pump priming was performed as we have previously described. Critically, this dosage of AngII was used based on prior literature demonstrating it is a subpressor dose (i.e., does not result in a robust or sustained MAP increase in WT mice). Thus, it is an effective method to assess potential sensitization to hypertensive stimuli after RSDS.

**Results**

**RSDS increases MAP transiently and sensitizes mice AngII**

In order to assess the effect of psychological trauma on hypertensive responses, RSDS was conducted after the implantation of radiotelemeters. We observed robust increases in resting MAP during the 10-day RSDS period (Figure 40). Following completion of the RSDS paradigm, we found a rapid and sustained return to baseline resting MAP (Figure 40), which is consistent with the current literature which demonstrates no rodent model of PTSD results in chronically elevated baseline blood pressure. However, upon infusion with a subpressor dose (200 ng/kg/min) of the hypertensive peptide AngII, we observed a significantly potentiated response in RSDS-
exposed animals as compared to control (Figure 40). This increase in resting MAP was maintained after an initial lag and maintained through complete AngII infusion from the osmotic pumps (i.e., 14 days).

**AngII sensitization after RSDS is lymphocyte dependent**

Following these investigations, we became interested in what could drive this potentiated response to a hypertensive stimulus exclusively in the RSDS cohort. Based on previous literature demonstrating a role for T-lymphocytes in hypertension, and our own work demonstrating T-lymphocyte-driven inflammation in RSDS, we then completed the same paradigm with RAG2-/- animals. RAG2-/- mice lack T and B lymphocytes due to their inability to properly recombine the variable regions of the TCR and BCR, respectively. When compared to WT controls, WT RSDS animals again demonstrated a robust increase in MAP, as represented by the change in MAP (ΔMAP) after 10 days of AngII infusion (Figure 41). Importantly, RAG2-/- animals demonstrated no heightened sensitivity to AngII compared to WT RSDS remained non-significantly
changed from other groups (Figure 41). This data suggests a lymphocyte-dependent mechanism for RSDS-induced hypertension sensitization to AngII.

**Discussion**

This data represents a novel finding where RSDS is able to sensitize animals to AngII through a lymphocyte-dependent mechanism. This increased AngII sensitization was similarly recently demonstrated in a rat model of PTSD\textsuperscript{420}. Xue et al. found that pre-treatment with angiotensin-converting enzyme (ACE) or tumor necrosis factor (TNF) inhibitors resulted in reversal of the sensitization, and further demonstrated a role for increased inflammatory and RAS signaling pathways within circumventricular organs. While interesting, this type of pre-treatment paradigm has little utility to any externally valid or clinical setting. Importantly, this investigation validates and corroborates our initial findings here, while pointing to neuroimmune mechanisms which might control this phenomenon.
As discussed, there is no preclinical model of PTSD which alone results in increased MAP after the completion of the trauma paradigm. Therefore, assessments of the influence of psychological trauma on hypertension necessitate the introduction of a "second hit", which can be a number of different hypertensive stimuli. While there are a number of different models of hypertension with the same output of increased MAP, the literature seems to indicate they may potentially display differential neuroimmune pathway activation (discussed in introduction). Knowing the intimate interconnection between the renin-angiotensin-aldosterone system (RAAS), sympathoexcitation, and T-lymphocyte immunity, we utilized the AngII infusion model. Future works will focus on other methods of hypertension induction which may result in different findings but will ultimately contribute to our understanding of the lymphocyte-driven mechanism of PTSD and hypertension. Additionally, these findings are not without limitation. While this functional data (i.e., ΔMAP) is critical to overall causality, further work investigating immune signatures within pressure-controlling regions (e.g., kidney, vasculature, brain) would be of great interest to the overall mechanism. Furthermore, RSDS and hypertensive paradigms could be combined with splenic denervation studies, which based on prior literature showing a role for the splenic nerve in hypertension\textsuperscript{412, 421}, would elevate the impact of these works. Overall, these lines of research align critically with the ultimate goal of all research—to comprehend the disease state in order to utilize this understanding to reduce morbidity and mortality. Hypertension is quantitatively the most important modifiable risk factor for cardiovascular disease\textsuperscript{422}, and mechanistic study of the complex comorbidity of PTSD and hypertension is a basic science question that is clinically pertinent. By further elucidating the neuroimmune interactions that govern the epidemiological data which drives mortality, we can in the future provide promising, rational approaches to prevent and treat both hypertension and PTSD more effectively.
### APPENDIX B: OLIGONUCLEOTIDES

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<th>Gene symbol</th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
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Table 1. Oligonucleotide primers for real time qPCR.