THE EFFECTS OF CHRONIC STRESS ON CD8 T CELLS IN HUMAN ADULTS: AN EXAMINATION FROM BENCH TO BEDSIDE

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Dedication

For my parents, Peter and Marla Slota

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ABSTRACT

THE EFFECTS OF CHRONIC STRESS ON CD8 T CELLS IN HUMAN ADULTS: AN EXAMINATION FROM BENCH TO BEDSIDE

Christina Marie Slota

Connie Ulrich

Life is full of stressors that challenge our health; recent evidence suggests that chronic stress leads to altered immune outcomes in humans. While the literature illustrates the chronic stress-induced changes in immune outcomes, there is limited understanding of the impact of a chronic stress response on specific immune cell subsets. The primary objective of this dissertation is to examine the effect of a stress hormone and the stress of caregiving on immune cell subsets, specifically CD8 T cells. To address this objective, we perform an in vitro experiment with isolated CD8 T cell subsets (naïve, central memory, effector memory) from human adults and treated them with stress-related hormone, norepinephrine (NE). We also perform a cross-sectional matched study of CD8 T cell subsets from twenty-one family caregivers of stem cell transplant recipients and twenty age- and gender-matched controls at the National Institutes of Health Clinical Center. In CD8 T cell subsets, we conducted flow cytometry analysis for immunophenotyping, RT-qPCR and a microarray for gene expression changes, and ELISA for protein level assessments on the impact of NE. We examined differences using t-tests and gene set enrichment analysis (GSEA) for global gene expression analysis of mRNA changes in NE treated and untreated CD8 central memory T cells. We were the first to report that memory CD8 T cells express higher levels of the NE receptor, beta-2 adrenergic receptor, compared to naïve cells. We found memory CD8 T cells had an increase in the mRNA levels of pro-inflammatory cytokines and chemokines before and after activation and a decrease in proliferation-related cytokines after activation. Protein levels were also significantly increased in pro-inflammatory cytokines and decreased in proliferation-related cytokines. Individuals with high levels of NE in their serum, or were family caregivers, had a pro-inflammatory state before and after antigenic

challenge of memory CD8 T cells. These findings suggest chronically stressed individuals may be more susceptible to previously encountered antigenic challenges compared to novel challenges. Future research should explore the exact mechanisms behind these stress-related changes in memory CD8 T cells and the longitudinal consequences on immunity in chronically stressed populations.

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CHAPTER 1

Introduction

Stress is a common aspect of modern life and can be perceived as a burden when extended over long periods of time. The definition of stress used for this dissertation is a constellation of events, consisting of a stimulus (stressor) that precipitates a reaction in the brain (perception), which activates a physiologic fight-or-flight response by the body (stress response) (Dhabhar, Malarkey, Neri, and McEwen, 2012). Stress-induced biological changes in the immune system can include: decreased cytokine, lymphocyte and immunoglobulin counts, decreased immune cell proliferation and functionality, increased infection rates, impaired responses to vaccines, and dysregulation in the inflammatory response (Cohen, Doyle, and Skoner, 1999;Glaser, Kiecolt-Glaser, Malarkey, and Sheridan, 1998;Gouin, Hantsoo, and Kiecolt-Glaser, 2008;Kiecolt-Glaser, Glaser, Shuttleworth, Dyer, Ogrocki, and Speicher, 1987;Vedhara, Shanks, Anderson, and Lightman, 2000). These cellular impairments in immune health are the result of a stressful environment that puts individuals at risk for infection or physical and psychological illnesses; thus, it is important to study the relationship between stress and immune function in greater depth.

A growing body of evidence shows the moderating effect of the sympathetic nervous system (SNS) on the immune system (Elenkov, Wilder, Chrousos, and Vizi, 2000;Sanders and Straub, 2002). Communication between the nervous and immune system is integral to the stress-induced changes seen in human adults since the SNS releases catecholamines that interact with surface receptors on immune cells to modulate their function (Kavelaars, 2002;Khan, Sansoni, Silverman, Engleman, and Melmon, 1986;Kin and Sanders, 2006;Rohleder, Marin, Ma, and Miller, 2009). Although over 150 clinical trials and animal studies have shown that environmental stressors can negatively impact immune function (Murgatroyd, Wu, Bockmuhl, and Spengler, 2010;Padgett and Glaser, 2003;Romana-Souza, Assis de Brito, Pereira, and Monte-Alto-Costa, 2014;Zieker, Zieker, Jatzko, Dietzsch, Nieselt, Schmitt, Bertsch, Fassbender, Spanagel, Northoff,

and Gebicke-Haerter, 2007), there is a gap in our understanding of how prolonged stress and the subsequent nervous-immune communication impacts the function of specific immune cells on the molecular, transcriptional, translational, and cellular levels. Specifically, we lack information related to: 1) potential molecular mechanisms behind stress-induced changes in immune outcomes seen in stressed populations, like family caregivers; 2) the effects of stress hormones, like norepinephrine, on human CD8 T cells' and their subsets' (naïve, central memory, effector memory) function; 3) the effect of chronic psychological stress, like caregiving, on naïve and memory CD8 T cells' function. Better understanding of how a prolonged stress response can influence the CD8 T cells of human adults could provide insight into the changes seen in the immune outcomes of chronically stressed populations, including family caregivers.

Purpose and Organization

The purpose of this research is to elucidate the impact of stress on the immune cells of human adults and family caregivers by examining changes in CD8 T cell subsets' transcription and translation of cytokines and chemokines, and CD8 T cell function. **The primary objective of this dissertation is to examine the effect of psychological stress or stress released hormones on the function of immune cell subsets, specifically CD8 T cells (naïve and memory).** This dissertation is comprised of five chapters and uses the three-article dissertation format. The first chapter includes an explanation of the significance of the problem and a comprehensive review of the literature that explores the following: nervous-immune communication via norepinephrine and its impact on T cell function; the potential role of epigenetics in moderating immune function in humans; and the impact of caregiving on immune health outcomes with a focus on family caregivers of stem cell transplant recipients.

Chapter 2 is the first article in the three article series. It is a theoretical paper exploring the literature related to stress-induced lifestyle changes, epigenetic mechanisms (DNA methylation and histone modifications) and immune outcomes, and proposes a framework for

examining the potential role of epigenetic changes as a mediator between stress-induced behavioral and psychological changes, and poor immune function in family caregivers. **The specific aims of this paper are: 1) to review the existing literature on the relationship between stress-induced lifestyle changes, epigenetic mechanisms, and immune function in humans; 2) to propose a framework for examining the potential mediating impact of epigenetic regulation between stress-induced behavioral and psychological factors, and immune function in family caregivers and 3) to lay out potential areas for future research and challenges to conducting this type of research.** This work merges the caregiving, immunology, behavioral and psychological literature to illustrate the potential benefit of conducting this type of research. This article will be submitted for publication in *Biological Research for Nursing* in Spring 2015.

Chapter 3, the second article of the three-article dissertation, is a primary, data-based paper that utilized human blood to conduct an *in vitro* investigation of the impact of norepinephrine on CD8 T cell subsets (naïve, central memory, effector memory) at resting, and 24 and 72 hours after activation with antibodies for CD3 and CD28. In addition, we used blood samples and norepinephrine measured in the serum of human adults to examine the impact of high levels of norepinephrine (>150 pg/mL) compared to low levels (<150 pg/mL) of norepinephrine on CD8 T cell subsets (naïve and memory). **The specific aims of this paper are: 1) To examine the expression of norepinephrine's receptor (beta-2 adrenergic receptor) on CD8 T cell subsets (naïve, central and effector memory); 2) To examine the impact of norepinephrine exposure on global gene expression changes in central memory CD8 T cells; 3) To examine the gene expression changes of cytokines and chemokines in CD8 T cell subsets treated with norepinephrine before and after activation; 4) To examine the protein level changes in cytokines and chemokines in CD8 T cell subsets and 5) To examine changes in identified cytokines and chemokines altered with norepinephrine treatment in individuals with high levels of norepinephrine compared to the low level group.** In this paper, we examine the effect of norepinephrine in CD8 T cell subsets *in vitro,* as

well as *in vivo* with human adults to understand if norepinephrine has a similar or different impact on the three major CD8 T cell subsets. This article was submitted for publication to *Brain Behavior Immunity* in Fall 2014 and received reviewer comments to revise and resubmit.

Chapter 4, the third article of the three-manuscript dissertation, is also a primary-data based study and uses a cross-sectional design of family caregivers of hematopoietic stem cell transplant recipients and their age-, gender- and ethnicity-matched non-caregiver controls to examine the impact of caregiving stress on cytokine and chemokine gene expression, and protein levels in CD8 T cell subsets (naïve and memory). **The specific aims are: 1) To examine the effect of caregiving on leukocyte composition compared to non-caregivers; 2) To examine the effect of caregiving on intracellular cytokine production of CD8 T cells compared to non-caregivers by flow cytometry analysis; and 3) To examine gene expression changes in inflammation-related cytokines and chemokines in caregivers compared to controls.** In this paper we explore the differences in immune cell composition and CD8 T cell expression of important cytokines and chemokines in family caregivers of stem cell transplant recipients compared to their matched controls. This article will be submitted for publication in *Psychosomatic Medicine* by Spring 2015.

Chapter 5 is the final chapter of this dissertation and presents a discussion of findings from all three manuscripts (one theoretical and two data-based papers), and the implications of these findings for understanding how stress influences subsets of immune cells differently, and recommendations for future research on this important area of research.

Significance

Previous literature demonstrates conflicting reports in immune cells after exposure to an environmental stressor including whether stress is immune-enhancing, immune-suppressive, or has no significant impact; a majority of these studies examined changes in whole blood or peripheral mononuclear cells (PBMC) (Gouin *et al.*, 2008;Hu, Wan, Chen, Caudle, LeSage, Li,

and Yin, 2014;Kiecolt-Glaser, Glaser, Gravenstein, Malarkey, and Sheridan, 1996;Torres, Antonelli, Souza, Teixeira, Dutra, and Gollob, 2005;Vedhara, McDermott, Evans, Treanor, Plummer, Tallon, Cruttenden, and Schifitto, 2002;Zieker *et al.*, 2007). Studies on the SNS-released hormones and neurotransmitters have also reported a contradictory impact on immune cells (Elenkov *et al.*, 2000;Kin *et al.*, 2006;Rohleder *et al.*, 2009;Sanders, Baker, Ramer-Quinn, Kasprowicz, Fuchs, and Street, 1997;Straub, Schaller, Miller, von, Jessop, Falk, and Scholmerich, 2000). In addition, several studies have examined epigenetic regulation and its role in mediating immune function, or its relationship to psychological and behavioral-induced changes, but not in relation to each other.

This dissertation contributes to the field of nursing by providing exploratory insight into understanding caregiving as a stressor and how it impacts specific immune cells; in addition, we take a first step in understanding the potential link between epigenetic regulation, psychological and behavioral factors in caregiving, and immune alterations in family caregivers. We also reveal the susceptibility of memory CD8 T cells to norepinephrine (an important hormone released during the stress response) exposure, as well as pro-inflammatory and proliferation-related cytokines and chemokines altered by norepinephrine treatment. This information will lead to a better understanding of how chronic stress, including norepinephrine exposure, can influence specific immune cell subsets in a heterogeneous manner. The direct benefits of this study include identifying immune cell subsets most susceptible to the effects of caregiving stress or stress hormones (norepinephrine), which subsequently leads to the production of a pro-inflammatory state. This will help identify individuals at risk for weakened immunological memory responses and in the long-term provide future guidance on interventions aimed at stress-reduction in family caregivers.

Background

In this section, I present an overview of background information related to: nervous-immune communication via norepinephrine and its previously reported impact on T cell function; the role of epigenetic regulation on impacting immune function; and finally a review of family caregiver immune outcomes with a specific focus on hematopoietic stem cell transplant family caregivers.

Nervous-immune communication via norepinephrine and its impact on T cell function

T cells are critical to the adaptive immune response as they are major effectors of cell-mediated responses. There are several subsets of T cells including CD4, CD8 and natural killer T cells; each has their own subsets including naïve and memory; for this dissertation, we focus on CD8 T cell subsets (naïve, central memory and effector memory). T cell function is mediated by the nervous system by the binding of hormones and/or neurotransmitters released by the nervous system to interact with surface receptors on T cells, such as adrenergic receptors. This nervous-immune relationship is of great importance during a stress response, when the somatic effects of stress are mediated by the neuroendocrine system. Two major neuroendocrine systems include the sympathetic nervous system (SNS) and hypothalamic pituitary adrenal (HPA) axis. The SNS releases catecholamines while the HPA axis releases glucocorticoids; immune cells have receptors for both catecholamines and glucocorticoids. Catecholamines tend to elicit a pro-inflammatory response, while glucocorticoids elicit an anti-inflammatory effect on immune cells (Chambers, Cohen, and Perlman, 1993;Sperner-Unterweger, Kohl, and Fuchs, 2014).

While the SNS can release several hormones and neurotransmitters during a stress response, its primary catecholamine is norepinephrine (NE), which binds to T cells by the beta-2 adrenergic receptor. The SNS and NE, in particular, have been associated with the “fight or flight” response and contribute significantly to immune function (Kohm and Sanders, 2001). NE is produced by chromaffin cells, and released from the sympathetic nerve terminals located in lymphoid tissues, and also from the adrenal gland into the blood stream which influences T cells both in lymphoid organs and in the periphery (Kohm and Sanders, 2000;Sanders *et al.*, 2002).

This interaction is important during a stress response since the SNS and its release of NE and stimulation of the beta-2 adrenergic receptor can regulate the magnitude and intensity of an immune response (Sanders *et al.*, 2002). Activation of the SNS and subsequent release of catecholamines, including NE, have been shown to affect the health of humans in several ways, including: contributing to the development of pro-inflammatory diseases, the effectiveness of vaccination protocols by altering immunological memory and alterations in immune status of depressed individuals (Castle, Wilkins, Heck, Tanzy, and Fahey, 1995;Elenkov *et al.*, 2000;Glaser *et al.*, 1998;Gouin *et al.*, 2008;Mills, Adler, Dimsdale, Perez, Ziegler, Ancoli-Israel, Patterson, and Grant, 2004). Binding of NE and activation of the beta-2 adrenergic receptor leads to the release of certain cytokines from T cells, which creates a feedback loop to the SNS and brain; this bidirectional communication between the nervous system and the immune system has significant influence on immune function, particularly during chronic stress experiences (Kohm *et al.*, 2001). Since the majority of our stress today is psychological and chronic, we need to learn more about the interaction between the SNS and immune system, and the impact of prolonged exposure of immune cells to stress hormones, like norepinephrine.

The majority of studies examining the effects of NE on T cells have been conducted in NK or CD4 T cells. These studies have found that NE can elicit a transient increase in NK number and activity and a decrease in CD4 activity and proliferation (Khan *et al.*, 1986;Sanders, Kasprowicz, Swanson-Mungerson, Podojil, and Kohm, 2003). The Th_2 subset of CD4 cells are unaffected by NE since they lack the beta-2 adrenergic receptor on their cell surface, unlike their Th_1 and naïve counterparts; as for other T cells, the number of receptors differs depend on the cell type, with CD8 T cells having more receptors than CD4 T cells (Ramer-Quinn, Baker, and Sanders, 1997;Sanders, 2012;Sanders *et al.*, 2003); yet, it is unknown about the expression of beta-2 adrenergic receptor on CD8 T cell subsets. NE and beta-2 adrenergic receptor stimulation also significantly influence the cytokine production of T cells, though this has primarily been studied in CD4 T cells (Kohm *et al.*, 2001). Many of these studies show conflicting findings; some report increased production of IL1-α, IL-6, IL-10 and IFN-γ, while others show a decrease in these

cytokines (Elenkov *et al.*, 2000;Swanson, Lee, and Sanders, 2001;Van Tits, Michel, Grosse-Wilde, Happel, Eigler, Soliman, and Brodde, 1990). The inconsistency in findings suggests stimulation of the beta-2 adrenergic receptor by NE may be regulated by additional factors, such as cell subset.

Epigenetic regulation of T cell function

Epigenetics serve as one molecular mechanism that can influence the expression of immune-related genes through environmental factors, including modifications in gene expression without altering the DNA sequence (Allis, Jenuwein, and Reinberg, 2013). Broadly speaking, epigenetics is the study of how the environment impacts gene activity without alterations in DNA. The epigenome acts as a gatekeeper of gene expression determining when to turn genes 'on' or 'off,' impacting transcription of genes. These epigenetic alterations can be long-term and heritable, resulting in disease development (Santos-Reboucas and Pimentel, 2007). Initially, DNA is tightly wound into coils around proteins called histones (H2a, H2b, H3, and H4 in octomers). This coiled combination of a histone plus DNA is called chromatin (colloquially pictured as beads on a string), and these chromatin structures are then coiled into chromosomes (Allis et al., 2013). Epigenetic marks regulate when and where these coils are unraveled, determining which genes can be expressed. For example, if a piece of DNA is wound tightly around a histone, the transcription machinery cannot bind to the DNA and express the gene (i.e. transcribe the gene into mRNA and then into protein). It is therefore necessary to open up the coil of DNA on the histone to access the desired gene. There are many types of epigenetic modifications; a frequently studied epigenetic mechanism includes acetylation of the N-terminal of the histone. Acetylation of the histone tail at specific sites causes the shape of the histone to change and allows for the unraveling of the DNA exposing certain genes at the unraveled spot to be transformed into proteins.

Recent studies have discovered that one form of epigenetic changes, histone modifications, are critical for proper activation and functioning of the immune system, including T

cells (Araki, Fann, Wersto, and Weng, 2008;Araki, Wang, Zang, Wood, III, Schones, Cui, Roh, Lhotsky, Wersto, Peng, Becker, Zhao, and Weng, 2009;Avni, Lee, Macian, Szabo, Glimcher, and Rao, 2002;Chang and Aune, 2007). Epigenetic regulation via DNA methylation and/or histone modifications are critical for proper T cell development, differentiation and function during a healthy human immune response and in an aged immune response (Araki *et al.*, 2009;Fann, Godlove, Catalfamo, Wood, III, Chrest, Chun, Granger, Wersto, Madara, Becker, Henkart, and Weng, 2006;Fitzpatrick and Wilson, 2003). These epigenetic alterations are important to allow for immune response genes to be activated during an antigenic challenge. Emerging evidence suggests that epigenetic alterations at the chromatin level play an important role in controlling the distinct transcriptional profiles of memory T cells and their subsequent ability to function (Weng, Araki, and Subedi, 2012). A genome-wide analysis of histone methylation on two histone lysine sites (H3K4me3 and H3K27me3) and gene expression profiles of naïve and memory $CD8^+$ T cells found that a positive correlation exists between gene expression and the amount of H3K4 methylation, and a negative correlation exists between gene expression and H3K27 methylation (Araki et al., 2009). In summary, this study concluded transcription is highly controlled at the chromatin level and specifically relevant to the function of memory $CD8^+$ T cells. These findings also suggest that histone modifications impact the function of memory $CD8^+$ T cells, and different epigenetic mechanisms (e.g. methylation, acetylation) impact transcriptional capability and thus functionality of naïve and memory $CD8^+$ T cells. For immune function, this means that histone modifications impact the ability of memory $CD8^+$ T cells to recognize antigens and mount a rapid, strong cellular response. Another study found T helper cell differentiation is associated with histone acetylation and chromatin remodeling for the genes encoded for IL4 and IFNG (Avni et al., 2002). This means that histone acetylation may be a major mechanism of controlling cytokine genes and thus transcription of cytokines in naïve and helper T cells. On a broader level, if inappropriate cytokine genes are repressed by this epigenetic mechanism (histone acetylation), then it will reflect poorly on overall immune function. One study showed that histone methylation is highly dynamic for the locus encoding IFN-G, an important cytokine for T cell functioning and

proliferation (Chang *et al.*, 2007). While many studies focus on epigenetic changes in early life on immune cells (King, Ismail, Davis, and Karp, 2006;Murgatroyd, Patchev, Wu, Micale, Bockmuhl, Fischer, Holsboer, Wotjak, Almeida, and Spengler, 2009;Murgatroyd and Spengler, 2011;Murgatroyd *et al.*, 2010), it is important to examine later life changes in adulthood to understand mechanisms behind immune function changes in the aging.

Family caregiver immune outcomes and hematopoietic stem cell transplant caregivers

This section reviews the literature pertaining to immunological outcomes in the family caregiver population with a specific focus on T cells, as well as a background on hematopoietic stem cell transplant (HSCT) family caregivers.

A large body of literature has identified an association between caregivers and altered immunological outcomes (i.e. natural killer (NK) cell cytotoxicity, T cell function, vaccination titers and cytokine production). While numerous cell types have been examined, here we will focus on the influence of caregiving on lymphocytes. Studies have shown that the chronic psychological stress of caregiving leads to significantly lower percentage of T cells overall, but higher percentages of CD8 T cells and lower percentages of suppressor and helper T cells (Pariante, Carpiniello, Orru, Sitzia, Piras, Farci, Del Giacco, Piludu, and Miller, 1997;Vitaliano, Scanlan, Ochs, Syrjala, Siegler, and Snyder, 1998). Alterations in the composition of immune cells in the periphery can negatively impact the immune system; for example, fewer helper T cells may mean the immune system has a lower capacity to activate cytotoxic T cells, while a higher percentage of these cells may be useless if they are not able to be notified by the helper T cells or turned off by suppressor T cells. Additional results showed caregivers who reported higher levels of psychological stress correlated positively with T regs and T cytotoxic cells, and correlated negatively with T helper/suppressor ratio (Pariante et al., 1997), suggesting the severity of perceived psychological stress moderates the influence of stress on immune changes. Investigations into immune alterations in family caregivers of Alzheimer's patients found a cytokine shift from CD4 Th_1 subtype to CD4 Th_2 as well as an increase in pro-inflammatory

cytokines like TNF and a decrease in proliferation related cytokines like IL-2 and IFN-γ (Glaser *et al.*, 1998;Kiecolt-Glaser *et al.*, 1996;Kiecolt-Glaser *et al.*, 1987). Studies on caregivers of patients with other forms of dementia found caregivers who reported high stress had reduced T cell proliferation and an increase in the percentage of CD8 T cells (Castle et al., 1995). Several studies found caregivers of cancer patients and dementia patients had an increase in pro-inflammatory cytokines, slower wound healing, and poorer response to vaccination (Glaser *et al.*, 1998;Kiecolt-Glaser *et al.*, 1996;Kiecolt-Glaser *et al.*, 1987;Kiecolt-Glaser, Marucha, Malarkey, Mercado, and Glaser, 1995;Kiecolt-Glaser, Preacher, MacCallum, Atkinson, Malarkey, and Glaser, 2003).

Clearly, the literature demonstrates that caregivers of the elderly, those with Alzheimer's disease or cancer patients have numerous altered immunological outcomes. Yet, other caregiver populations like HSCT caregivers are not as well understood in terms of immunological changes due to caregiving stress. According to a study by Gratwohl and colleagues, the use of HSCT has rapidly expanded and as of 2006, over 50,417 HSCTs were reported worldwide, including 21,516 allogeneic transplants (Gratwohl, Baldomero, Aljurf, Pasquini, Bouzas, Yoshimi, Szer, Lipton, Schwendener, Gratwohl, Frauendorfer, Niederwieser, Horowitz, and Kodera, 2010). HSCT caregivers endure what is arguably one of the most intense treatments available for cancer patients which can result in physical, psychological and social challenges (Beattie and Lebel, 2011). In addition, the need to decrease inpatient healthcare costs has motivated the move of HSCT and recovery to outpatient settings, requiring caregivers to assume care that would otherwise be provided in a supervised medical setting (Rizzo, Vogelsang, Krumm, Frink, Mock, and Bass, 1999).

In order to understand how allogeneic HSCT caregiver is an ideal subject to evaluate chronic stress of caregiving, it is important to understand the transplantation process itself. Allogeneic HSCT is when hematopoietic stem cells from a histologically compatible relative or unrelated donor are collected either from peripheral blood while an autologous HSCT is when the

patient's own cells are harvested, stored, and then re-infused during transplant (Shelburne and Bevans, 2009). Despite the growth in transplantations and responsibilities caregivers assume during the intense HSCT transplant and post-transplantation process, there is limited research on the family caregivers of HSCT recipients. The two major types of allogeneic HSCT include: standard treatment which includes chemotherapy and/or radiation given before transplantation and 2) reduced intensity conditioning which is less intense and given to adults over the age of 55 to improve survival (Shelburne *et al.*, 2009). While the HSCT transplantation process itself can be stressful, it is the recovery period that presents a significant challenge to family caregivers due to the serious and sometimes life-threatening toxicities that can arise from any of the following: chemo- and radio-therapy; graft versus host disease, or medication side-effects from immunosuppression (Bevans, Wehrlen, Prachenko, Soeken, Zabora, and Wallen, 2011). Even under the best conditions, HSCT caregivers experience significant chronic stress in their role; HSCT typically requires a month hospital stay with three to four months of intense outpatient care. During this time, family caregivers face a variety of stressors that may negatively impact their health. For example, during the pre-transplantation timeframe caregivers must coordinate transportation to the clinic, find new long-term housing near the clinic, and potentially face emotional distress from the uncertainty of the effectiveness of the transplantation; pre-transplantation has been reported as the most stressful time for caregivers (Bevans et al., 2011). After transplantation, family caregivers are the main care providers of the transplant recipients; they complete everyday tasks like preparing meals, bathing, and house cleaning, to medical tasks of assessing their care-recipient for potential medical complications and declines in health. With the significant time and attention dedicated to the transplant recipient, HSCT caregivers have little time or energy left to focus on their own emotional, behavioral, and physical health. In 2006, an alarming report found 53% of family caregivers of mixed patient populations reported a decline in their health, affecting their ability to provide care (National Alliance for Caregiving, 2009).

The majority of literature on transplant caregivers examines the effects of caregiving on psychosocial and emotional health outcomes (Beattie *et al.*, 2011;Bevans *et al.*, 2011;Frey,

Stinson, Siston, Knight, Ferdman, Traynor, O'Gara, Rademaker, Bennett, and Winter, 2002;Northouse, Williams, Given, and McCorkle, 2012;Shelburne *et al.*, 2009;Siston, List, Daugherty, Banik, Menke, Cornetta, and Larson, 2001). Two studies reported high perceived stress in HSCT caregivers, but made no correlations between high reported stress and changes in immune function (Beattie *et al.*, 2011;Northouse *et al.*, 2012). There is little knowledge on the immune outcomes of this population of caregivers. One study by Futterman et al. (1996), examined the impact of bone marrow transplantation on caregivers' immunological status and found there was a significant negative correlation between caregivers' reported stress and the total percentage of T cells and $CD4^+$ T cells (Futterman, Wellisch, Zighelboim, Luna-Raines, and Weiner, 1996). The immune function of HSCT caregivers requires further investigation; we were able to address this topic since we had access to blood samples from this particular group of caregivers. Understanding of HSCT caregiver immunity can aid in illustrating further the immune health outcomes of caregivers, particularly in a population that is chronically stressed and may undergo an event that can serve as an acute stressor.

Summary

This dissertation is a preliminary, exploratory step in filling knowledge gaps in how CD8 T cell subsets (naïve and memory) are influenced by norepinephrine and caregiving stress. This work explores functional changes of CD8 T cells *in vitro* and *in vivo* by studying isolated CD8 T cell subsets in culture with the catecholamine released during stress, norepinephrine, and in vivo by examining a chronically stressed population: family caregivers of stem cell transplant recipients. The knowledge generated from this project will include identification of areas for further research, potential obstacles that may be encountered in this field, and promising areas for examining the effects of stress on immunological function, particularly on immunological memory. Chapter 2 explores the recent literature surrounding epigenetics as a potential mechanism behind stress-induced changes in the immune system, and offers a proposed framework for study in family caregivers, as well as potential obstacles encountered in this area

of research. Chapter 3 and 4 consist of two primary data-based papers based on an *in vitro* and *in vivo* study on the function of CD8 T cell subsets in humans altered by norepinephrine or caregiving. This research explores the impact of stress on important cells to the adaptive immune response (CD8 T cells) in humans with a translational approach from bench (*in vitro*) to bedside (*in vivo*).

CHAPTER 2

Examining the role of epigenetic modifications in stress-induced immune changes in family caregivers: A literature review and proposed framework

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Abstract

Introduction: Research has demonstrated a link between poor immune outcomes and the stress of family caregivers; however, there is a gap in the understanding of potential molecular mechanisms underlying the link between caregiving stress and immune dysfunction. **Aims:** To review the literature on how epigenetic changes may serve as a mediator between stress-induced psychological and behavioral changes, and T cell function in family caregivers. **Methods:** The review was conducted by a search of published studies in electronic databases. **Results:** Nine articles met our review criteria. The reviewed literature clearly illustrated a relationship between stress-induced psychosocial and behavioral changes, epigenetic mechanisms (DNA methylation and histone modifications), and altered T cell function in humans. No literature discussed the potential role of epigenetics in mediating stress-induced changes in family caregivers' immune outcomes. **Conclusion:** We identify the technical, methodological and practical challenges of conducting epigenetic studies on humans, including family caregivers. Despite these challenges, identification of epigenetic mechanisms mediating stress-induced changes in T cell function of family caregivers could assist in the development of targeted stress-reduction interventions. Finally, we propose a framework in which epigenetic regulation may serve as a mediator between stress-induced psychological and behavioral factors, and altered immune function in the family caregiver population.

Keywords: epigenetics, family caregivers, stress, immune health, literature review

Introduction

Previous evidence has shown the negative impact of chronic stress on health, particularly related to immune health outcomes (Bonneau, Kiecolt-Glaser, and Glaser, 1990;Kemeny and Schedlowski, 2007;Kiecolt-Glaser *et al.*, 1996;Kiecolt-Glaser *et al.*, 1987;Kiecolt-Glaser *et al.*, 1995;Pariante *et al.*, 1997). Much of this research has been conducted in family caregivers since they are an ideal population to study the effects of chronic stress; caregivers are generally stressed for extended periods of time, and suffer worse health outcomes and increased mortality and morbidity compared to non-caregivers (Rohleder *et al.*, 2009;Schulz and Beach, 1999). Although the detrimental effect of stress on immune function has been reported extensively in family caregivers, there is less understanding of the mechanisms underlying stress induced changes in immunity, such as neuroendocrine mechanisms or molecular mediators (Segerstrom and Miller, 2004).

Epigenetics is one molecular mechanism that controls the expression of genes without altering the DNA sequence (Allis et al., 2013). The field of epigenetics holds promise for elucidating how lifestyle factors, like a stressful environment, can influence the expression of immune-related genes. Emerging evidence suggests epigenetic regulation may serve as a potential mediator of stress-induced psychosocial and behavioral changes, and alterations in immune function (Mathews and Janusek, 2011;Mathews, Konley, Kosik, Krukowski, Eddy, Albuquerque, and Janusek, 2011;Mifsud, Gutierrez-Mecinas, Trollope, Collins, Saunderson, and Reul, 2011). Several excellent reviews have discussed the connection between stress-induced lifestyle changes and epigenetic modifications, particularly on stress-induced psychosocial and behavioral alterations which impact the epigenome (Alegria-Torres, Baccarelli, and Bollati, 2011;Mathews *et al.*, 2011;McEwen, 2012). Reviews have also been conducted on epigenetic regulation of immune cell genes, including T cells (Kondilis-Mangum and Wade, 2013;Lim, Li, Holloway, and Rao, 2013;Reiner, 2005;Weng *et al.*, 2012).

Despite these findings, a review of the potential relationship between chronic-stress induced psychosocial and behavioral changes, epigenetic regulation, and immune alterations has not been completed. Several lifestyle factors such as diet, physical activity, smoking, alcohol use, and stress have been identified to potentially modify epigenetic patterns (Alegria-Torres *et al.*, 2011;Lee, Sahoo, and Im, 2009). While the lifestyle factors and immune outcomes of family caregivers have been examined previously, no work has yet examined the potential role of epigenetic changes mediating caregiving stress-induced immune dysfunction.

The purpose of this review is to explore how epigenetic changes may serve as a mediator between stress-induced behavioral and psychological changes, and poor immune function in family caregivers. Here, we review the literature on human studies of behavioral and psychological changes common to family caregivers and their connection to epigenetic changes (DNA methylation and histone modifications) in T cells. We focus on T cells since they are critical players in the adaptive immune response and have been previously shown to be partly regulated by epigenetic mechanisms (Lee et al., 2009; Bowen, Kelly, Lee, & Lavender, 2008; Weng et al., 2012; Sanders, 2006; Bandyopadhyay, Montagna, & Macian, 2012). Table 1 provides definitions for epigenetic-related terms cited in this review. We identify the technical, methodological and practical challenges of conducting human epigenetic studies, particularly in family caregivers. Finally, a proposed framework is presented in which epigenetic regulation serves as a mediator between stress-induced psychological and behavioral factors, and biological immune responses in the family caregiver population.

Background

Epigenetic mechanisms, including DNA methylation, and histone methylation and acetylation, play a role in altering the function of T cells by changing the gene expression of cytokines and chemokines important to T cells, and thus impacting the quality of the adaptive immune response. Previous reviews have extensively covered the various types of epigenetic modifications that impact the gene expression of T cells (Kondilis-Mangum *et al.*,

2013;Kouzarides, 2007;Lim *et al.*, 2013;Mathews *et al.*, 2011;Mifsud *et al.*, 2011), however this review will focus on DNA methylation, and histone methylation and acetylation since these epigenetic marks tend to be heritable and long-term (Greer and Shi, 2012).

DNA methylation is one form of a covalent modification and involves the addition of a methyl group onto cytosines at CpG dinucleotides which have significant influence over transcription (Lee et al., 2009). Little to no methylation of the CpG islands is typically associated with genes that are actively transcribed, compared to methylation of the CpG islands that are associated with gene silencing (Weng et al., 2012). Methylation can also occur at interspersed repetitive sequences, including long interspersed nuclear elements (LINEs) and Alu (Sukapan, Promnarate, Avihingsanon, Mutirangura, and Hirankarn, 2014). LINE-1 and Alu are considered representative of genome-wide methylation marks and considered as proxies for global DNA methylation status in T lymphocytes (Kitkumthorn and Mutirangura, 2011;Nakkuntod, Avihingsanon, Mutirangura, and Hirankarn, 2011;Sukapan *et al.*, 2014).

While DNA methylation is involved in the repression of transcription, post-translational histone modifications can activate or repress transcription depending on the position and type of modification (Johnson and Dent, 2013;Northrup and Zhao, 2011). Histones are the basic components where the DNA wraps around two copies of the four core histones (H2A, H2B, H3 and H4) to form nucleosomes or 'beads on a string' that together form chromatin (Lee et al., 2009). The methylation or acetylation of histones are two forms of posttranslational histone modifications that can define the chromatin structure and transcriptional capacity of a gene; H3 and H4 being the most commonly methylated or acetylated on lysine (K) residues (Lee et al., 2009). Acetylation involves the addition of an acetyl group ($COCH_3$) to lysine (K) residues and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Gong and Miller, 2013); histone acetylation is related to activation of transcription, while histone deacetylation is associated with repression of transcription (Falvo, Jasenosky, Kruidenier, and Goldfeld, 2013).

The biological function of T cells, including the release of cytokines and chemokines has been extensively studied for many years; however the molecular mechanisms, including chromatin remodeling, is in its infancy (Lee *et al.*, 2009;Reiner, 2005). The cytokine environment and interaction with other immune cells, such as CD4 T cells can induce epigenetic modifications in CD8 T cell subsets (naïve and memory). For example, CD4 T cells influence the epigenetic remodeling of the IL2 and IFNG loci in activated memory CD8 T cells (Northrop, Thomas, Wells, and Shen, 2006). IL2, IFNG, and IL4 have been studied extensively in both CD4 and CD8 T cells for expression changes controlled by epigenetic mechanisms (Fitzpatrick *et al.*, 2003). Histone methylation and acetylation also influences the chromatin states of many important genes, including cytokines, that are characteristic of CD4 memory T cells (Weng et al., 2012). In CD8 memory T cells, genes associated with effector function, including IFN-G, have high levels of H3K4me3, but low levels of H3K427me3 (Weng et al., 2012). In addition, studies on T cell subsets have focused on the potential of epigenetic regulation of important functional genes (Singh, de Camargo, Zhang, Foley, Hedrick, and Farber, 2010;Yano, Ghosh, Kusaba, Buchholz, and Longo, 2003).

Method

We utilized an integrative literature review approach to provide a new framework on the topic of stress-induced changes in immune function in family caregivers. The integrative literature review was completed on a search of electronic databases including PubMed, Medline, CINAHL, Ovid and Cochrane Review with no date limit since there was limited information available. Search terms used were: “stress” or “stress response” or “chronic stress”; “family caregivers” or “caregiving”; “perceived stress”; “health behaviors” or “health practices”; “psychological pathways” or “psychosocial”; “epigenetics”; “immune function” or “immunity” or “T cells,” in various combinations. References of identified articles were also searched for additional relevant articles. Articles were selected if they met three criteria: a) discussed stress-induced psychosocial or behavioral changes that could also be found in the family caregiver population, including: distress

(Goossens, Van, Knoppert-van Der Klein EA, and Van, 2008;Khoo, Chen, Ang, and Yap, 2013;Mourik, Rosso, Niermeijer, Duivenvoorden, Van Swieten, and Tibben, 2004;Pellegrino, Formica, Portarena, Mariotti, Grenga, Del, and Roselli, 2010), sleep disturbances , PTSD (Jacobson, 2010;Perreira and Ornelas, 2013;Stukas, Jr., Dew, Switzer, DiMartini, Kormos, and Griffith, 1999;Teixeira and Pereira, 2014), diet (Abu-Farha, Tiss, Abubaker, Khadir, Al-Ghimlas, Al-Khairi, Baturcam, Cherian, Elkum, Hammad, John, Kavalakatt, Warsame, Behbehani, Dermime, and Dehbi, 2013;McGuire, Bouldin, Andresen, and Anderson, 2010;Ronn, Volkov, Davegardh, Dayeh, Hall, Olsson, Nilsson, Tornberg, Dekker, Eriksson, Jones, Groop, and Ling, 2013;Zhang, Cardarelli, Carroll, Zhang, Fulda, Gonzalez, Vishwanatha, Morabia, and Santella, 2011), physical exercise (von, Mausbach, Dimsdale, Mills, Patterson, Ancoli-Israel, Ziegler, Roepke, Harmell, Allison, and Grant, 2011;Zhang *et al.*, 2011), or alcohol/smoking use (Alegria-Torres *et al.*, 2011;Lazarus, 1974;McGuire *et al.*, 2010;Trunzo, Pinto, and Chougule, 2014); b) discussed behavioral or psychological pathways related to DNA methylation, or histone methylation or acetylation, and c) discussed epigenetic regulation of transcription in T cells or their related cytokines. This review excluded unpublished studies, commentaries, and studies not conducted in humans.

Results

Notably, the majority of epigenetic studies on psychological or behavioral induced changes in T cells focus on the neuroendocrine system, early life or pregnancy, and have been conducted in mouse and rat models (Champagne and Curley, 2009;Hunter, 2012;Jensen, Monk, and Champagne, 2012;Murgatroyd, 2014;Murgatroyd *et al.*, 2011;Murgatroyd and Spengler, 2014;Murgatroyd *et al.*, 2010); there are excellent reviews on these systems (Mathews *et al.*, 2011;Mifsud *et al.*, 2011). Here, we review human studies (N=9) on psychosocial (n=4) (**Table 2**) and behavioral (n=5) changes (**Table 3**), and T cell and related cytokines changes via epigenetic modifications relevant to the family caregiver population.

Psychological models and epigenetic mediation of stress-induced immune changes

Post-traumatic stress disorder (PTSD) develops from a maladaptive stress response resulting in poor health outcomes (Yehuda and LeDoux, 2007), yet the molecular mechanisms of PTSD are partially unknown. Previous microarrays from PTSD individuals have illustrated differential gene expression compared to non-PTSD sufferers (Segman, Shefi, Goltser-Dubner, Friedman, Kaminski, and Shalev, 2005;Zieker *et al.*, 2007) suggesting subsequent pathophysiology of PTSD. Since alterations in immune function are a feature of PTSD (Segman *et al.*, 2005;Zieker *et al.*, 2007), one study examined the role of methylation in distinct-immune related gene expression patterns (Uddin, Aiello, Wildman, Koenen, Pawelec, de los, Goldmann, and Galea, 2010). This study found uniquely unmethylated genes that were related to immune function, especially inflammatory cytokine genes produced by T lymphocytes, and innate immunity genes in PTSD individuals compared to controls. The affected genes were significantly negatively correlated with traumatic exposure and were also associated with differences in the ability to mount an immune response to cytomegalovirus. DNA methylation patterns correlated with immune dysregulation in a PTSD study focused on traumatized African American adults from Atlanta (Smith, Conneely, Kilaru, Mercer, Weiss, Bradley, Tang, Gillespie, Cubells, and Ressler, 2011). Global methylation was increased in PTSD individuals and several genes associated with inflammation were methylated in individuals with high levels of total life stress; furthermore, T lymphocyte cytokine levels of TNF, IL4, and IL2 were associated with PTSD (Smith et al., 2011). Another study of methylation patterns in PTSD U.S. military service members found significant increase in T lymphocyte produced IL18 promoter region methylation and protein levels in PTSD subjects compared to controls (Rusiecki, Byrne, Galdzicki, Srikantan, Chen, Poulin, Yan, and Baccarelli, 2013).

Distress related to poor health can impact immune function via epigenetic changes. Mathews and colleagues examined the effects of psychosocial distress on immune function in women (n=33) with a new breast cancer diagnosis at the time of diagnosis and four months later

after completion of treatment (Mathews et al., 2011). Mathews made a similar case as Uddin for the connection between distress and its negative impact on immune health, and both populations reported alterations in immune function in their stressed populations of women with a new breast cancer diagnosis and PTSD affected individuals, respectively. A correlation was found between the perceived stress survey and immune outcomes at the first time point in this study. In association with H4K8 acetylation of CD56 lymphocytes, individuals with higher levels of perceived stress had lower levels of CD56 lymphocytes as measured by mean fluorescent index (MFI). Furthermore, immune dysregulation was associated with a decrease in acetylation of H4K8 and H4K12. When the stress was reduced at the second time point, immune function was improved and no correlations among the variables were significant.

Behavioral models of epigenetic modulation of stress-induced immune changes

The role of nutrition and exercise in modifying epigenetic mechanisms has been examined in multiple studies since epigenetic changes are potentially reversible, and modifiable factors such as diet and exercise provide hope for healthy intervention strategies to buffer the effects of stress (Abu-Farha *et al.*, 2013;McGee, Fairlie, Garnham, and Hargreaves, 2009;Ronn *et al.*, 2013;Zhang *et al.*, 2011). In a human study on postmenopausal women, DNA hypomethylation was associated with folate depletion, which was reversible with folate repletion(Jacob, Gretz, Taylor, James, Pogribny, Miller, Henning, and Swendseid, 1998). In a previous investigation, men did not have hypomethylation with a low folate diet indicating gender and/or age may impact the pathways of epigenetic changes from folate deficiency (Jacob, Pianalto, Henning, Zhang, and Swendseid, 1995).

In examining the impact of physical exercise, a recent study assessed whether DNA methylation was associated with different levels of physical activity by measuring global genomic DNA methylation for LINE-1 in PBMC of participants (n=161) of the North Texas Healthy Heart Study (Zhang et al., 2011). The investigators found physical activity of 26-30min/day was associated with hypermethylation in PBMC LINE-1 elements compared to those with less than

10min/day of physical activity (Zhang et al., 2011). In another study, the ASC gene, which is important for control of IL-1B and IL-18 secretion, was methylated after moderate physical exercise (Nakajima, Takeoka, Mori, Hashimoto, Sakurai, Nose, Higuchi, Itano, Shiohara, Oh, and Taniguchi, 2010).

Excessive alcohol consumption may serve as a negative health behavior that can alter the epigenome. In an analysis of DNA methylation by PCR-pyrosequencing of data from five individual studies, alcohol consumption was inversely associated with Alu methylation, but no association was found with smoking; in addition, the percent of lymphocytes in blood counts were negatively associated with LINE-1 methylation (Zhu, Hou, Bollati, Tarantini, Marinelli, Cantone, Yang, Vokonas, Lissowska, Fustinoni, Pesatori, Bonzini, Apostoli, Costa, Bertazzi, Chow, Schwartz, and Baccarelli, 2012).

Sleep disturbances have also been examined as a potential inducer of epigenetic change. In an examination of DNA methylation of Alu, LINE-1, GCR, TNF, and IFNG, shiftworkers had significantly different levels of methylation (Bollati, Baccarelli, Sartori, Tarantini, Motta, Rota, and Costa, 2010). Furthermore, job seniority was significantly negatively correlated with IFNG hypomethylation, and shiftworkers who were described as the 'morning type' showed TNF hypomethylation which may be related to increased TNF gene expression and subsequent increased risk for inflammatory-related diseases (Bollati et al., 2010).

Discussion

Apparent from this review of the literature, little evidence currently exists in humans that chronic stressors impact psychosocial and behavioral factors which can lead to epigenetic changes to impact immune function. Furthermore, even though epigenetic changes can occur throughout life, little work has focused on modifications in adulthood where long-term exposure to stressors such as poverty, poor health behaviors, poor psychosocial factors, or environmental traumas may be present.

Studies cited in this review were cross-sectional or retrospective, providing a snapshot of epigenetic changes and altered T cell function or cytokine gene expression. Longitudinal studies, particularly those starting in early life, could provide more information about how the epigenome changes over time and the impact of various environmental stressors on T cell function and cytokine expression; yet, longitudinal studies are challenging to design and implement. Still, it is important to develop longitudinal studies to understand the dynamic nature of epigenetic changes, immune functions, and stressors over time (Ng, Barrett, Wong, Kuh, Smith, and Relton, 2012). The reviewed studies also varied substantially in sample size, ranging from 8 participants to over 1000 participants. It is important to recruit large sample sizes, particularly for global methylation analyses, since a variety of factors *in vivo* can alter the epigenome and influence the gene expression patterns in immune cells. Designing a human epigenetic study controlling for confounding factors is crucial to eliminating variables that can limit the validity and reliability of reported findings.

All of the studies in this review utilized samples from peripheral blood or peripheral mononuclear cells (PBMCs). While this sample collection method provides a large amount of immune cells, it makes it impossible to identify the exact locus of epigenetic change and identification of the immune cell subset where functional changes occurred. PBMC contains a mixture of immune cells and each individual has different percentages of these cells circulating in their blood; thus, it is difficult to tell from these studies if the epigenetic modifications proposed are due to actual epigenetic changes or because of variability of cell composition between donors. Thus, the ideal study will need to study the epigenetic modifications in isolated defined cell populations and subsets, which require even higher number of cells or blood.

While epigenetic studies are not a problem for *in vitro* studies utilizing cell lines or even animal models, the large amount of blood necessary for the currently available methodologies examining epigenetic change on specific immune cell subsets presents another challenge to conducting epigenetic studies in humans. All of the studies cited in this literature review examined global methylation patterns via LINE-1 or Alu; this provides a general picture for the methylation status

of immune cells, including T cell-related inflammatory cytokine genes. With improvement of new technologies such as ChIP-Seq, microarray and RNA-seq, it becomes possible to analyze the epigenetic modifications and gene expression at the genome-wide level (Northrup *et al.*, 2011). These new developments could potentially reduce the requirement of cells, improved coverage and sensitivity, and reduced cost. Still, it is important to conduct follow-up validation studies on specific immune cell subsets and epigenetic loci to understand the exact location and molecular modification occurring.

In conclusion, several models of stress and health (Ingram and Luxton, 2005;Lazarus and Folkman, 1984;Lutgendorf and Costanzo, 2003;McEwen, 1998) lack the concept of epigenetics as a potential factor mediating the relationship between stress and health outcomes. Furthermore, in models of caregiving and health outcomes (Gonzalez, Polansky, Lippa, Walker, and Feng, 2011;Pearlin, Mullan, Semple, and Skaff, 1990;Vitaliano, Zhang, and Scanlan, 2003) little attention is paid to molecular pathways that may mediate stress-induced psychological or behavioral changes, and biological outcomes in health. We understand the negative effects of chronic stressors, such as caregiving, on clinical outcomes but face a gap in our knowledge of the molecular mechanisms behind stress-induced changes in health. The proposed framework (**Figure** 1) illustrates the potential mediator of epigenetic regulation between stress-induced psychosocial and behavioral changes, and immune alterations in family caregivers. This can help frame future studies examining the role of epigenetic changes in family caregivers.

While the studies discussed in this literature review are preliminary, they provide interesting findings and support for future investigations to incorporate an epigenetic component when examining the impact of psychological and behavioral factors on immune outcomes. Since the psychological and behavioral factors examined are also seen in family caregivers, we argue future research should be conducted in family caregivers who serve as an ideal clinical model to study the effects of stress-induced psychosocial and behavioral changes, and altered immune outcomes. Now, as the push to translate bench to bedside science is increasing, it becomes important that the role of epigenetic modifications as a mediator between stress-induced

psychosocial and behavioral factors, and immune health outcomes are proposed in a framework that can be further developed and tested in human populations.

Table 1. Definitions of Epigenetic-Related Terminology.

Term	Definition
Epigenetics	Epigenetics is one molecular mechanism that controls the transcription of genes without altering the DNA sequence (Allis, Jenuwein, & Reinberg, 2013)
DNA methylation	DNA methylation is one form of a covalent modification and involves the addition of a methyl group onto cytosines at CpG dinucleotides. Methylation of cytosines primarily occurs at groupings of CpG rich areas called CpG islands; often these islands exist upstream from a transcriptional start site giving them significant influence over transcription (Lee et al., 2009).
Histones	Histones are the basic components where the DNA wraps around two copies of the four core histones (H2A, H2B, H3 and H4) to form nucleosomes or 'beads on a string' that together form chromatin (Lee et al., 2009).
Histone modifications	Post-translational histone modifications can activate or repress transcription depending on the position and type of modification (Johnson & Dent, 2013; Northrup & Zhao, 2011).
Acetylation	Acetylation involves the addition of an acetyl group ($COCH_3$) to lysine (K) residues and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Gong & Miller, 2013); histone acetylation is related to activation of transcription, while histone deacetylation is associated with repression of transcription (Falvo, Jasenosky, Kruidenier, & Goldfeld, 2013).

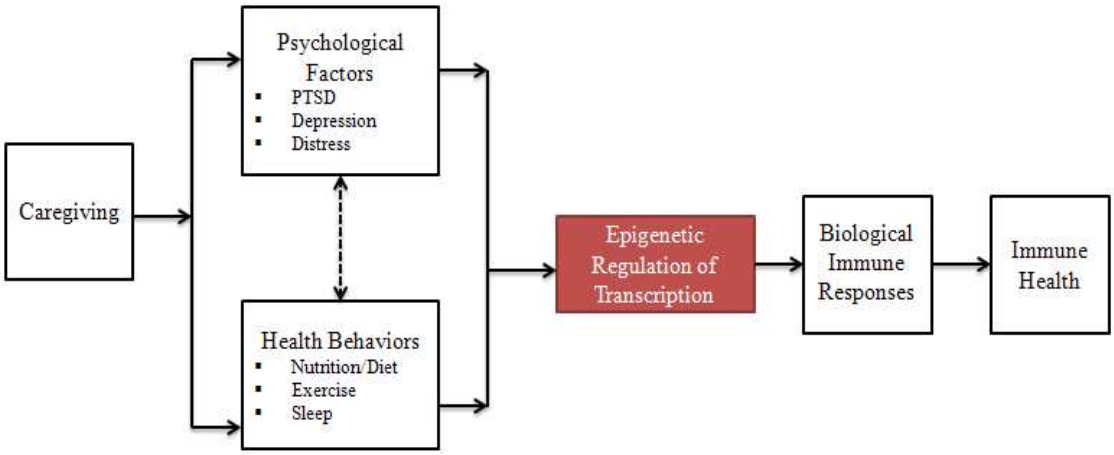
Table 2. Summary of represented epigenetic studies of psychosocial factors.

	Population	n	Sample Collection	Methylation Analysis	Immune Outcome
PTSD	African American adults	110	Peripheral Blood	Human Methylation27 BeadChip	Global methylation was increased for PTSD subjects and inflammatory genes were differentially methylated including IL4, IL2, TNFa
	Detroit Neighborhood Health Study	100	Peripheral Blood	HM27 DNA Analysis BeadChip Array	PTSD subjects had unmethylated genes related to immune function and inflammation. Affected genes were negatively correlated with trauma exposure and associated with differences in ability to mount immune response to CMV
	U.S. Army and Marine service members	75	Serum	Pyrosequence	Increased IL18 promoter region methylation and protein levels in PTSD subjects
Distress	Early diagnosed breast cancer subjects	33	Peripheral Blood	Immuno-fluorescence	Reduced nuclear acetylation of H4-K8 and H4-K12 and reduced phosphorylation of H3-S10 and altered IFNG activity in stressed diagnosed breast cancer subjects

Table 3. Summary of represented epigenetic studies of health behaviors

	Population	n	Sample Collection	Methylation Analysis	Immune Outcome
Physical Exercise	North Texas Healthy Heart Study	161	Peripheral blood	MethyLight	Methylation of proinflammatory markers
	Health-Promotion Program for Elderly People in Matsuomoto	436	Peripheral blood	Pyrosequencing	Methylation of ASC was higher in older exercise group compared to older control group
Diet	Postmenopausal women	8	Peripheral blood	DNA incorporation of methyl groups inversely related to endogenous DNA methylation, Method of Balaghi and Wagner (1993)	Folate deficiency resulted in significantly elevated plasma homocysteine and lymphocyte DNA hypomethylation
Alcohol /Smoking	Five studies of healthy subjects	1465	Peripheral blood	Pyrosequencing	Decreased Alu methylation with alcohol consumption. No associations with smoking and methylation. Percent of lymphocytes in blood were negatively associated with LINE-1 methylation
Irregular Sleep	Shiftwork employees from Northern Italy	150	Peripheral blood	Pyrosequencing	Significant difference in Alu and gene-specific methylation of IFNG and TNF promoters in morning versus evening shiftwork

Figure 1. Proposed framework of caregiving stress-induced psychosocial and behavioral changes with epigenetic modifications as a mediator of biological immune outcomes.



CHAPTER 3

Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation

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Abstract

Background

Norepinephrine (NE) is one of the primary catecholamines of the sympathetic nervous system released during a stress response and plays an important role in modulating immune function. NE binds to the adrenergic receptors on immune cells, including T cells, resulting in either suppressed or enhanced function depending on the type of cell, activation status of the cell, duration of NE exposure and concentration of NE. Here, we aim to analyze the effects of NE on the functionality of naïve (Tn), central memory (Tcm) and effector memory (Tem) CD8 T cells.

Methods

We isolated CD8 T cell subsets from healthy human adults and treated cells *in vitro* with NE ($1x10^{-6}$ M) for 16 hours; we then stimulated NE treated and untreated CD8 T cell subsets with antibodies for CD3 and CD28 for 24 and 72 hours. We assessed the level of beta-2 adrenergic receptor (ADRB2) expression in these cells as well as global gene expression changes in NE treated Tcm cells by microarray analysis. Altered expressed genes after NE treatment were identified and further confirmed by RT-qPCR, and by ELISA for protein changes. We further determined whether the observed NE effects on memory CD8 T cells are mediated by ADRB2 using specific adrenergic receptor agonist and antagonists. Finally, we examined the levels of mRNA and protein of the NE-induced genes in healthy adults with high serum levels of NE (>150 pg/mL) compared to low levels (<150 pg/mL).

Results

We found that memory (Tcm and Tem) CD8 T cells expressed a significantly higher level of ADRB2 compared to naïve cells. Consequently, memory CD8 T cells were significantly more sensitive than naïve cells to NE induced changes in gene expressions *in vitro*. Global gene expression analysis revealed that NE induced an elevated expression of inflammatory cytokines and chemokines in resting and activated memory CD8 T cells in addition to a reduced expression of growth-related cytokines. The effects of NE on memory CD8 T cells were primarily mediated by

ADRB2 as confirmed by the adrenergic receptor agonist and antagonist assays. Finally, individuals with high serum levels of NE had similar elevated gene expressions observed *in vitro* compared to the low NE group.

Conclusions

Our results demonstrate that NE preferentially modulates the functions of memory CD8 T cells by inducing inflammatory cytokine production and reducing activation-induced memory CD8 T cell expansion.

Keywords: norepinephrine; CD8 T cells; stress; inflammation

1. Introduction

A growing body of evidence indicates that the sympathetic nervous system (SNS) modulates functions of the immune system (Elenkov *et al.*, 2000;Kin *et al.*, 2006;Sanders *et al.*, 2002). This nervous-immune communication is illustrated during a stress response as the SNS releases catecholamines, such as norepinephrine (NE), which interact with surface receptors on lymphocytes and modulate their functions (Khan *et al.*, 1986;Kohm *et al.*, 2000;Sanders, 2012). Chronic stress has detrimental effects on the immune system and to some degree resembles the immune changes seen in aging (Gouin *et al.*, 2008;Hu *et al.*, 2014;Kiecolt-Glaser *et al.*, 1996). NE is the primary catecholamine released by the SNS and has been previously found to significantly impact lymphocytes, including T cells, natural killer (NK) cells and B cells (Kin *et al.*, 2006;Kohm *et al.*, 2001;Lang, Drell, Niggemann, Zanker, and Entschladen, 2003;Sanders *et al.*, 2002;Wahle, Hanefeld, Brunn, Straub, Wagner, Krause, Hantzschel, and Baerwald, 2006). The effect of NE on immune cells appears complex as NE can have either a stimulatory or inhibitory effect depending on the type of immune cell, activation status of the cell, duration of exposure, and dosage (Kalinichenko, Mokyr, Graf, Jr., Cohen, and Chambers, 1999;Kin *et al.*, 2006;Kohm *et al.*, 2001;Levite, 2000). For example, NE simulates the migratory activity of naïve CD8 T cells but inhibits the migration of activated CD8 T cells (Strell, Sievers, Bastian, Lang, Niggemann, Zanker, and Entschladen, 2009). Furthermore, NE exposure reduces IL-2 production and upregulates chemokine receptors such as CXCR1 and CXCR2 during CD8 T cell activation (Strell *et al.*, 2009).

CD8 T cells are a heterogeneous population consisting of naïve (Tn) cells and two major memory cell populations: central memory (Tcm), which provide a memory reservoir for the rapid response to stimulation, and effector memory (Tem), which provide immediate effector functions and protective immunity (Sallusto, Geginat, and Lanzavecchia, 2004). It is unknown if the effect of NE is similar or different across the CD8 T cell subsets (Tn, Tcm and Tem), which may have in different implications on one's overall immune function.

NE binds to the adrenergic receptors expressed on the surface of a variety of immune cells. The beta-2 adrenergic receptor (ADRB2) is believed to be the primary receptor on T and B cells through which NE directly modulates cellular activity (Padgett *et al.*, 2003;Sanders, 2012;Sanders *et al.*, 2003;Sanders *et al.*, 2002). Signaling through the ADRB2 on lymphocytes is one way the nervous system regulates the immune system (Nakai, Hayano, Furuta, Noda, and Suzuki, 2014). Previous studies have shown the heterogeneous expression of the ADRB2 within peripheral blood mononuclear cells (PBMCs) (Anstead, Hunt, Carlson, and Burki, 1998;Khan *et al.*, 1986;Van Tits *et al.*, 1990), as well as the expression of the ADRB2 on T_{h1} cells but not on T_{h2} CD4 T cells (Kohm *et al.*, 2001;Ramer-Quinn *et al.*, 1997;Sanders, 2012). However, it is unknown whether the expression of the ADRB2 is also heterogeneous within CD8 T cell subsets (Tn, Tcm, and Tem), or if NE signals mainly through the ADRB2 on CD8 T cells.

To determine whether NE has a similar or different effect on CD8 T cell subsets and to identify the specific changes that occur in CD8 T cells, we compared the ADRB2 expression on CD8 T cell subsets (Tn, Tcm and Tem cells) of healthy human donors. In addition, we examined the consequences of NE exposure on isolated CD8 T cell subsets' gene expressions and corresponding protein levels, as well as functional changes. To verify these changes were a result of NE binding with the ADRB2, we isolated ADRB2 positive and negative memory CD8 T cells to examine the impact of NE on cytokine gene expression. We also utilized adrenergic receptor agonist and antagonists to determine if the NE effect on gene expression changes in memory CD8 T cells are mediated by ADRB2. Finally, we examined this phenomenon in adults with either high or low levels of NE in their serum by measuring changes in gene expressions and intracellular cytokines of memory CD8 T cells.

2. Materials and Methods

2.1 Human subjects and blood collection

Peripheral blood was collected at the clinic of the National Institute on Aging (NIA), National Institutes of Health (NIH) from healthy adult donors (N=63) (Supplementary Table 1). PBMCs were isolated from the blood and used for the experiment immediately or stored at -80°C for later experimental use. To examine potential differences in individuals with either high or low levels of NE, we utilized frozen PBMCs from an investigation at the NIH Clinical Center examining physiological changes in family caregivers compared to age-, gender- and ethnicity-matched normal volunteers (N=32) (Supplementary Table 2) under an Internal Review Board approved protocol at the NIH.

2.2 Flow cytometry analysis

For the ADRB2 cell surface staining, freshly isolated PBMCs were incubated with either an unlabeled monoclonal antibody against ADRB2 (Abnova) followed by a secondary goat anti-mouse IgG conjugated with FITC, or by using the same anti-ADRB2 mAb by custom conjugation with PerCP/Cy5.5 using a kit (Lightning-Link, Innova Biosciences) according to the manufacturer's instructions. When the secondary goat anti-mouse IgG was used, a non-specific mouse IgG was used as a control. Other antibodies used for flow cytometry staining included CD8a (APC), CD45RA (PE) and CD62L (FITC) (Biolegend). Samples were analyzed on the BD Accuri™ C6 Flow Cytometer. An example of the flow cytometry staining and gating strategy can be found in Supplementary Fig. 1.

For intracellular cytokine staining, frozen PBMCs were thawed and incubated for 3 hours in an incubator containing 5% O_2. Next, PMA (50ng/mL, Sigma), Ionomycin (80ng/mL, EMD), and a Golgi Blocker (Monensin, 1 µg/million cells, BD Biosciences) were added to the cells and incubated for 4 hours. Cells were stained with a viability dye (e506) and antibodies, including CD8 (PeCy7), CD4 (Pacific Blue), CD3 (ApcCy7), CD28 (APC), CD45RA (FITC) from Biolegend and ADRB2 (PerCP/Cy5.5) from Abnova. Cells were washed and then 0.5 ml of fixation buffer (Biolegend) was added for overnight incubation at 4°C in the dark. The next day, cells were

washed with 1 ml permealization buffer (Biolegend). Intracellular staining for IL-1A (PE) and TNF (PerCP/Cy5.5) from Biolegend were completed in one tube, and IL-2 (PerCP/Cy5.5, Biolegend) and CCL-2 (PE, eBioscience) in a separate tube. Isotype and fluorescent dye matched non-specific mouse IgG were used as controls for cytokine staining. Samples were collected on the Canto II Flow Cytometer (BD Biosciences). Data and mean fluorescent intensity (MFI) were further analyzed using FloJo V10 software.

We used an antibody against Annexin V and a DNA binding dye, 7-AAD (Biolegend), staining to assess cell death and apoptosis at 24 and 72 hours after activation. Samples were analyzed on the BD Accuri^{TM} C6 Flow Cytometer, as previously described.

2.3 Isolation and culture of human CD8 T cell subsets from adults with NE

The procedure for isolating naïve and memory CD8 T cells was described previously (Araki *et al.*, 2009). Briefly, PBMCs were isolated by the Ficoll (GE Healthcare) gradient centrifugation. Enrichment of CD8 T cells (naïve and memory) was followed by a negative immunomagnetic separation process. Briefly, the removal of other cell types in PBMCs through incubation with a panel of mouse mAbs including: CD4, CD11b, CD19, CD14, CD16, MHC class II, erythrocytes, platelets, and CD45RO (for naïve cell enrichment) or CD45RA (for memory cell enrichment). The antibody bound cells were subsequently removed by the anti-mouse IgG magnetic beads (BioMag Goat Anti-Mouse IgG beads, Qiagen). Isolated CD8 T cells were incubated in the presence or absence of $1x10^{-6}$ M NE (catalog #A7256, Sigma-Aldrich), which was dissolved in PBS before being immediately added into human culture media (RPMI1640 with 10% FBS and penicillin (10 U/ml)/streptomycin (10 µg/ml)) (Life Technologies) for 16 hours. A NE concentration of $1x10^{-6}$ M is considered physiologically relevant (Sanders, 2012;Strell *et al.*, 2009;Torres *et al.*, 2005;Wahle *et al.*, 2006). The next day, NE-treated or untreated CD8 T cells were further sorted into naïve ($CD45RA^+$, $CD62L^+$), central memory cells ($CD45RA^-$, $CD62L^+$) and effector memory cells ($CD62L^-$, $CD45RA^-$) by a cell sorter (MoFlo, Dako Cytomation). The purity of sorted naïve

and memory CD8 T cells was >96%. Isolated CD8 T cell subsets were either used for gene expression analysis immediately or incubated at 5% O_2, with anti-CD3/28-coupled beads at a cell:bead ratio of 1:1 in human culture media, and harvested at the indicated time for analyses of mRNA. In addition, the culture supernatant was collected for cytokine protein analysis.
For isolation of naïve and memory CD8 T cells from cryopreserved PBMCs of human adults, PBMCs were thawed in a 37°C water bath, washed and resuspended in RPMI. Naïve and memory (Tcm+Tem) CD8 T cells were isolated by a cell sorter (MoFlo, Dako Cytomation) using the following staining: Viability dye (e506), $CD8^+$, Naive ($CD45RA^+$, $CD28^+$), and memory ($CD28^+$ $^-$). The purity of sorted naïve and memory CD8 T cells was >96%.

2.4 Gene expression analysis by microarray

Genome-wide gene expression analysis was performed on NE-treated and untreated CD8 Tcm cells before and after anti-CD3/CD28 beads stimulation (24 and 72 hours) using Agilent's whole genome array (SurePrint G3 Human GE 8x60K V2 Microarray Kit, Agilent Technologies). Three biological repeated samples were used for the resting and 24 hour time points, and two biological repeated samples for 72 hours were used. Each sample consisted of pooled RNA from 3 different donors, based on the manufacturer's instruction. Briefly, RNA was isolated from cells (RNeasy Mini Kit, Qiagen) and the integrity and quality of the RNA was tested using the Bioanalyzer Chip (Agilent).

Next, a two-color microarray-based gene expression analysis of ~60,000 genes/transcripts was conducted according to the manufacturer's instructions. We started with 100 ng of total RNA of the sample and human universal reference RNA for labeling using the Low Input Quick Amp Labeling Kit based on the manufacturer's instructions. The labeled probes were quantified by NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (G4851B, Agilent). Samples were hybridized onto whole human genome 8x60K array slides for 17 hours at 65°C in a rotator oven,

followed by washing with appropriate Wash Buffers (Agilent). Hybridization signals were extracted via the Agilent Feature Extraction Software.

Two-color microarray data was first extracted from the Agilent reader, and was log-normalized relative to the reference color. Data was batch normalized and significant outliers were filtered using custom 'perl' scripts. Determination of the most significantly different gene ontology (GO) groups was done through Broad Institute's Gene Set Enrichment Analysis (GSEA) tool (Mootha, Lindgren, Eriksson, Subramanian, Sihag, Lehar, Puigserver, Carlsson, Ridderstrale, Laurila, Houstis, Daly, Patterson, Mesirov, Golub, Tamayo, Spiegelman, Lander, Hirschhorn, Altshuler, and Groop, 2003;Subramanian, Tamayo, Mootha, Mukherjee, Ebert, Gillette, Paulovich, Pomeroy, Golub, Lander, and Mesirov, 2005). We utilized the Biological Processes sub-category of the GO groups provided by Broad Institutes' Molecular Signature Database. Groups were deemed significant at a FDR q-value of 0.25 or below. Genes of interest for each group were extracted based on the core enrichment value provided by GSEA. The pre-activation time point was studied alone; however, the post-activation 24-hour and 72-hour time points were combined into a single group during GSEA analysis. The entire microarray data set was deposited in the GEO database (GSE64635).

2.5 Quantitative RT-PCR of mRNA of human donors

The procedure for real time quantitative PCR (RT-qPCR) was described previously (Araki *et al.*, 2009). Briefly, RNA was isolated using the RNeasy Mini Kit with Qiacube (Qiagen) and cDNA was synthesized with oligo-dT and random hexamers (Life Technologies) with 60ng of RNA. Primer sequences can be found in the Supplementary material (Supplementary Table 3). The mRNA levels were determined by quantitative RT-PCR using 2x SYBR Green PCR Master Mix (Fisher Scientific) and normalized to a lymphocyte housekeeping gene, acyl-Coenzyme A oxidase 1 (ACOX1), as described previously (Araki *et al.*, 2009). PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems).

2.6 Measurement of cytokine protein by ELISA

Culture supernatants from CD8 T cell subsets were collected before, 24 hours, and 72 hours after anti-CD3/CD28 stimulation. The amount of cytokine proteins was determined by an ELISA kit (ELISA Max Deluxe Set Human: IL-2, IL-6, MCP-1, IL-1A, TNF, IFN-γ, Biolegend) according to the manufacturer's instructions. Concentrations were calculated according to the standard, and normalized to the number of cells among different samples.

2.7 Assay for Adrenergic Receptor agonist and antagonists

To verify if the ADRB2 is the primary receptor for NE on memory CD8 T cells, we isolated $ADRB2^+$ and $ADRB2^-$ memory CD8 T cells by a cell sorter using anti-ADRB2 mAb by custom conjugation with PerCP/Cy5.5, as described in the above section. We also isolated memory CD8 T cells via a negative immunomagnetic separation described in Methods 2.3 for testing the ADRB2 agonist and antagonists. The purity of isolated memory CD8 T cells was ~82% (no detectable naïve CD8 or CD4 T cells) and had comparable changes in cytokine expression by NE and its agonist and antagonists when memory CD8 T cells were isolated by a cell sorter (data not shown).

To further determine that ADRB2 is the primary receptor for NE, we added Terbutaline (ADRB2 agonist, catalog #T2528, Sigma-Aldrich) at a concentration of 10^{-5} M and incubated for 16 hours before activation. For the antagonist experiments, Nadolol (beta-adrenergic receptor antagonist (ADRB), catalog #N1892, Sigma-Aldrich) or Phentalomine (alpha-adrenergic receptor (ADRA) antagonist, catalog #P7547 Sigma-Aldrich) were added at 10^{-5} M at the same time as NE. We did serial dilutions (10^{-3}, 10^{-5} and 10^{-8} M) of the agonist and antagonists based on previous reports and found 10^{-5} M to be the most effective dose after titrating the agonist and antagonists (data not shown). The treated CD8 T cells were stimulated with CD3/CD28 antibodies. Treated cells were

harvested and the culture supernatants were collected before and 24 hours after activation for RT-qPCR and ELISA analyses, respectively.

2.8 NE measurements in serum

NE was measured in the serum of all donors as described previously (Eisenhofer, Goldstein, Stull, Keiser, Sunderland, Murphy, and Kopin, 1986). Briefly, peripheral blood was collected after a 15 minute rest period and placed on ice until processed and stored at -80°C (Eisenhofer *et al.*, 1986). When thawed, NE was measured using standard high-performance liquid chromatography (HPLC) with electrochemical detection that has been previously established for simultaneous measurement of the concentrations of catecholamines; quantification of NE was detected with a triple-electrode system. The designation of 150 pg/mL for the high or low NE level in serum was reached by averaging the 3 time points donors had their NE levels measured during a 3 month timeframe and finding the approximate median among the donors. NE levels did not differ significantly between time points in the donors. Utilization of these samples and the primary investigation where the samples originated was approved by the Internal Review Board at the National Heart Lung and Blood Institute (NHLBI), NIH and the University of Pennsylvania.

2.9 Statistics

Data are expressed as the mean ± SEM and significance was assessed using the paired Student's t-test. $p < 0.05$ was considered significant.

3. Results

3.1 ADRB2 is highly expressed in the memory subsets compared to the naïve subset of CD8 T cells

Previous studies have found that ADRB2 is heterogeneously expressed on different immune cells (Anstead *et al.*, 1998;Kohm *et al.*, 2001;Sanders, 2012), yet the expression of ADRB2 on CD8 T cell subsets is not known. We first assessed the surface expression of ADRB2 by flow cytometry on the 3 subsets of CD8 T cells (Tn, Tcm and Tem) in the PBMCs of healthy human adults (**Fig**. 1A). We found that the memory populations (Tcm and Tem) of CD8 T cells expressed a significantly higher percentage (~40%) of ADRB2 compared to the Tn population (~10%) (**Fig**. 1B). Furthermore, memory CD8 T cells also expressed significantly more ADRB2 on average compared to Tn cells as measured by MFI with flow cytometry (**Fig**. 1C). To further determine if ADRB2 expression is regulated by transcription, we assessed the mRNA level of *ADRB2* in Tn, Tcm and Tem and found greater expression (0.61 fold higher) in memory CD8 T cells (Tcm and Tem) compared to Tn cells (**Fig**. 1D). Together, our findings show that ADRB2 is highly expressed in memory CD8 T cell populations compared to the Tn population.

3.2 NE induces expression of inflammatory cytokines and chemokines in memory CD8 cells

The effect of NE on the expression of several cytokines in CD8 T cells has been reported (Kalinichenko *et al.*, 1999;Sanders, 2012;Strell *et al.*, 2009), but a genome-scale assessment of NE-induced changes in CD8 T cells has not been conducted. We focused on Tcm cells because of their critical role in the recall response for adaptive immunity and their high level of ADRB2 expression. To determine the overall impact of NE on human CD8 T cells, we conducted a genome-wide analysis of gene expression changes in CD8 Tcm cells after NE exposure using a microarray. CD8 Tcm cells were isolated from healthy adults and treated with NE for 16 hours before stimulation with antibodies against CD3 and CD28 (anti-CD3/CD28), and harvested before

and 24, 72 hours after stimulation for gene expression analyses. Genes with the greatest degree of changes after NE treatment were identified (Supplemental Table 4).

A functional assessment using GSEA of altered gene expressions by NE treatment revealed important biological and immunological functions, including regulation of cell differentiation, cell cycle process and MAPK activity (**Fig**. 2A). Among the NE induced genes that were identified based on GSEA and fold changes, we focused on the inflammatory cytokines and relied on RT-qPCR method to confirm and extend our analysis to other inflammatory cytokines in all CD8 T cell subsets (Tn, Tcm and Tem). We found that Tcm and Tem exhibited a similar upregulation of *IL1A* and *IL6,* while Tn cells did not show a significant difference in expression between NE treated and untreated cells (**Fig**. 2B). Both *IL1A* and *IL6* have multiple, important functions in inflammation (Ershler and Keller, 2000). In addition, several chemokines related to the inflammatory and chemoattraction processes were also upregulated in the NE treated cells, including *CXCL1, CXCL2, CXCL3* and *CCL2,* as determined by the RT-qPCR method (**Fig**. 2C). Next, we assessed whether the NE induced changes observed at the mRNA level correlate with the protein level. We then measured protein levels of selected cytokines and chemokines in the culture supernatant of the memory CD8 T cells by ELISA. Since NE treated Tn cells did not show any significant gene expression changes, we did not further investigate this population. A similar increase in the protein levels of IL-1A and CCL-2, but not IL-6 were observed (**Fig**. 2D). Together, these results demonstrate that memory CD8 T cells were more susceptible to the effects of NE than the naïve CD8 T cell subset, and suggest that NE exposure induces a pro-inflammatory state in memory CD8 T cells.

3.3 Activation induces greater expression of inflammatory cytokines and chemokines in NE treated memory CD8 cells

We next asked what impact NE would have on memory CD8 T cells in response to activation and again found several cytokines and chemokines significantly upregulated (top 100 most altered

genes after activation are identified in Supplemental Table 5). Using GSEA, we identified the altered biological and immunological functions in NE-treated Tcm CD8 cells (**Fig**. 3A). We again focused on the inflammatory cytokines and relied on RT-qPCR method to confirm and extend our analyses to other inflammatory cytokines in memory CD8 T cells (Tcm and Tem).

Among the altered expressed genes, *IL6, CXCL2, CXCL3,* and *CCL2* were upregulated with NE treatment before activation and remained upregulated after activation compared to controls (**Fig**. 3B). Furthermore, two pro-inflammatory cytokines (*TNF* and *IL36G*) and a chemokine (*CCL8*) were upregulated in NE treated memory CD8 T cells only after activation (**Fig**. 3C). We examined *TNF* specifically since it is a well-known pro-inflammatory cytokine involved in inflammatory-related diseases. *CCL8*, a chemokine involved in several immune-regulatory and inflammatory processes, exhibited a similar enhanced expression pattern to *TNF*, whereas *IL36G*, participating in local inflammatory responses, showed an increased enhancement of expression from 24 to 72 hours compared to the control (**Fig**. 3C).

Protein levels of TNF, IL-6 and CCL-2 in the culture supernatant correlated with the gene expression changes at 24 and 72 hours after activation (**Fig**. 3D). We observed an average four-fold increase in the protein level of TNF compared to a two-fold difference in IL-6 and CCL-2. In 5 of the 6 cases, the most significant difference in protein levels between untreated and treated memory CD8 T cells was observed at 24 hours.

3.4 Activation induces lower expression of growth-related genes in NE treated memory CD8 T cells and consequently reduces activation-induced expansion of CD8 T cells

In contrast to the enhanced expression of inflammatory cytokines in NE-treated memory CD8 T cells before and after stimulation, we also identified two growth-related cytokines (IL2 and IFNG) whose expression levels were reduced in NE-treated memory but not naïve CD8 T cells after stimulation. *IL2* met both criteria of the altered gene expression by the microarray and RT-qPCR; however, *IFNG* did not meet the criteria of our microarray, but was confirmed by RT-qPCR

as significantly altered with NE treatment (**Fig**. 4A). IL2 is an important growth factor and IFNG has been previously shown to promote the growth of memory T cells (Asao and Fu, 2000;Kryczek, Wei, Gong, Shu, Szeliga, Vatan, Chen, Wang, and Zou, 2008;Zhang, Sun, Hwang, Tough, and Sprent, 1998). The reduced expressions of IL2 and IFNG were further confirmed at the protein levels in the culture supernatant of stimulated memory CD8 T cells (**Fig**. 4B). These findings suggest that NE has an immunosuppressive effect on memory CD8 T cells by significantly down-regulating cytokines critical for the proliferation of CD8 T cells.

To determine the impact of the reduced expression of growth-related cytokines such as IL-2 and IFN-γ, we assessed the activation-induced expansion of memory CD8 T cells and observed a significant reduction in the cell number of NE treated CD8 Tcm cells 24 hours after activation compared to the NE untreated CD8 Tcm cells (**Fig**. 4C). To rule out the potential role of cell death in the differences in activation-induced expansion, we analyzed cell viability and found no significant difference in the viability of Tcm cells between NE treated and untreated Tcm cells at baseline, 24 or 72 hours after activation (**Fig**. 4D).

3.5 NE effect on memory CD8 T cells is mediated by ADRB2

NE can bind to other adrenergic receptors aside from the ADRB2 (Ramer-Quinn *et al.*, 1997). To determine if ADRB2 was responsible for the observed NE effects on memory CD8 T cells, we isolated $ADRB2^{+}$ and $ADRB2^{-}$ CD8 memory T cells with a cell sorter. The effects of NE on the expression of *IL1A* and *IL6* (before activation), and *IL2* and *TNF* (after 24 hour activation) were significantly greater in the $ADRB2^{+}$ than in the $ADRB2^{-}$ memory CD8 T cells (**Fig**. 5A). To further determine whether the effects of NE were through ADRB2 but not ADRB1, we treated resting memory CD8 T cells with an ADRB2 agonist, Terbutaline, and found it induced an indistinguishable level of changes in mRNA and protein levels of IL1A and IL6 compared to the NE treated memory CD8 T cells (**Fig**. 5B and 5C). We also determined if NE stimulates the ADRB2 specifically by exposing memory CD8 T cells to NE in the presence or absence of either

the beta-adrenergic receptor (ADRB) antagonist (Nadolol) or the alpha adrenergic receptor (ADRA) antagonist (Phentalomine). Blocking ADRB with Nadolol abolished the NE effect on IL1A and IL6 but blocking ADRA with Phentalomine did not have an obvious impact on NE's effect on IL1A and IL6 expression (**Fig.** 5B and C), suggesting NE's effect on memory CD8 T cells is primary mediated by ADRB2, but not by ADRA. We extended the antagonist analysis to activation-induced changes in gene expression affected by NE and observed a similar response: Terbutaline mimicked NE's effect; Nadolol blocked NE's effect, and Phentalomine did not block NE's effect on IL2 and TNF expression (**Fig**. 5D and E).

3.6 High blood NE levels are associated with enhanced expression of inflammatory cytokines in memory CD8 T cells

Our *in vitro* experiments illustrate a previously unknown effect of NE inducing greater expression of inflammatory cytokines in memory CD8 T cells. We next asked if these findings would exist in an *in vivo* model by analyzing the relationship of blood NE levels and inflammatory cytokine expressions in memory CD8 T cells in 32 adults participating in a primary investigation examining the effects of chronic stress on health outcomes. Subjects who participated in this study had their serum NE level measured at each visit with a total of 3 measurements over approximately 3 months. Based on the approximate median of the blood NE levels, we grouped subjects into two groups: high (>150 pg/mL) and low NE levels (<150 pg/mL) (**Fig**. 6A). We isolated memory and naïve CD8 T cells from frozen PBMCs of these subjects by a cell sorter and analyzed the expression of inflammatory cytokines identified from our *in vitro* work with RT-qPCR and flow cytometry.

We found that *IL1A* and *TNF* were significantly higher in memory CD8 T cells of the high NE group than the low NE group, whereas *IL2* was significantly lower in memory CD8 T cells of the high NE group compared to the low NE group (**Fig**. 6B). We then measured the protein levels of these 3 cytokines via intracellular staining with flow cytometry. We found adults in the high NE

group had significantly higher percentages of memory CD8 T cells expressing IL-1A and TNF, but a significantly lower percentage of memory CD8 T cells expressing IL-2 (**Fig**. 6C).

4. Discussion

Here we conducted a comprehensive assessment of how NE modulates CD8 T cell subsets by examining ADRB2 expression, transcriptional and protein level alterations, and activation-induced expansion in both an *in vitro* condition using a physiological relevant concentration of NE, and in an *in vivo* setting using a study cohort with known serum NE levels. Our results demonstrate that ADRB2 is highly expressed in memory CD8 T cells, revealing the preferential effect of NE on memory CD8 T cells. Furthermore, we found that NE induces inflammatory cytokine production while simultaneously reducing production of growth-related cytokines, leading to a reduced activation-induced expansion of memory CD8 T cells. Ultimately, this indicates a two-sided effect of NE on memory CD8 T cells. Finally, we show that a high serum concentration of NE is associated with a high expression of inflammatory cytokines and a low expression of growth-related cytokines in the memory CD8 T cells of stressed adults. NE's preferential impact on memory CD8 T cells may help improve our understanding of the mechanisms of NE in chronic stress-associated immune-related disorders such as viral and bacterial infections (Farias, Teixeira, Moreira, Oliveira, and Pereira, 2011;Kemeny *et al.*, 2007).

ADRB2 is known to be expressed differently in different type of cells within PBMCs and believed to be the main receptor for NE (Sanders, 2012). Our study extends these findings by showing ADRB2 is differentially expressed on naïve and memory CD8 T cells. Fewer naïve CD8 T cells express ADRB2, but significantly more antigen-experienced memory CD8 T cells express ADRB2. Furthermore, ADRB2 is significantly more abundant on memory than naïve CD8 T cells. Previous studies also show that ADRB2 plays a critical role in regulating the immune function of lymphocytes (Anstead *et al.*, 1998;Bonneau *et al.*, 1990;Khan *et al.*, 1986;Mills *et al.*, 2004;Mills, Ziegler, Patterson, Dimsdale, Hauger, Irwin, and Grant, 1997). Here, we demonstrate memory

CD8 T cells expressing ADRB2 respond to NE, but memory CD8 T cells that are ADRB2 negative do not respond to NE. The effect of NE on memory CD8 T cells is primarily through ADRB2, as supported by the results from the receptor agonist and antagonists assays. The ADRB2 agonist was able to mimic the changes induced by NE at the mRNA and protein level. The alpha antagonist showed no effect on NE-induced changes in expression of the four cytokines tested, while the beta adrenergic antagonist almost completely blocked NE's effect on these cytokines. Our findings suggest that ADRB2 is the primary receptor for NE, and ADRA does not play a measurable role in NE-induced memory CD8 T cell changes. Our findings demonstrate for the first time that ADRB2 expression in CD8 T cells is differentially regulated and the impact of NE is preferentially on memory CD8 T cells. Further studies need to be conducted to provide further insight into the mechanisms behind altered cytokine production in memory CD8 T cells following NE binding to the ADRB2.

Chronic stress and inflammation is intricately linked via a network of interactions mediated by neurotransmitters and hormones (Carlson, Brooks, and Roszman, 1989;Levite, 2000;Straub, Westermann, Scholmerich, and Falk, 1998). NE is also implicated in the inflammatory response through a variety of pathways, one of them being the regulation of IFN-γ production in immune cells (Dhabhar *et al.*, 2012;Dimsdale, Mills, Patterson, Ziegler, and Dillon, 1994;Mausbach, Dimsdale, Ziegler, Mills, Ancoli-Israel, Patterson, and Grant, 2005;Sperner-Unterweger *et al.*, 2014). However, the global scope and the type of T cell subsets that respond to NE have not been previously addressed. Our global gene expression analysis of memory CD8 T cells before and after activation reveals some unexpected and rich findings of NE-induced production of a large panel of inflammatory cytokines by memory CD8 T cells. Interestingly, we found NE induced an inflammatory state in memory CD8 T cells even before the cells were activated. Cytokines genes such as *IL6* and *TNF* play an important role in the pro-inflammatory response as well as a variety of other immune responses. Chemokines discussed such as *CCL2, CXCL1* and *CXCL3* also mediate inflammatory responses as well as having important chemotactic activity for lymphocyte and monocyte migration. After activation, some of the same

inflammatory cytokines and chemokines were still significantly increased as well as some new inflammatory markers.

Previous studies have shown the inhibitory impact of NE on immune cells (Kalinichenko *et al.*, 1999;Kohm *et al.*, 2000;Kohm *et al.*, 2001;Lang *et al.*, 2003;Malarkey, Wang, Cheney, Glaser, and Nagaraja, 2002;Ramer-Quinn, Swanson, Lee, and Sanders, 2000;Swanson *et al.*, 2001), including CD8 T cells and the alteration in IFN-□ and IL-2 production (Strell *et al.*, 2009;Torres *et al.*, 2005;Wahle *et al.*, 2006). Our study supports these findings by showing a reduction in the expression of IFN-□ and IL-2 in NE treated memory CD8 T cells. The decrease in IL-2 and IFN-□ production explains, in part, the modest decrease in cell number found in NE treated cells after stimulation, which may play a role in altering the proliferation of NE treated compared to untreated memory CD8 T cells. However, the precise intracellular pathways leading to these transcriptional and protein changes requires further investigation.

Findings from our study of CD8 T cell subsets may help explain the conflicting findings of previous studies looking at the effect of NE on immune cells to be immune suppressive, immune-enhancing, or null (Dhabhar *et al.*, 2012;Kin *et al.*, 2006;Strell *et al.*, 2009). By studying immune cells in PBMCs rather than specific cell types, or even subsets (naïve or memory), the actual effects of NE on a subset of lymphocytes can be masked by the larger group of other types of immune cells. Future studies will benefit from utilizing defined types of immune cells and their subsets to draw out the impact of NE on these cells. Memory CD8 T cell sensitivity to NE may have clinical relevance since memory cells are responsible for recall immune responses as opposed to naïve cells that fight off new challenges. An individual with high levels of NE may be more compromised in terms of fighting off recall infections rather than new antigenic challenges.

Compared to our *in vitro* findings under controlled conditions, *in vivo* changes are likely influenced by multiple factors. It is therefore reassuring to find that some inflammatory-related cytokines and chemokines in adults with high blood levels of NE are also elevated in memory CD8 T cells compared to low blood levels of NE; these findings are similar to the inflammatory response we observed *in vitro*. We also examined other cytokines and chemokines (*CCL2, IFNG,*

IL6, CXCL1, and *CCL8*) but did not find significant differences between the high and low groups. There was also no difference in ADRB2 expression between the high or low NE groups in lymphocytes, Tn, or Tm cells (Supplementary Fig. 2). It is important to note that the effects observe *in vivo* could be a result of a combined influence from hormones and neurotransmitters including NE directly or indirectly (Straub *et al.*, 2000;Straub *et al.*, 1998). Nevertheless, NE appears to play an important role in modulating CD8 T cell function, particularly in memory cells. The impact of chronic stress on immune function is undoubtedly complex. More work needs to be done to better understand the impact of NE on CD8 T cells as well as on other types of lymphocytes such as CD4 and B cells. Investigating the effect of NE under defined *in vitro* conditions and some suitable *in vivo* settings will help to elucidate the role of NE in modulating immune function. Future work should also focus on the clinical implications of high NE levels, particularly on immune health outcomes as well as interventions to alleviate the effects of stress on the immune system.

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Conflict of Interest

The authors declare no conflicts of interest.

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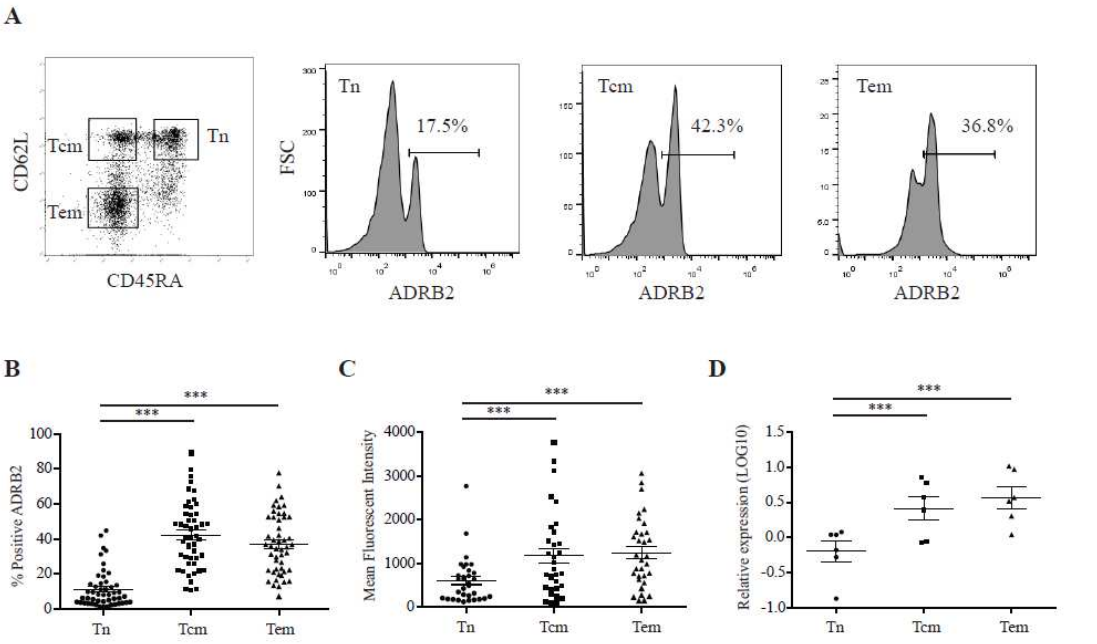


Figure 1. ADRB2 is highly expressed in the memory subsets compared to the naïve CD8 T cells (A) Representative figures of the flow cytometry staining for ADRB2 expression on CD8 T cell subsets. Lymphocytes were gated from the peripheral mononuclear cell (PBMC) sample followed by a $CD8^+$ T cell (APC) gate. CD8 T cell subsets, naïve ($CD45RA^+CD62L^+$), central memory ($CD45RA^-CD62L^+$) and effector memory ($CD45RA^-CD62L^-$) cells were gated for measure of ADRB2 expression. Staining for ADRB2 was described in the Methods. **(B)** ADRB2 expression in individual CD8 T cell subsets. ADRB2 expression is presented as a percentage for each CD8 T cell subset: naïve (Tn), central memory (Tcm) and effector memory (Tem). There was significant difference in ADRB2 percentage of expression between Tn and memory (Tcm and Tem) T cells (N=50, $p<0.001$). **(C)** Mean fluorescent intensity (MFI) of ADRB2 expression in CD8 T cell subsets. The MFI of each CD8 T cell subset was measured to examine ADRB2 on the average of each type of cells. Per subject, memory CD8 T cells expressed significantly more ADRB2 compared to naïve cells (N=50, $p<0.001$). **(D)** ADRB2 expression on the mRNA level of Tn, Tcm and Tem subsets in healthy human adults by RT-qPCR. Data is presented as the relative mRNA expression in the LOG10 value (N=6). Figures throughout this manuscript

illustrated the results with the mean and SEM. Significance is identified as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

C. Slota, Norepinephrine effects on CD8 T cell subsets (2 column fitted image) Fig. 2

A

Functional group	Notable Enriched Genes	NE-effect	NES*	FDR q-value
Regulation of Cell Differentiation	*EREG, IL4, NANOG*	Enhanced	-1.52	0.162
Activation of MAPK Activity	*PROK2, ADRB2, PIK3CB*	Reduced	1.98	<0.001
Cell cycle process	*CDKN3, BUB1B, BRCA2, TIMELESS*	Reduced	1.83	0.078

*NES=Normalized Enrichment Score

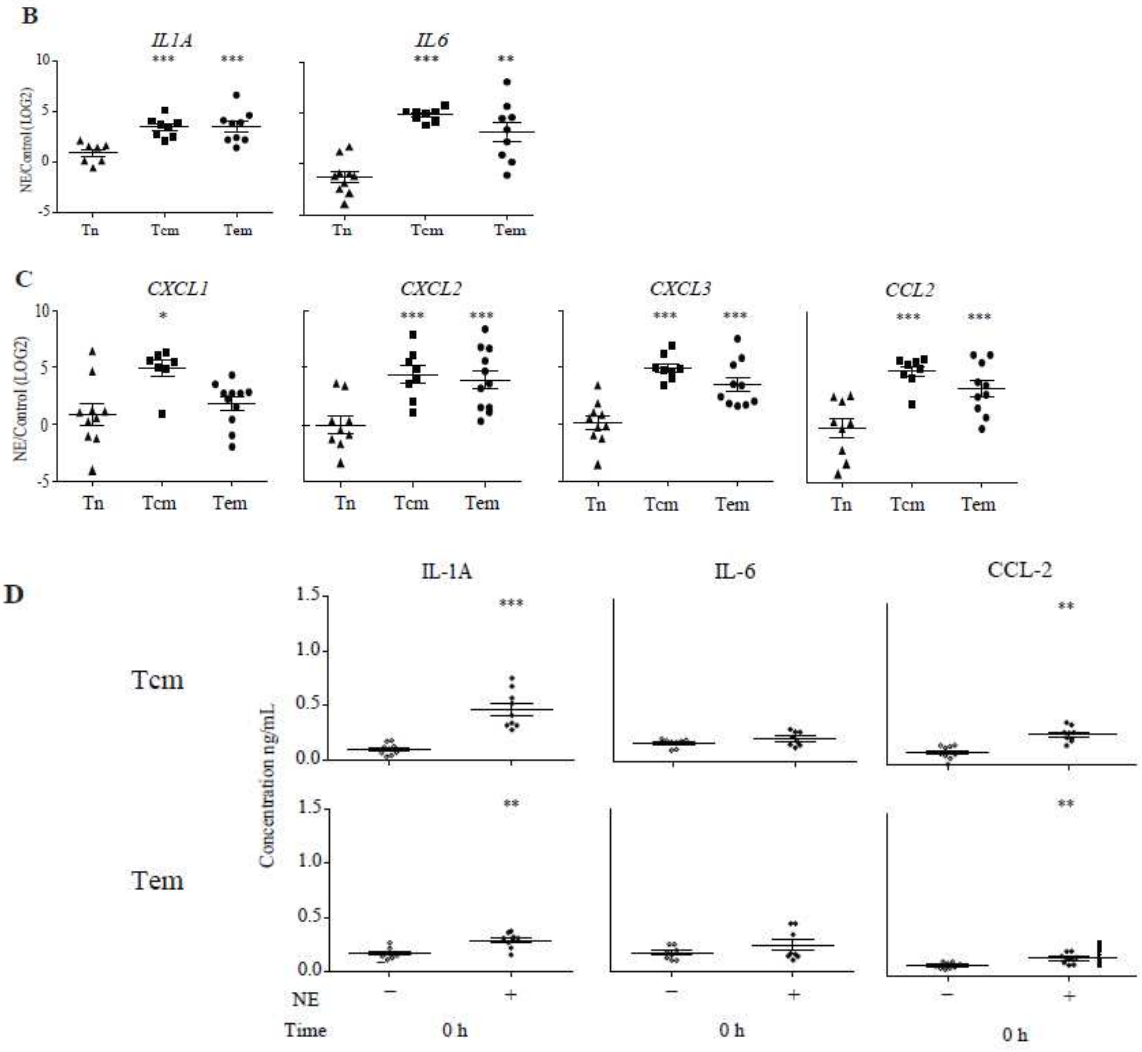


Figure 2. Increased gene expression of inflammatory cytokines in CD8 Tcm cells treated with norepinephrine. (A) Relevant Gene Ontology (GO) groups extracted from GSEA comparison between NE treated and untreated CD8 Tcm cells before activation. These groups had significant

FDR q-values (<0.25), and representative genes were chosen from the set of core enriched genes, also derived from GSEA. (B) and (C) Significantly increased inflammatory cytokines and chemokines (mRNA) after NE treatment. RT-qPCR was done based on the microarray results for the selected cytokine genes significantly increased by NE treatment. Results are presented as a ratio (NE/Control) on the Log_2 scale for the naïve (Tn), central memory (Tcm) and effector memory (Tem) subsets of CD8 T cells (N=7-11). (D) Protein levels of the selected cytokines and chemokines altered by NE treatment examined via ELISA. Results are presented as the concentration (ng/mL) of the cytokine or chemokine in the controls (untreated) and NE treated central memory (Tcm) and effector memory (Tem) cells at 0 hours (N=6-14).

C. Slota, Norepinephrine effects on CD8 T cell subsets (2 column fitted image) Fig. 3

A

Functional group	Notable Enriched Genes	NE-effect	NES*	FDR q-value
Inflammatory and Humoral Response	*CCL22, IL8, CXCL1, CXCL2, CCL2, IL6*	Enhanced	-2.15	<0.001
Positive Regulation of Cytokine Biosynthethic Process	*TLR8, EREG, TLR1*	Enhanced	-1.88	<0.001
Mitosis	*TPX2, MADL2, CDCA5, CCNA2*	Reduced	1.69	0.121
Chromosome segregation	*TOP2A, NEK6, INCENP, BRCA1*	Reduced	1.69	0.091

*NES=Normalized Enrichment Score

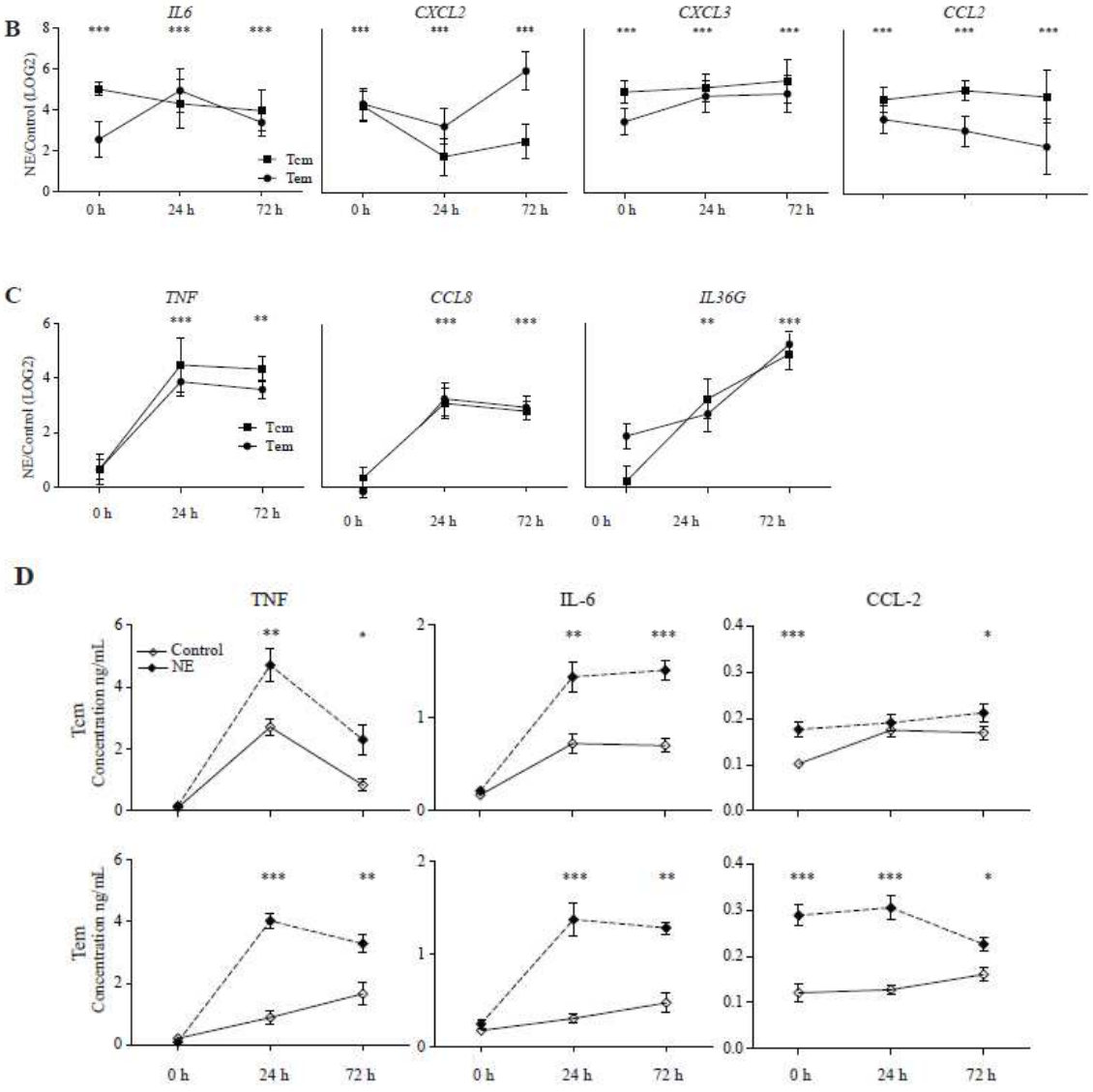


Figure 3. Activation induces greater expression of inflammatory cytokines and chemokines in norepinephrine treated memory CD8T cells. (A) Relevant Gene Ontology (GO) groups extracted

from GSEA comparison between treated and untreated CD8 Tcm cells after activation. These groups had significant FDR q-values (<0.25), and representative genes were chosen from the set of core enriched genes, also derived from GSEA. (B) Significantly increased inflammatory cytokines and chemokines (mRNA) in NE treated CD8 T cells before and activation. Results are presented as a ratio (NE/Control) on a LOG_2 scale for 0, 24, and 72 hours in Tcm and Tem cells (N=6-14). (C) Significantly increased inflammatory cytokines and chemokines (mRNA) in NE treated CD8 T cells only after activation. Data is presented as a ratio (NE/Control) on a LOG_2 scale for 0, 24, 72 hours in Tcm and Tem cells (N=6-14). (D) Protein level expression of selected cytokines and chemokines at 0, 24, 72 hours after activation in untreated (control) and NE treated Tcm and Tem cells. Data is presented as the concentration (ng/mL) (N=6-12).

C. Slota, Norepinephrine effects on CD8 T cell subsets (1 column fitted image) Fig. 4

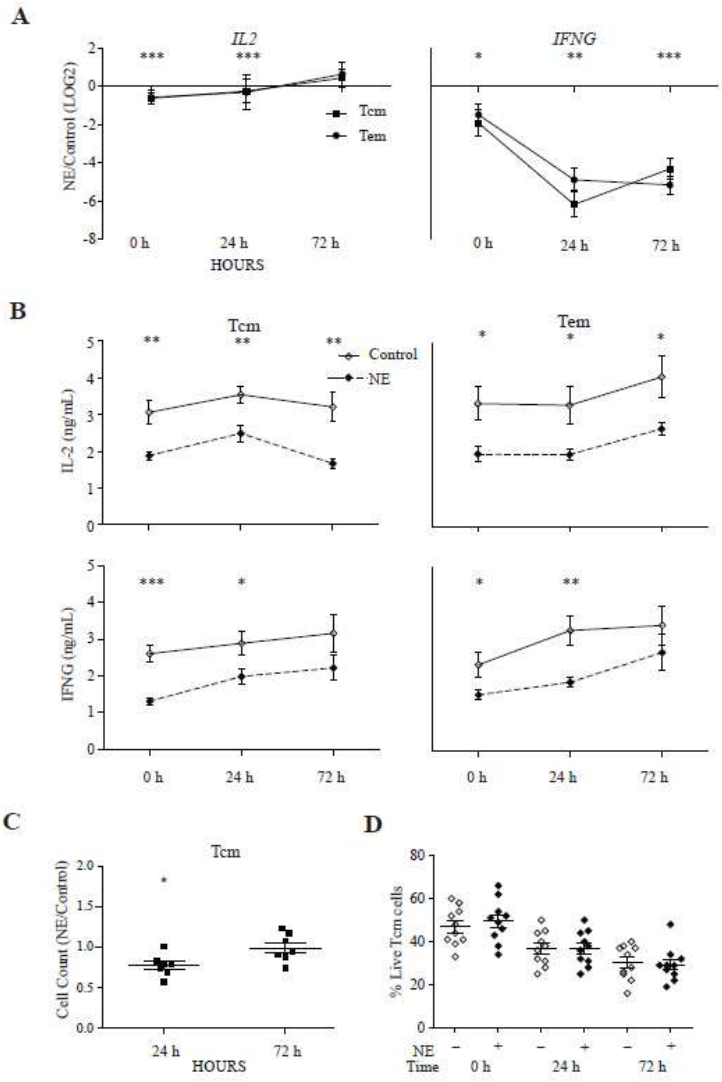
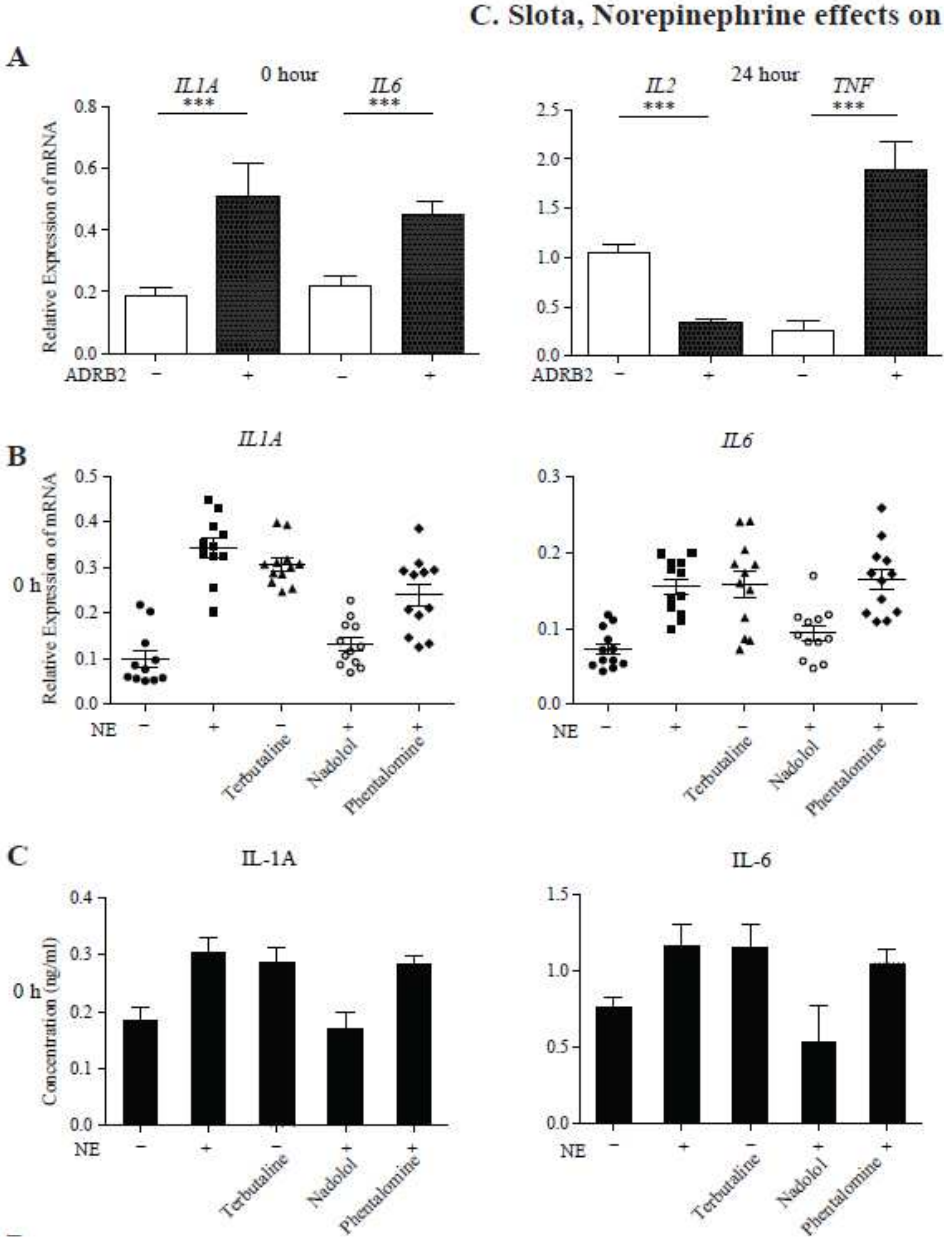


Figure 4. Activation of NE treated memory CD8 T cells led to decreased expression of *IL2* and *IFNG* in the memory subsets resulting in a reduced activation-induced expansion. (A) Decrease in proliferation-related cytokines, *IL2* and *IFNG*, at the mRNA level in memory CD8 T cells. RT-qPCR was used for confirmation of microarray results as well as examining gene expression changes in the naïve (Tn) and effector memory (Tem) subsets. Results are presented as a ratio (NE/Control) in the LOG_2 value from baseline to 24 and 72 hours after activation (N=6-14). (B) Protein levels of memory subsets (Tcm and Tem) as measured by ELISA at baseline, 24 and 72

hours after activation in control and NE treated cells. Data is presented as the concentration (ng/mL) (N=7-12). (C) Cell counts of Tcm cells at 24 and 72 hours after activation. Results are presented as the ratio (NE/Control) at 24 and 72 hours after activation in central memory (Tcm) cells (N=15). (D) Viability of Tcm cells was examined by viability dye (7AAD) and apoptosis dye (Annexin V) at 0, 24 and 72 hours after activation. Data is presented as the percentage of live cells which was determined by the gated Tcm cells followed by $7AAD^-$ and Annexin V^- gated cells in flow cytometry of the NE treated and untreated cells (N=10).


C. Slota, Norepinephrine effects on
A
0 hour
IL1A
IL6
***
***
Relative Expression of mRNA
0.8
0.6
0.4
0.2
0.0
ADRB2 – + – +
24 hour
IL2
TNF
***
***
2.5
2.0
1.5
1.0
0.5
0.0
ADRB2 – + – +
B
IL1A
IL6
0 h
Relative Expression of mRNA
0.5
0.4
0.3
0.2
0.1
0.0
NE – + – + +
Terbutaline
Nadolol
Phentalomine
0.3
0.2
0.1
0.0
NE – + – + +
Terbutaline
Nadolol
Phentalomine
C
IL-1A
IL-6
0 h
Concentration (ng/ml)
0.4
0.3
0.2
0.1
0.0
NE – + – + +
Terbutaline
Nadolol
Phentalomine
1.5
1.0
0.5
0.0
NE – + – + +
Terbutaline
Nadolol
Phentalomine

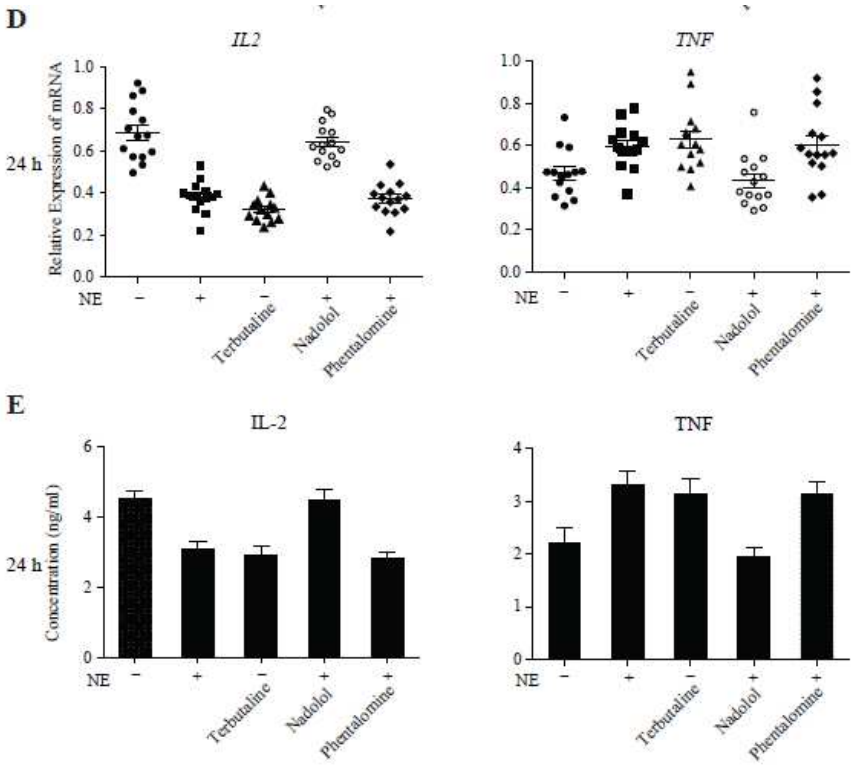


Figure 5. NE action in memory CD8 T cells is mediated by ADRB2. (A) ADRB2^{+} CD8 Tcm cells respond to NE treatment. We isolated ADRB2^{+} and ADRB2^{-} CD8 Tcm cells from the blood of human donors. Relative mRNA levels of cytokines in the ADRB2^{+} cells compared to ADRB2^{-} cells before (*IL1A* and *IL6)* and 24 hours after activation *(IL2* and *TNF*) are presented (N=6). (B) The effect of ADRB2 agonist and antagonists on NE-induced cytokine expression (mRNA) before activation. Memory CD8 T cells were isolated and cultured with NE or an ADRB2 agonist (Terbutaline) alone or NE plus an antagonist (either an ADRB antagonist (Nadolol) or an ADRA antagonist (Phentalomine)). Relative expression changes of *IL1A* and *IL6* under these treatments are presented (N=12). (C) The effect of the adrenergic receptor agonist and antagonists on NE-induced cytokine expression (protein) before activation. Similar changes of IL-1A and IL-6 protein concentration levels (ng/ml) in the culture supernatant under the treatments of the agonist (Terbutaline) and antagonists (Nadolol or Phentalomine) are presented (N=8). (D) The effect of the adrenergic receptor agonist and antagonists on NE induced cytokine expression (mRNA)

after activation. Relative levels of *IL2* and *TNF* mRNA changes after 24 hours of activation under NE alone, Terbutaline alone, NE plus Nadolol, and NE plus Phentalomine are presented (N=12). (E) The effect of the adrenergic receptor agonist and antagonists on NE induced cytokine expression (protein) after activation. IL-2 and TNF protein concentration levels (ng/ml) in the culture supernatant under the treatment of the agonist (Terbutaline) and antagonists (Nadolol or Phentalomine) are presented (N=8).

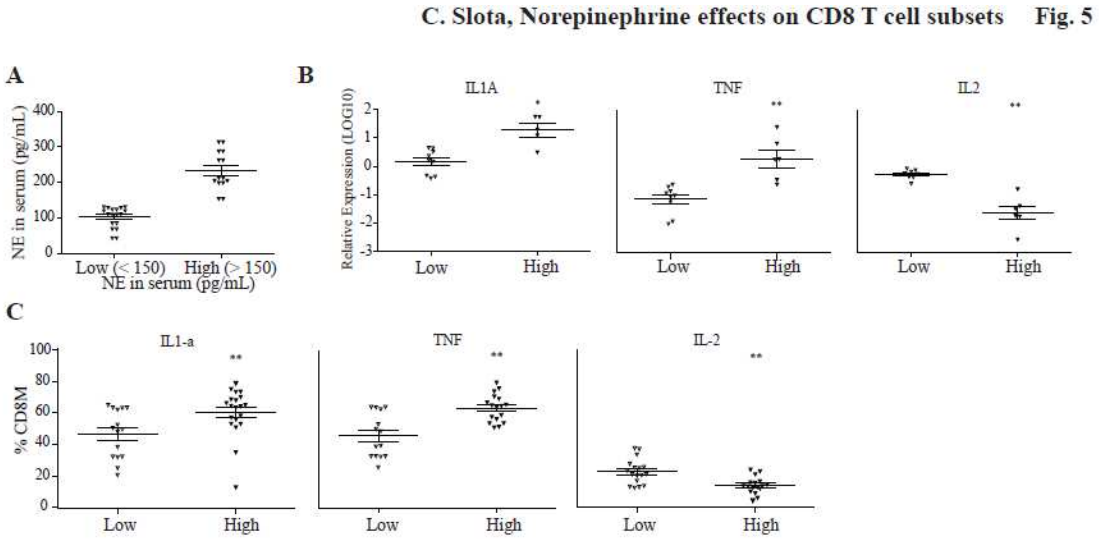


Figure 6. Altered inflammatory cytokines in adults with higher levels of norepinephrine. (A) Blood NE levels in study subjects. Blood NE levels are separated into two groups based on the approximate median: low NE levels (<150 pg/mL) (N=18) and high NE levels (>150 pg/mL) (N=14). Data is shown as NE levels measured in the serum (pg/mL) in the two groups (low and high) in which the following analyses were conducted. **(B)** Gene expression changes between the low NE group and the high NE group examined using RT-qPCR. Data is presented as the LOG_{10} value (N=9 for high and N=6 for the low blood NE group). **(C)** Cytokine protein levels (IL-2, IL1-α, TNF) in memory CD8 T cells measured by intracellular staining between the low and high NE groups. Data is presented as the percentage of cells in each group (N=14-17 for high and N=17-22 for the low NE group).

Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation

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Supplemental materials

Table 1. List of donors for the *in vitro* portion of this study with their age, gender and experimental use.

Table 2. List of donors for the *in vivo* portion of this study with their age, gender, and experimental use.

Table 3. Primer sequences used for RT-qPCR analyses of gene expression.

Table 4. List of top 50 most upregulated and top 50 most down-regulated genes when comparing pre-activated CD8+ Tcm with overnight NE treatment.

Table 5. List of top 50 most upregulated and top 50 most down-regulated genes when comparing activated CD8+ Tcm with overnight NE treatment.

Figure 1. Gating strategy for norepinephrine receptor staining on CD8 T cell subsets.

Figure 2. Beta-2 adrenergic receptor expression in individuals with high and low levels of norepinephrine in their serum.

Supplemental Table 1. List of donors for the *in vitro* portion of this study with their age, gender and experimental use. An 'X' indicates the donor was used for the experiment.

Donor ID	Gender	Age	Experiment				
			ADRB2 Staining	Array	RT-qPCR	Elisa	Agonist/ Antagonist
1	F	22	X				
2	M	23	X				
3	F	25	X		X	X	X
4	M	25	X				X
5	M	25	X				
6	F	27	X				
7	F	28	X	X	X	X	
8	F	28	X				
9	M	29			X	X	
10	F	29	X				
11	M	30	X				
12	F	30	X				
13	F	32			X	X	X
14	F	32		X		X	X
15	M	34	X	X	X	X	
16	F	34	X				
17	M	35		X	X	X	
18	F	35	X		X	X	
19	M	35	X				
20	M	35	X				
21	M	36	X				
22	F	37	X		X		X

23	F	37			X	X	
24	M	38	X	X	X	X	
25	F	39	X		X	X	
26	M	39	X	X			
27	F	39	X				
28	M	40	X		X	X	X
30	F	40	X				
31	F	42			X	X	
32	M	43			X	X	X
33	F	43			X	X	X
34	M	43	X				
35	M	45			X	X	
36	M	44			X	X	X
37	M	47			X	X	
38	F	49	X		X	X	
39	F	49	X		X	X	
40	F	49	X	X	X	X	
41	M	53	X		X	X	
42	F	57	X	X	X	X	X
43	F	57	X				X
44	M	59	X		X	X	
45	M	60	X		X	X	
46	F	64	X		X		
47	M	66	X	X	X	X	
48	M	67	X		XX		X
49	M	68	X		X		
50	M	69	X		X	X	
51	M	69	X		X		

52	M	70	X	X	X	X	
53	M	71	X		X		
54	F	72	X	X	X		
55	M	72	X		X		
56	F	73	X		X	X	
57	F	73	X	X	X		
58	M	74	X		X	X	
59	M	75	X		X		
60	M	78	X	X	X	X	
61	M	81	X		X		
62	F	82	X		X		
63	F	85	X	X	X		

Supplemental Table 2. List of donors for the *in vivo* portion of this study with their age, gender, and experimental use. An 'X' indicates donor was used for the experiment.

Donor ID	Age	Gender	Experiments	
			Cell sorted/ RT-qPCR	Flow Cytometry
1	30	F	X	X
2	31	M	X	X
3	32	F		X
4	33	M		X
5	39	F	X	X
6	41	F		X
7	42	F		X
8	43	F		X
9	44	F	X	X
10	44	F	X	X
11	45	F		X
12	47	F	X	X
13	48	F	X	X
14	49	F	X	X
15	49	M	X	X
16	51	M	X	X
17	51	M	X	X
18	52	M	X	X
19	53	F		X
20	53	M	X	X
21	53	M	X	X
22	54	F	X	X
23	55	F	X	X

24	56	F	X	X
25	57	M		X
26	61	M	X	X
27	61	M	X	X
28	65	M		X
29	67	F	X	X
30	70	M		X
31	74	M	X	X
32	74	M	X	X

Supplemental Table 3. Primer sequences used for RT-qPCR analyses.

Primer	Sequence
IL1A	Ft: AGCATGGTGGTAGTAGCAACCA Bt: BTTGGCTTAAACTCAACCGTCTCT
IL6	Ft: AATTCGGTACATCCTCGACGG Bt: GGTTGTTTTCTGCCAGTGCCT
CXCL1	Ft: CCCCAAGAACATCCAAAGTGTG Bt: GCAGGATTGAGGCAAGCTTTC
CXCL2	Ft: TCATCGAAAAGATGCTGAAAAATG Bt: GAACAGCCACCAATAAGCTTCCT
CXCL3	Ft: GCATCCCCCATGGTTCAG Bt: TCAGTTGGTGCTCCCCTTGT
CCL2	Ft: AAGATCTCAGTGCAGAGGCTCG Bt: CACAGATCTCCTTGGCCACAA
TNF	Ft: TGGCCCAGGCAGTCAGA Bt: GGTTTGCTACAACATGGGCTACA
CCL8	Ft: CTCATGGCAGCCACTTTCAG Bt: GCAGGTGATTGGAATGGAAACT
IL36G	Ft: GGGTCAGAACCTTGTGGCAG Bt: TAGCTGCAATGTCGGCTGTT
IL2	Ft: AAGAATCCCAAACTCACCAGGAT Bt: TAGACACTGAAGCTGTTTCAGTTCTG
IFNG	Ft: ACTCATCCAAGTGATGGCTGAA Bt: AACAGCATCTGACTCCTTTTTCG
ADRB2	Ft: CGCTTCCATGTCGAACCT Bt: TCTTGAGGGCTTTGTGCTCC
ACOX1	Ft: TGCTTTGGTTGATGCATTTGA Bt: CATAGCGGCCAAGCACAGA

Supplemental Table 4. List of top 50 most upregulated and top 50 most down-regulated genes when comparing pre-activated CD8+ Tcm with overnight NE treatment. The log ratio differences were used to rank the genes. Genes are listed in alphabetical order.

Gene symbol	Gene name	Ratio (WT/NE)	p-value
HBB	hemoglobin, beta	-0.96	0.03
C8orf29	AGENCOURT_7912774 NIH_MGC_72 cDNA clone	-0.80	0.04
FAM104B	family with sequence similarity 104, member B	-0.79	0.03
TLR8	toll-like receptor 8	-0.78	0.06
CXCL2	chemokine (C-X-C motif) ligand 2	-0.77	0.22
DNASE1L3	deoxyribonuclease I-like 3	-0.76	0.04
ARPP21	cAMP-regulated phosphoprotein, 21kDa	-0.72	0.01
FAM186B	family with sequence similarity 186, member B	-0.70	0.07
FAM43B	family with sequence similarity 43, member B	-0.70	0.00
FLJ31485	uncharacterized LOC440119	-0.69	0.09
HOXB7	homeobox B7	-0.69	0.08
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	-0.68	0.00
RAMP3	receptor (G protein-coupled) activity modifying protein 3	-0.66	0.09
GAL3ST4	galactose-3-O-sulfotransferase 4	-0.66	0.01
GPR17	G protein-coupled receptor 17	-0.63	0.10
SLC22A7	solute carrier family 22 (organic anion transporter), member 7	-0.63	0.05
KLHL14	kelch-like 14 (Drosophila)	-0.63	0.01
PLA2G4D	phospholipase A2, group IVD (cytosolic)	-0.62	0.09
KIAA1875	KIAA1875	-0.62	0.01
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	-0.62	0.07
TMEM156	transmembrane protein 156	-0.62	0.02
TMEM231	transmembrane protein 231	-0.62	0.15
FLJ42709	uncharacterized LOC441094	-0.60	0.11
CXCL1	chemokine (C-X-C motif) ligand 1	-0.60	0.42
NXNL1	nucleoredoxin-like 1	-0.60	0.05
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	-0.60	0.10
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	-0.60	0.12
TCHH	trichohyalin	-0.59	0.11
IL1B	interleukin 1, beta	-0.59	0.15

FBN2	fibrillin 2	-0.59	0.06
TBX10	T-box 10	-0.59	0.00
ENO4	enolase family member 4	-0.59	0.05
UROS	uroporphyrinogen III synthase	-0.59	0.01
TLR4	toll-like receptor 4	-0.59	0.16
USH2A	Usher syndrome 2A	-0.58	0.13
EMR4P	egf-like module containing, mucin-like, hormone receptor-like 4 pseudogene	-0.58	0.07
DKFZP761C1711	mRNA; cDNA DKFZp761C1711	-0.58	0.08
FBN1	fibrillin 1	-0.58	0.11
NRXN2	neurexin 2	-0.58	0.02
DEFA3	defensin, alpha 3, neutrophil-specific	-0.57	0.22
TSC1	tuberous sclerosis 1	-0.57	0.05
PCDH7	protocadherin 7	-0.57	0.03
PAGE1	P antigen family, member 1	-0.57	0.08
TFAP2A	transcription factor AP-2 alpha	-0.56	0.03
SNORA14A	small nucleolar RNA, H/ACA box 14A	-0.56	0.03
FLJ43663	uncharacterized LOC378805	-0.56	0.06
GREM1	gremlin 1	-0.55	0.09
HERC2P4	hect domain and RLD 2 pseudogene 4	-0.55	0.01
TRPM4	transient receptor potential cation channel, subfamily M, member 4	-0.55	0.12
SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, secreted	-0.54	0.06
AFAP1L2	actin filament associated protein 1-like 2	0.97	0.00
CDADC1	cytidine and dCMP deaminase domain contain 1	0.47	0.00
GXYLT2	glucoside xylosyltransferase 2	0.48	0.02
C15orf41	chromosome 15 open reading frame 41	0.48	0.00
TMC8	transmembrane channel-like 8	0.48	0.04
CCDC112	coiled-coil domain containing 112	0.48	0.01
SIRPG	signal-regulatory protein gamma	0.48	0.05
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa	0.49	0.00
SEC24A	SEC24 family, member A (S. cerevisiae)	0.49	0.02
GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2	0.49	0.01
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	0.49	0.00
MAP7	microtubule-associated protein 7	0.49	0.01
NOG	noggin	0.50	0.05

TMPO	thymopoietin	0.50	0.02
NRTN	neurturin	0.50	0.02
TIMD4	T-cell immunoglobulin and mucin domain containing 4	0.50	0.04
CCL27	chemokine (C-C motif) ligand 27	0.51	0.00
HYDIN2	hydrocephalus inducing homolog 2	0.53	0.02
GGT8P	gamma-glutamyltransferase 8 pseudogene	0.54	0.00
CXCR7	chemokine (C-X-C motif) receptor 7	0.54	0.00
IFI44	interferon-induced protein 44	0.54	0.00
SUGT1P3	suppressor of G2 allele of SKP1 (S. cerevisiae) pseudogene 3	0.54	0.05
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	0.55	0.01
OIP5	Opa interacting protein 5	0.56	0.05
ZDHHC21	zinc finger, DHHC-type containing 21	0.56	0.00
Q5D1D6	Q5D1D6_CERAE, Guanylate binding protein 1	0.56	0.05
C14orf102	chromosome 14 open reading frame 102	0.56	0.00
PRKCE	protein kinase C, epsilon	0.57	0.01
SLCO4C1	solute carrier organic anion transporter family, member 4C1	0.58	0.01
ELOVL7	ELOVL fatty acid elongase 7	0.59	0.01
IL20RA	interleukin 20 receptor, alpha	0.60	0.04
FGFBP2	fibroblast growth factor binding protein 2	0.61	0.03
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.62	0.01
MTSS1	metastasis suppressor 1	0.62	0.01
C15orf37	chromosome 15 open reading frame 37	0.63	0.00
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	0.65	0.02
TTC16	tetratricopeptide repeat domain 16	0.66	0.01
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	0.66	0.03
DCD	dermcidin	0.67	0.03
RSAD2	radical S-adenosyl methionine domain contain 2	0.67	0.05
PTPN3	protein tyrosine phosphatase, non-receptor type 3	0.67	0.04
CD244	CD244 molecule, natural killer cell receptor 2B4	0.69	0.01
PLLP	plasmolipin	0.70	0.00
HPS4	Hermansky-Pudlak syndrome 4	0.72	0.00

FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor	0.73	0.03
IFI44L	interferon-induced protein 44-like	0.76	0.00
UBL4A	ubiquitin-like 4A	0.80	0.00
PROK2	prokineticin 2	0.80	0.04
N4BP3	NEDD4 binding protein 3	0.81	0.00
MYL2	myosin, light chain 2, regulatory, cardiac, slow	0.84	0.03

Supplemental Table 5. List of top 50 most upregulated and top 50 most down-regulated genes when comparing 24h and 72h post-activated CD8+ Tcm with overnight NE treatment. The maximum of either the log ratio of the 24h and 72h comparisons were used to rank the genes.

Gene symbol	Gene name	Ratio 24h (WT/NE)	p-value (24h)	Ratio 72h (WT/NE)	p-value (72h)
PSPHP1	phosphoserine phosphatase pseudogene 1	-1.33	0.00	-0.906	0.082
CXCL2	chemokine (C-X-C motif) ligand 2	-1.12	0.07	-1.128	0.143
CSF3	colony stimulating factor 3, granulocyte	-1.12	0.00	-0.427	0.219
CXCL3	chemokine (C-X-C motif) ligand 3	-0.86	0.13	-0.957	0.173
ANK3	ankyrin 3, node of Ranvier	-0.82	0.00	0.031	0.919
CDH1	cadherin 1, type 1, E-cadherin	-0.80	0.04	-0.636	0.185
IL36G	interleukin 36, gamma	-0.79	0.01	-0.771	0.032
SNORD98	small nucleolar RNA, C/D box 98	-0.76	0.00	-0.119	0.669
CXCL1	chemokine (C-X-C motif) ligand 1	-0.76	0.31	-1.398	0.124
LUZP1	leucine zipper protein 1	-0.69	0.05	-0.050	0.908
HEATR1	HEAT repeat containing 1	-0.68	0.00	-0.041	0.862
ARNTL	aryl hydrocarbon receptor nuclear translocator-like	-0.67	0.00	-0.506	0.050
B3GAT2	beta-1,3-glucuronyltransferase 2	-0.67	0.00	-0.211	0.421
GABRB3	gamma-aminobutyric acid	-0.67	0.00	-0.115	0.638
SNORD114-3	small nucleolar RNA, C/D box 114-3	-0.66	0.01	-0.086	0.789
TRIM62	tripartite motif containing 62	-0.66	0.00	-0.207	0.452
IL4	interleukin 4	-0.66	0.01	0.043	0.885
RAB19	RAB19, member RAS oncogene family	-0.66	0.00	-0.008	0.972
CRHR1	corticotropin releasing hormone receptor 1	-0.65	0.01	-0.076	0.793
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	-0.64	0.03	-0.689	0.059
USP45	ubiquitin specific peptidase 45	-0.64	0.00	-0.211	0.423

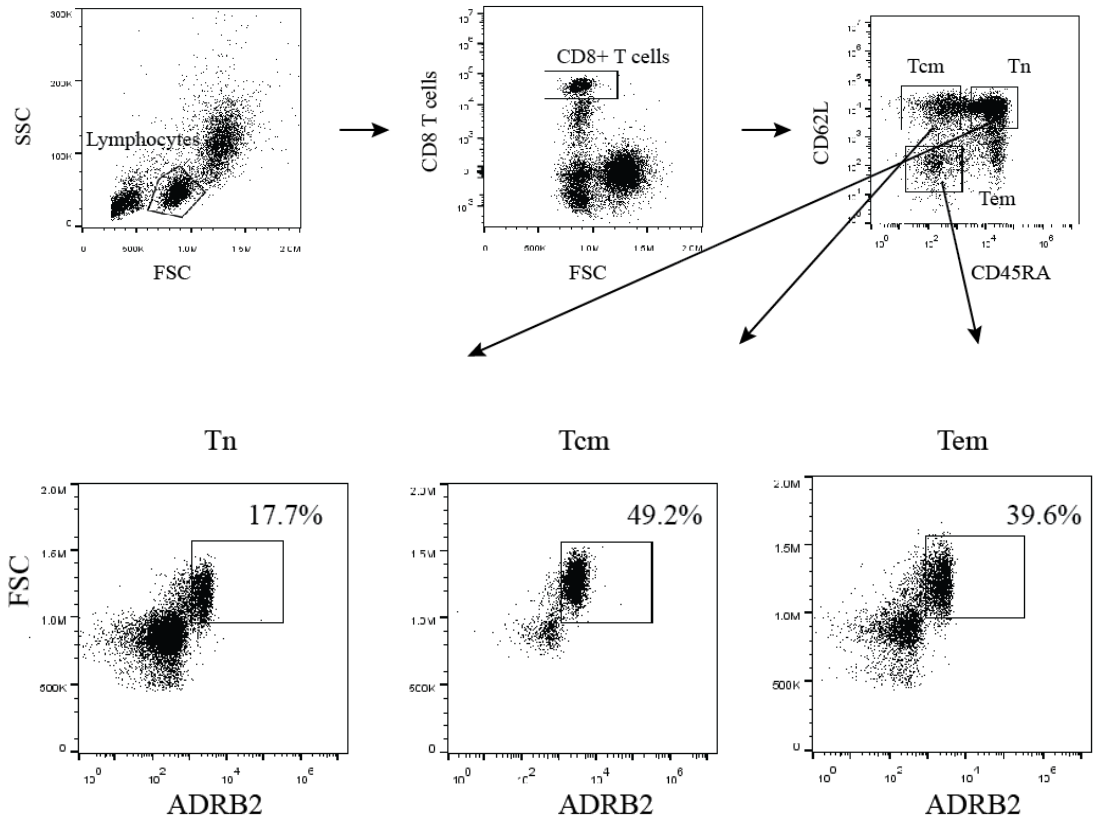
SHANK1	SH3 and multiple ankyrin repeat domains 1	-0.64	0.03	-0.086	0.812
HAS3	hyaluronan synthase 3	-0.64	0.02	-0.080	0.813
WDR33	WD repeat domain 33	-0.64	0.03	-0.056	0.877
IL28RA	interleukin 28 receptor, alpha (interferon, lambda receptor)	-0.63	0.00	-0.125	0.626
FLJ40536	cDNA FLJ40536 fis, clone TESTI2047930	-0.63	0.00	-0.110	0.686
PFN1P2	profilin 1 pseudogene 2	-0.62	0.01	-0.050	0.871
GZF1	GDNF-inducible zinc finger protein 1	-0.60	0.07	-0.152	0.705
GPR15	G protein-coupled receptor 15	-0.60	0.03	-0.326	0.350
SLC4A5	solute carrier family 4, sodium bicarbonate cotransporter, member 5	-0.60	0.04	-0.178	0.620
AMICA1	cDNA FLJ33028 fis, clone THYMU2000140	-0.59	0.01	0.002	0.995
SNORD66	small nucleolar RNA, C/D box 66	-0.59	0.00	-0.076	0.724
ALDOAP2	Human aldolase pseudogene	-0.58	0.01	-0.427	0.130
C17orf105	chromosome 17 open reading frame 105	-0.58	0.01	-0.218	0.450
OR1N2	olfactory receptor, family 1, subfamily N, member 2	-0.57	0.02	-0.012	0.967
Q07610	Q07610_RAT	-0.57	0.03	-0.466	0.135
ACP1	acid phosphatase 1, soluble	-0.56	0.01	-0.195	0.458
FLJ42351	uncharacterized LOC400999	-0.56	0.01	-0.126	0.644
KIAA1486	KIAA1486	-0.56	0.02	0.038	0.900
TMSB4Y	thymosin beta 4, Y-linked	-0.56	0.01	-0.737	0.004
SNX29	sorting nexin 29	-0.55	0.01	-0.243	0.370
TTC23L	tetratricopeptide repeat domain 23-like	-0.55	0.04	-0.235	0.482
ARAP1	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	-0.55	0.01	0.002	0.993
ANKRD43	ankyrin repeat domain 43	-0.55	0.01	-0.076	0.779
RHBDL3	rhomboid, veinlet-like 3	-0.55	0.01	-0.252	0.344
SCD5	stearoyl-CoA desaturase 5	-0.55	0.02	-0.101	0.735

C21orf15	CU015_HUMAN	-0.54	0.01	0.013	0.961
FSIP2	fibrous sheath interacting protein 2	-0.54	0.05	-0.138	0.687
TTC16	tetratricopeptide repeat domain 16	-0.54	0.03	-0.226	0.446
SNORA22	DKFZp686E1139_r1 686	-0.54	0.01	-0.227	0.387
HOXB9	homeobox B9	0.52	0.00	0.302	0.034
IL26	interleukin 26	0.51	0.02	-0.011	0.966
GPR124	G protein-coupled receptor 124	0.52	0.03	0.256	0.394
NFAM1	NFAT activating protein with ITAM motif 1	0.52	0.04	0.012	0.970
CALCA	calcitonin-related polypeptide alpha	0.53	0.08	0.345	0.345
KIF7	kinesin family member 7	0.53	0.01	0.085	0.742
TUBB6	tubulin, beta 6 class V	0.53	0.01	0.261	0.280
ULBP1	UL16 binding protein 1	0.54	0.01	0.438	0.069
ITGAD	integrin, alpha D	0.54	0.06	0.015	0.967
CHD5	chromodomain helicase DNA binding protein 5	0.55	0.06	0.232	0.522
OIP5	Opa interacting protein 5	0.55	0.06	0.044	0.901
RBFOX2	RNA binding protein, fox-1 homolog	0.55	0.11	0.873	0.037
BTLA	B and T lymphocyte associated	0.56	0.07	0.290	0.449
PGBD1	piggyBac transposable element derived 1	0.57	0.01	0.307	0.257
SCHIP1	schwannomin interacting protein 1	0.58	0.04	0.307	0.362
DIXDC1	DIX domain containing 1	0.58	0.02	0.105	0.739
GPR133	G protein-coupled receptor 133	0.58	0.02	0.282	0.363
IL23R	interleukin 23 receptor	0.59	0.01	0.053	0.854
NEXN-AS1	chromosome 1 open reading frame 118	0.60	0.01	0.070	0.796
C10orf92	chromosome 10 open reading frame 92	0.60	0.00	0.073	0.712
GDF10	growth differentiation factor 10	0.60	0.01	0.230	0.430
CD38	CD38 molecule	0.60	0.02	0.127	0.694
RPL37A	BROAD Institute lincRNA	0.61	0.00	0.301	0.251
CCNA2	cyclin A2	0.61	0.08	0.042	0.922

CCL4	chemokine (C-C motif) ligand 4	0.61	0.01	0.063	0.826
ZNF788	zinc finger family member 788	0.62	0.05	0.272	0.476
TK1	thymidine kinase 1, soluble	0.62	0.01	0.034	0.910
CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)	0.63	0.06	0.472	0.247
BCAT1	branched chain amino-acid transaminase 1, cytosolic	0.63	0.01	-0.007	0.982
ECSCR	endothelial cell-specific chemotaxis regulator	0.64	0.05	0.160	0.686
DGKI	diacylglycerol kinase,	0.65	0.05	0.508	0.218
ZNF365	zinc finger protein 365	0.65	0.02	0.218	0.516
CCL3L3	chemokine (C-C motif) ligand 3-like 3	0.66	0.05	0.271	0.515
C10orf105	chromosome 10 open reading frame 105	0.66	0.03	0.452	0.214
CTH	cystathionase (cystathionine gamma-lyase)	0.68	0.01	0.532	0.121
ABLIM3	actin binding LIM protein family, member 3	0.68	0.05	0.441	0.304
CKAP2L	cytoskeleton associated protein 2-like	0.70	0.03	0.059	0.885
CD200	CD200 molecule	0.71	0.02	0.453	0.217
DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	0.71	0.04	0.381	0.373
SPC24	SPC24, kinetochore complex component, homolog	0.71	0.06	0.003	0.995
IL2	interleukin 2	0.72	0.11	0.472	0.391
GPT2	glutamic pyruvate transaminase	0.72	0.07	0.116	0.811
NEIL3	nei endonuclease VIII-like 3 (E. coli)	0.73	0.03	0.000	1.000
CCL3	chemokine (C-C motif) ligand 3	0.74	0.03	0.168	0.679
G0S2	G0/G1switch 2	0.75	0.01	0.127	0.710
NUMB	BROAD Institute lincRNA	0.76	0.00	0.300	0.330
MYOF	myoferlin	0.81	0.07	0.374	0.486
ESCO2	establishment of cohesion 1 homolog 2	0.81	0.07	0.046	0.933

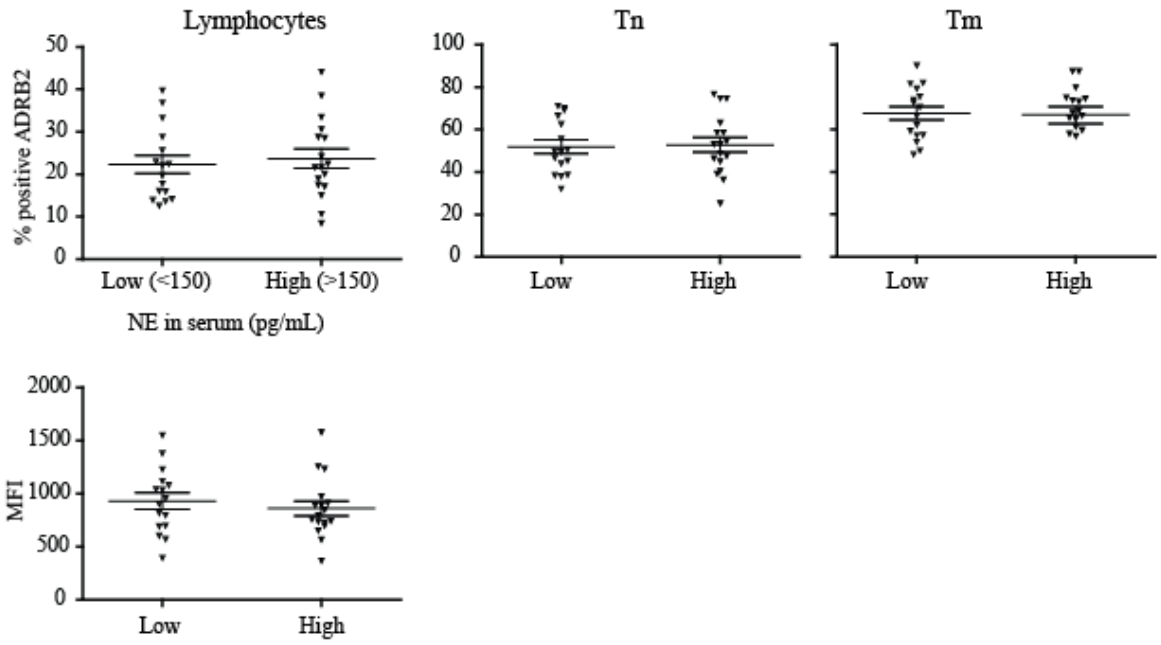
NHS	Nance-Horan syndrome (congenital cataracts and dental anomalies)	0.85	0.01	0.391	0.331
SHC4	SHC family, member 4	1.08	0.00	0.397	0.369

Figure S1. Gating strategy for ADRB2 staining on CD8 T cell subsets.



Gating strategy for ADRB2 staining on CD8 T cell subsets is illustrated. We first gated on live lymphocytes based on the FSC and SSC, followed by $CD8^+$ T cells (APC), and then gated on the subsets: naïve ($CD45RA^+$, PE), ($CD62L^+$, FITC), central memory ($CD45RA^-/CD62L^+$) and effector memory ($CD45RA^-/CD62L^-$). Finally, we gated on ADRB2 (PerCP/Cy5.5) positive cells within each CD8 T cell subset.

Figure S2. ADRB2 expression in individuals with high and low levels of NE in their serum.



ADRB2 expression in individuals with high and low levels of NE in their serum. ADRB2 expression in individuals with high levels of norepinephrine (>150pg/ml, N=17) compared to individuals with low levels of norepinephrine (<150pg/ml, N=15). There were no significant differences in the percentage or MFI of ADRB2 expressed CD8 T cell subsets (Tn, Tm) between individuals with high or low levels of NE on a population or individual cell level.

CHAPTER 4

Caregiving modulates memory CD8 T cell function inducing pro-inflammatory cytokine and chemokine production: A study of family caregivers of hematopoietic stem cell transplant patients

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Abstract

Objective: Previous studies have shown the detrimental impact of caregiving stress on different types of immune cells. This study examines the effects of caregiving for hematopoietic stem cell transplant recipients on the immune phenotype and the function of CD8 T cell subsets (naïve and memory).

Methods: We examine twenty-one caregivers' and twenty age-, gender- and ethnicity-matched controls' immune cell composition as well as naïve (defined by $CD45RA^{+}CD28^{+}$) and memory ($CD45RA^{-}CD28^{+/-}$) $CD8^{+}$ T cell cytokine gene and protein expression by RT-qPCR and flow cytometry.

Results: Caregivers of transplant patients showed an increase in gene expression of pro-inflammatory cytokines (TNF, CXCL1, IL1A) and a decrease in growth-related cytokines (IL2, IFNG) by CD8 Tm cells compared to matched controls.

Conclusions: Our results demonstrate memory CD8 are susceptible to the effects of caregiving stress by inducing a pro-inflammatory state while simultaneously inhibiting memory CD8 function by reducing proliferation-related cytokines. This data suggests that the stress of caregiving may have a detrimental impact on an individual's ability to respond to previous antigenic challenges and fight off infections.

Words: hematopoietic stem cell transplant caregivers, family caregivers, CD8 T cells, caregiving stress

Abbreviations: IL = interleukin; PBMC = peripheral mononuclear cells; HSCT = hematopoietic stem cell transplant; Tn = CD8 naïve T cell; Tm = CD8 memory T cell

Introduction

Chronic stressors are commonplace and challenge our psychological, behavioral and physiological health. Recent evidence has shown the detrimental impact of chronic stress on human health, including immune health outcomes. Chronic stress-induced biological changes in the immune system reveal the inhibited function of cytokines, decreased lymphocyte counts, and decreased immune cell proliferation and functionality; furthermore, these biological changes in immune function can lead to increased infection rates, impaired responses to vaccines, pre-mature aged immune system, or dysregulation in the inflammatory response (Cohen *et al.*, 1999;Gouin *et al.*, 2008;Kiecolt-Glaser *et al.*, 1996;Kiecolt-Glaser *et al.*, 2003;Padgett *et al.*, 2003).

Several human studies examining the effects of chronic stress on immune health have resulted in conflicting findings or observed no differences between the chronically stressed individual and their control (Bonneau *et al.*, 1990;Gouin *et al.*, 2008;Hu *et al.*, 2014;Padgett *et al.*, 2003). A majority of these studies have examined immune cell changes in whole blood or peripheral mononuclear cells (PBMCs)(Bauer, Vedhara, Perks, Wilcock, Lightman, and Shanks, 2000;Bonneau *et al.*, 1990;Castle *et al.*, 1995;Dhabhar *et al.*, 2012;Glaser *et al.*, 1998;Gouin *et al.*, 2008;Mills *et al.*, 1997;Vitaliano *et al.*, 1998)(Bauer, Vedhara, Perks, Wilcock, Lightman, and Shanks, 2000;Bonneau *et al.*, 1990;Castle *et al.*, 1995;Dhabhar *et al.*, 2012;Glaser *et al.*, 1998;Gouin *et al.*, 2008;Mills *et al.*, 1997;Vitaliano *et al.*, 1998) (Mills et al., 1997; Bauer et al., 2000; Glaser et al., 1998; Bonneau et al., 1990; Vitaliano et al., 1998); it is unknown if chronic psychological stress impacts CD8 T cell subsets in a homogeneous manner. Isolation of specific immune cell subsets may reveal differences induced by chronic stress that may otherwise be masked by a mixture of immune cells in whole blood; thus, recent studies have focused on specific immune cells subsets such as CD4 T cells, natural killer (NK) cells or B cells (Bauer *et al.*, 2000;Bonneau *et al.*, 1990;Castle *et al.*, 1995;Dhabhar *et al.*, 2012;Glaser *et al.*, 1998;Gouin *et al.*, 2008;Mills *et al.*, 1997;Vitaliano *et al.*, 1998). Less work has focused on the effects of

chronic stress on CD8 T cells despite their critical role in mounting an effective adaptive immune response (Araki *et al.*, 2008;Araki *et al.*, 2009;Fann *et al.*, 2006). CD8 T cells are cytotoxic to infected cells and are comprised of two major subsets: naïve (Tn), which have not yet encountered an antigenic challenge, and memory (Tm) that have already been exposed to an antigen and can respond more rapidly.

Family caregivers serve as an ideal clinical model to study the effects of chronic stress on immune cell function since they often perceive their role as stressful, function in their role for several years, and have impaired health outcomes, including impaired immunity (Bauer *et al.*, 2000;Castle *et al.*, 1995;Futterman *et al.*, 1996;Gouin *et al.*, 2008;Rohleder *et al.*, 2009;Schulz *et al.*, 1999;Vedhara *et al.*, 2002;Vitaliano *et al.*, 2003). A majority of the research on the biological impact of caregiving has focused on caregivers of dementia and Alzheimer's and cancer patients, or older adult caregivers. This research has shown a wide-range of effects on immune health including altered cytokine production, immune cell dysfunction, decreased immune cell proliferation and inhibited responses to vaccination (Glaser *et al.*, 1998;Gouin *et al.*, 2008;Kiecolt-Glaser *et al.*, 1996;Lutgendorf and Laudenslager, 2009;Mills, Yu, Ziegler, Patterson, and Grant, 1999;Vitaliano *et al.*, 2003).

Family caregivers of hematopoietic stem cell transplant (HSCT) recipients are an understudied population of caregivers; they often serve in their role for several years and accompany the HSCT patient through one of the most intense treatments available for cancer patients that can result in multiple physical, psychological and social challenges for the HSCT caregiver (Beattie *et al.*, 2011;Eldredge, Nail, Maziarz, Hansen, Ewing, and Archbold, 2006;Futterman *et al.*, 1996;Laudenslager, 2014;Rizzo *et al.*, 1999). This study aimed to determine whether there is a difference between HSCT caregivers and non-caregivers in terms of their adaptive immune cell composition as well as the transcription and translation of pro-inflammatory (IL1A, IL2, IL6, IL8) and growth-related cytokines (IL2, IFNG) and chemokines (CCL2, CCL8, CXCL1) of CD8 T cell subsets (Tn and Tm) compared to non-caregivers.

Methods

Human subjects and blood collection

Participants for this study (N=41) were drawn from a larger longitudinal study examining physiological and clinical markers of chronic stress in caregivers of HSCT recipients (Bevans under review) at the National Institutes of Health (NIH) Clinical Center. Blood was drawn from the participants at 3 time points: up to 2 weeks before transplantation, one week after transplantation and 6 weeks post-discharge. Blood samples collected at the 3 time points of the primary study were combined for this study in order to have an adequate cell number for experimental use. PBMCs were isolated from the peripheral blood of adult caregivers (N=21), and age-, gender- and ethnicity-matched non-caregiver controls (N=20) and frozen at -80°C for use in this study. Utilization of these samples and the primary investigation was approved by the Internal Review Boards at the National Heart Lung and Blood Institute (NHLBI), NIH and the University of Pennsylvania.

Flow cytometry for phenotype and intracellular cytokine analyses

PBMCs were stained for phenotyping and intracellular cytokines for flow cytometry analysis with the Canto II Flow Cytometer (BD Biosciences). Antibodies included: Viability (e506), CD8 (PeCy7, Biolegend), CD4 (Pacific Blue, Biolegend), CD3 (ApcCy7, Biolegend), CD28 (APC, Biolegend), CD45RA (FITC, Biolegend).

For intracellular cytokine staining, frozen PBMCs were thawed and incubated for 3 hours at 5% O_2. Next, PMA (50ng/mL, Sigma), Ionomycin (80ng/mL, Sigma), and a Golgi Block (1 µg/million cells, BD Biosciences) were added to the cells and incubated for 4 hours at 5% O_2. Cells were washed and fixation buffer added then incubated overnight at 4°C in the dark. The next day, cells were washed with permealization buffer and then antibodies added for intracellular staining for IL1-α (PE, Biolegend), TNF (PercpCy5.5, Biolegend), IL-2 (PercpCy5.5, Biolegend), and CCL-2 (PE, eBioscience). Isotype and fluorescent dye matched non-specific mouse IgG was

used as controls for cytokine staining. Samples were collected on the Canto II Flow Cytometer (BD Biosciences). Data and mean fluorescent intensity were further analyzed using FloJo V10 software.

Isolation and culture of human CD8 T cell subsets

For isolation of naïve and memory CD8 T cells from frozen PBMCS of caregivers and non-caregivers, PBMCs were thawed in a 37°C water bath, washed and resuspended in RPMI. Naïve and memory CD8 T cells were isolated by cell sorter (MoFlo, Dako Cytomation) using the following staining: Viability (e506), CD8+, naive ($CD45RA^+$, $CD28^+$), and memory ($CD28^{+-}$). The purity of sorted naïve and memory CD8 T cells was >96%.

Isolated CD8 T cell subsets were either used for gene expression analysis immediately or incubated at 5% O_2 with anti-CD3/28-coupled beads at a cell:bead ratio of 1:1 in RPMI1640 with 10% FBS and penicillin (10U/ml)/streptomycin (10µg/ml) and harvested at 24 hours for analyses of mRNA.

Quantitative RT-qPCR of mRNA

The procedure for quantitative RT-PCR (RT-qPCR) was described previously (Araki et al., 2009). Briefly, RNA was isolated with the RNeasy Mini Kit for Qiacube (Qiagen) and cDNA was synthesized with oligo-DT and random hexamers (Invitrogen) with 60ng of RNA. The mRNA levels were determined by RT-qPCR using 2x SYBR Green PCR Master Mix (Applied Biosystems) and normalized to a lymphocyte housekeeping gene, acyl-Coenzyme A oxidase 1 (ACOX1), as described previously (Araki et al., 2009). RT-qPCR was conducted for gene expression analysis of several cytokines and chemokines important to CD8 T cell function and related to inflammatory processes, including: IL2, CCL2, CXCL1, IL6, IL8, IFN-G, IL1A, CCL8 and TNF. The value of cytokine mRNA threshold cycle (Ct) was normalized to ACOX1. PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistics

Analyses were conducted comparing the HSCT caregiver group with the non-caregiver control group on immune cell composition, intracellular proteins and gene expression levels. Since this study controlled for covariates such as age, gender and ethnicity, no correlational statistics were conducted. Data are expressed as the mean ± SEM and we considered $p < 0.05$ as statistically significant.

Results

T lymphocyte composition is similar between controls and HSCT caregivers

First, an examination of potential phenotype changes in HSCT caregivers (N=21) compared to controls (N=20) was conducted by examining the composition of adaptive immune cells in PBMC. To address this, flow cytometry analysis was utilized with antibodies staining for various immune cell populations as illustrated by the gating strategy (Fig. 1A). Table 1 shows the immune cell composition percentages between caregivers and their controls. There was no significant difference between caregivers and controls in their adaptive immune cell composition (Fig. 1B). T lymphocyte composition was not significantly different between the HSCT caregivers and controls, however CD8 Tm cells were trending ($p<0.06$) toward a descriptively higher percentage in controls compared to the HSCT caregivers (Table 1).

Table 1. Immune cell composition between controls and family caregivers

	Lymphocytes	T cells	CD8 T cells	Tn	Tm	CD4 T cells
Control	62.9%	75.9%	18.0%	41.1%	40.0%	41.7%
Caregiver	60.4%	73.9%	16.6%	44.3%	33.1%	46.9%

Increased expression and intracellular cytokine proteins of pro-inflammatory cytokines and chemokines in caregivers compared to controls

While no changes were found at the cellular level in terms of immune cell composition between caregivers and controls, this study next asked whether the function of these immune cells might be altered in our chronically stressed population. From the 41 participants (caregivers N=21, controls N=20), 22 participant samples (caregivers N=13, controls N=9) were successfully isolated for the mRNA analyses. The results showed caregivers had significantly higher expression of pro-inflammatory cytokines including CXCL1 at baseline and TNF at 24 hours, and a lower expression of proliferation and growth related genes including IL2 at baseline and IFN-G at 24 hours in CD8 Tm cells (Fig. 2A).

This study next examined if changes at the gene level in family caregivers (N=21) compared to controls (N=20) were translated to the protein level by studying four intracellular proteins using flow cytometry. TNF levels were significantly increased in CD8 Tm cells which correlated with the increase in gene expression changes (Fig. 2B). There was no difference between controls and caregivers in the other cytokines (CXCL1, IFN-γ, IL-2) measured despite the changes seen at the mRNA level.

Discussion

This study examined the T lymphocyte composition, cytokine gene expression and intracellular proteins of CD8 T cell subsets (naïve and memory) in HSCT family caregivers and non-caregivers. There was no significant difference in immune cell composition of caregivers compared to controls, which was similar to findings in other studies of HSCT caregivers (Laudenslager, 2014), suggesting chronic stress does not significantly impact immune cell composition. However, the gene expression of certain cytokines and chemokines of CD8 Tm cells differed between groups: HSCT caregivers had increased expression of pro-inflammatory cytokines TNF and CXCL1 and decreased expression of activation induced proliferative cytokines IL2 and IFN-G. Intracellular TNF proteins were also significantly increased in CD8 Tm cells. This study concurs with the results of previous investigations in other immune cell types which examined caregivers and matched control subjects to find caregivers' illnesses lasted longer, they

had lower levels of IL-2 and IL-1β in CD4 T_h1 associated cytokines as well as an increase in IL-6 (Kiecolt-Glaser *et al.*, 1987;Kiecolt-Glaser *et al.*, 1995;Kiecolt-Glaser *et al.*, 2003). It appears that the chronic stress of caregiving impacts memory CD8 T cells more significantly than other cell types. Inhibited function of memory CD8 T cells presents a clinical challenge for individuals since they will be more compromised in fighting off recall infections rather than new immune challenges; this is especially important for elderly caregivers since aging is associated with an increased percentage and number of memory cells compared to naïve cells.

Caregivers of HSCT patients present a unique perspective on caregiving since their burden is typically shorter but more intense and patients have a better prognosis compared to caregivers of the more studied populations such as Alzheimer's (Laudenslager, 2014). In summary, our study shows HSCT caregivers exhibit a pro-inflammatory response and growth-related inhibitory response in CD8 Tm cells compared to non-caregivers, possibly contributing to an immunocompromised status for opportunistic infections that would typically be addressed by memory T cells. This study is unique since it is the first to examine the effects of HSCT caregiving on CD8 T cell subsets' transcription and translation of important inflammatory and growth-related cytokines and chemokines. There were a few limitations to this study; since this study was based off a primary investigation examining physiological changes in HSCT caregivers and their controls, we were limited by the original sample size and measures. In addition, given small amount of blood collected, we were limited in the number of analyses we could conduct for the gene expression and intracellular protein analyses.

Future work should address the possible mechanisms behind these changes in caregivers' CD8 Tm cells and isolate this subset over the pre, transplantation, and post-transplantation periods; previous studies have reported caregiver distress is highest pre-transplantation and decreases over time (Beattie *et al.*, 2011). This work is important to uncover the transcriptional and phenotypic changes that occur in caregivers despite the challenges of conducting these types of studies including: caregiver recruitment, attrition, collection of large volumes of blood for future isolation of immune subsets, and challenge of conducting longitudinal

studies. In general, our findings are consistent with prior studies showing the deficits in immune function of family caregivers and other chronically stressed populations, and suggest the importance of identifying specific immune subsets when examining stress-induced immunological changes.

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Conflict of Interest

The authors declare no conflicts of interest.

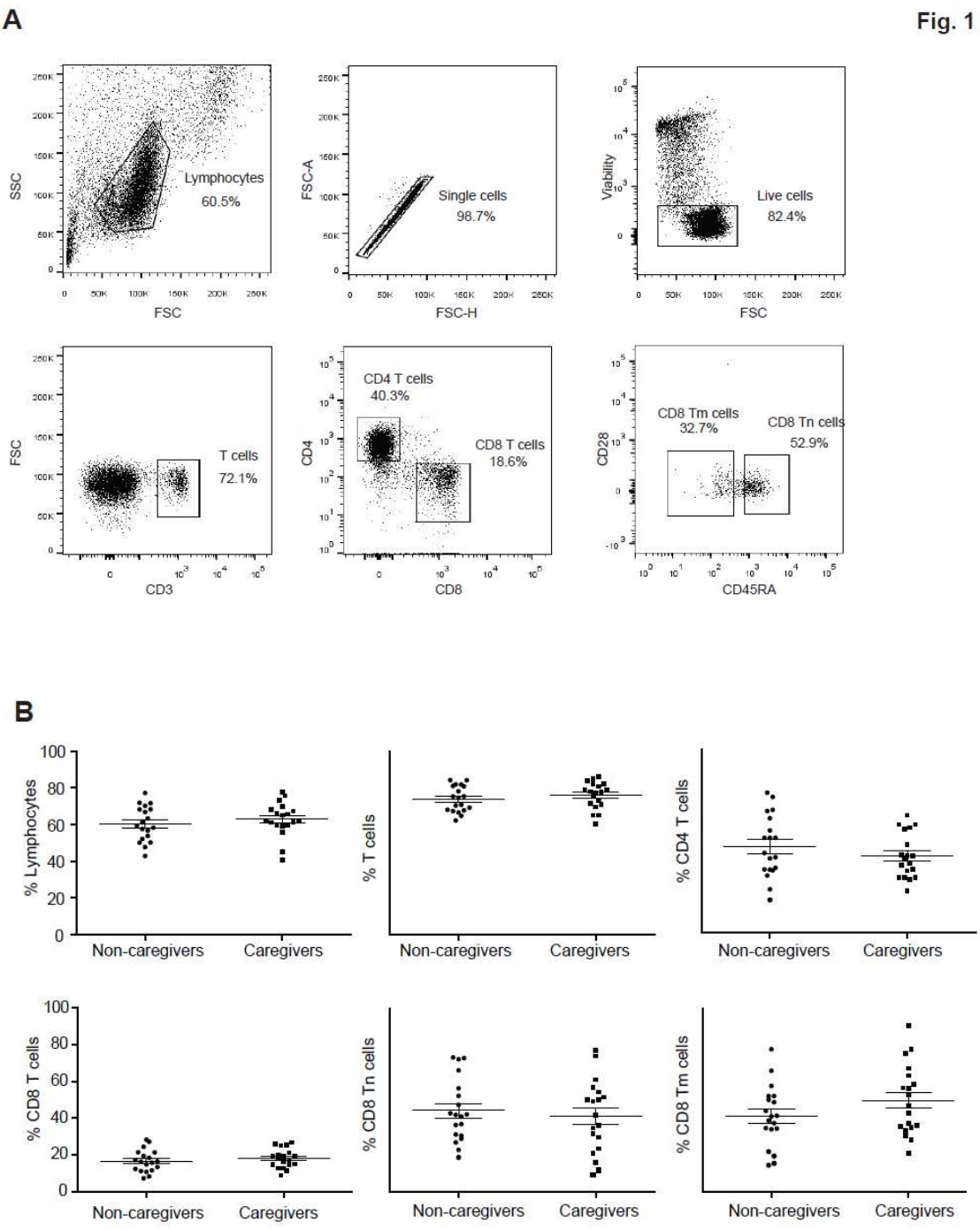

Figure 1. Adaptive immune cell composition is similar between controls and HSCT caregivers (A) A representative staining of PBMCs of controls and HSCT caregivers by flow

cytometry. Cells were gated first on lymphocytes followed by single cells, live cells, T cells, CD4 and CD8 T cells, and CD8 T cell subsets: naïve (Tn) and memory (Tm). **(B)** Comparison of immunophenotype of non-caregivers (N=20) and caregivers (N=21) included: lymphocytes, T cells, CD4 T cells, CD8 T cells, and naïve and memory CD8 T cells. Data illustrated as the percentage of the immune cell population in non-caregivers and caregivers. Results throughout the manuscript are presented as the mean ± SEM. * $p < 0.05$ was considered significant.

Fig. 2

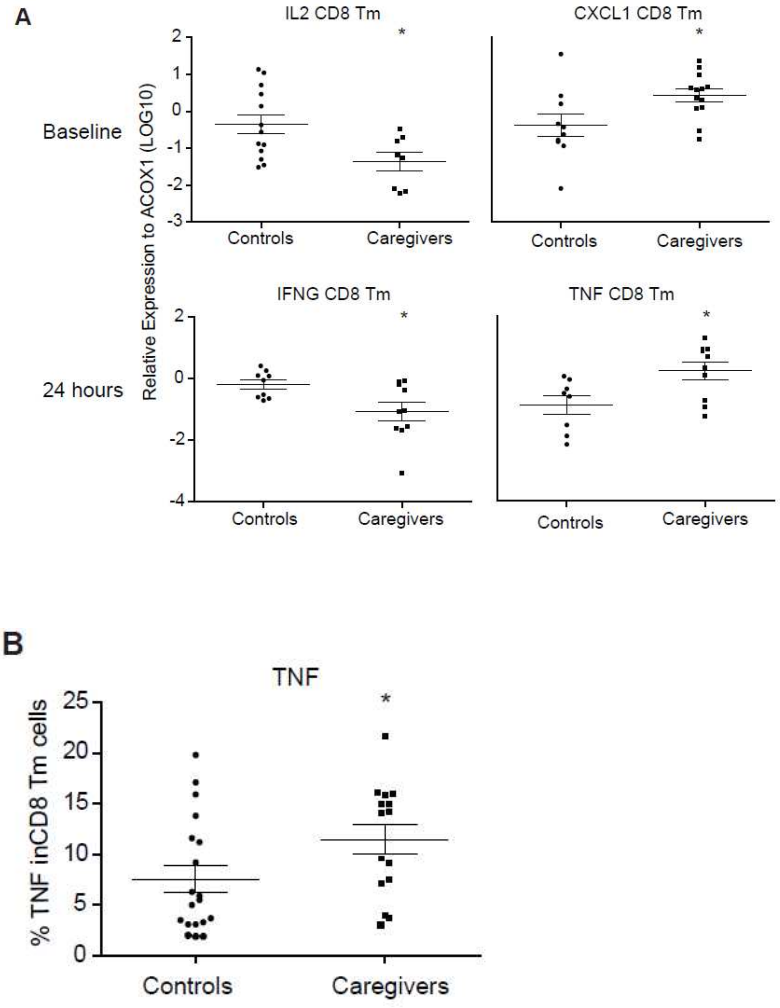

Figure 2. Caregivers show an increased pro-inflammatory gene expression and decreased proliferative related gene expression compared to controls (A) Gene expression changes in family caregivers (N=9-13) compared to their age- and gender-matched controls (N=7-9). Results are presented as the relative expression in LOG_{10}. **(B)** Intracellular level of TNF in CD8 Tm cells between controls (N=20) and caregivers (N=15). Data is presented as the percentage TNF in CD8 Tm cells.

CHAPTER 5

Overview

Provided is a summary of the study design and overall results for each article (Chapter 2, Chapter 3 and Chapter 4). Following this is a discussion of the major findings from this dissertation, including comparisons with relevant literature. Finally, provided is a discussion of the important areas for future research based on the findings.

Summary

This dissertation was a translational study from 'bench to bedside,' utilizing *in vitro* and *in vivo* models in order to explore the impact of stress via norepinephrine or caregiving on human CD8 T cell subsets (naïve, central memory and effector memory). We focused on the expression of the norepinephrine receptor (beta-2 adrenergic receptor) on CD8 T cell subsets, changes in cytokine and chemokine gene expression and protein level expression in each subset, and changes in proliferation. We utilized blood samples from apheresis donors at the National Institute on Aging (NIA) as well as PBMCs from 41 participants (21 HSCT family caregivers, 20 controls) of a primary investigation at the National Institutes of Health (NIH) Clinical Center.

Chapter 2 consisted of a comprehensive literature review exploring the literature regarding epigenetic regulation as a mediator of stress-induced psychological and behavioral factors, and immunological changes in T cell function. Results from this review support a new area of study for chronically stressed individuals, including family caregivers. Additionally, we propose a framework to examine the opportunity of incorporating epigenetic regulation as a mediator between psychological and behavioral factors, and T cell function in the stress paradigm. Finally, we offer advice for future research in this area and potential obstacles to consider when exploring these types of research investigations in human populations like family caregivers.

Chapter 3 assesses the effect of NE on the functionality of different CD8 T cell subsets (naïve, central memory and effector memory). Results showed that memory cells (central and effector memory) express more beta-2 adrenergic receptors compared to naïve cells; consequently, memory CD8 T cells were significantly more affected by NE induced changes of gene expression *in vitro*. We also found via global gene expression analysis that NE induced an elevated expression of inflammatory cytokines and chemokines in resting and activated memory CD8 T cells, as well as reducing the expression of growth-related cytokines. Finally, we found individuals with high levels of NE in their serum had similar gene expression changes seen in vitro compared to the low NE group.

Chapter 4 assessed the effect of caregiving for stem cell transplant recipients on immunophenotype and CD8 T cell subsets (naïve and memory)-related cytokines transcription and translation *in vivo*. Results showed that caregivers showed an increase in gene expression of pro-inflammatory cytokines and a decrease in growth-related cytokines by memory CD8 T cells compared to non-caregivers.

Major Findings

Epigenetic regulation may serve as a mediator of stress-induced psychological and behavioral changes and T cell function

In this review of the literature, we examine current findings in humans supporting a connection between epigenetic regulation of T cell function as well as epigenetic changes induced by behavioral and psychological factors. We argue that epigenetic regulation may serve as a mediator between the psychological and behavioral changes induced by caregiving stress and subsequent T cell functional changes. We also considered the challenges and possibility of examining epigenetic regulation as a potential pathway for stress-induced immune alterations in the family caregiver population and presented a theoretical model illustrating the relationship between caregiving, behavioral and psychological factors, epigenetic regulation, and immune outcomes.

We found significant literature currently exists in humans linking environmental stressors influencing psychosocial and behavioral factors that alter the epigenetic landscape (Alegria-Torres *et al.*, 2011;Brockie, Heinzelmann, and Gill, 2013;Essex, Boyce, Hertzman, Lam, Armstrong, Neumann, and Kobor, 2013); similarly, we found literature linking epigenetic modifications and immune cell function (Adachi and Rothenberg, 2005;Kondilis-Mangum *et al.*, 2013;Reiner, 2005). Many of the stress-related psychosocial and behavioral factors linked to epigenetic changes were also seen in family caregivers (Brockie *et al.*, 2013;Mifsud *et al.*, 2011;Uddin *et al.*, 2010). A majority of stress-induced changes in the epigenetic landscape were studied in early life or in utero, leaving a gap in our understanding of how stress in adulthood, like caregiving, can alter the epigenome (Essex *et al.*, 2013;McGowan, Sasaki, D'Alessio, Dymov, Labonte, Szyf, Turecki, and Meaney, 2009;Weaver, Cervoni, Champagne, D'Alessio, Sharma, Seckl, Dymov, Szyf, and Meaney, 2004). This gap presents an opportunity for future research to make the connection between stress-induced psychosocial and behavioral changes, as well as immune alterations in humans.

This review of the literature also revealed available methodologies for epigenetic studies and the associated potential challenges that can be encountered in conducting this type of research. The literature review showed utilization of whole blood or PBMCs, which limits the conclusions one can draw from the source of epigenetic changes since the exact immune cell subset is unknown (Hunter, 2012;Jensen *et al.*, 2012;Murgatroyd *et al.*, 2011). Conducting large data analyses of epigenetic and subsequent transcriptional changes in immune cells require large numbers of isolated cells from patients; this poses a significant obstacle for human studies since participants would need to donate large quantities of blood through donation methods such as apheresis donation, which require time and specialized supervision by certified nursing staff. In addition, future studies would need to be longitudinal since epigenetic changes can be transient (Madrigano, Baccarelli, Mittleman, Sparrow, Vokonas, Tarantini, and Schwartz, 2012). While there are several obstacles to conducting human research on epigenetic changes and immune

function, this is a necessary pursuit since the epigenetic pathways of stress-induced alterations in immune function remain to be elucidated.

Finally, we offered a framework linking stress-induced changes in psychosocial and behavioral factors with immune changes by epigenetic mediation. Several theoretical models have been presented in the literature review as models of stress and health, but they lack the modern understanding of molecular mechanisms, including epigenetics as a factor controlling gene expression in humans and how this might play a role in stress and health outcomes (Ingram *et al.*, 2005;Lutgendorf *et al.*, 2003;McEwen, 2012). This work draws attention to the potential role of epigenetics as a mediator of stress-induced immune changes and the potential obstacles one can face in conducting human studies to better understand how stress gets 'under the skin' to alter immune outcomes in stressed populations, such as family caregivers.

Memory CD8 T cells are more susceptible to norepinephrine than naïve cells

Chapter 3 of this dissertation explored the effects of norepinephrine (NE) on human CD8 T cell subsets (naïve, central memory, effector memory). Results provided evidence that memory subsets (central and effector memory) of CD8 T cells had greater expression (~40%) for the NE receptor, beta-2 adrenergic receptor, than naïve cells (~10%). Furthermore, we found NE induces a pro-inflammatory state by increasing inflammatory cytokine production while simultaneously decreasing activation-induced proliferation of memory CD8 T cells. We found these changes in gene expression were mimicked at the protein level in memory CD8 T cell subsets. Finally, we showed that a high level of NE in the serum of humans was associated with an increased expression of inflammatory cytokines and low expression of growth-related cytokines in memory CD8 T cells. We were the first to report memory CD8 T cells have greater expression of the NE receptor and are more susceptible to the effects of NE including a pro-inflammatory status before and after antigenic challenge.

Other studies have showed the differential expression of the beta-2 adrenergic receptor on immune cells (Anstead *et al.*, 1998;Bartik, Brooks, and Roszman, 1993); the majority of

studies have focused on CD4 and B cell expression of beta-2 adrenergic receptor while few have studied CD8 T cells (Kin *et al.*, 2006;Kohm *et al.*, 2001). Studies have also shown the link between chronic stress and inflammation, as mediated by neurotransmitters and hormones (Carlson *et al.*, 1989;Levite, 2000;Straub *et al.*, 1998). NE has been implicated in the inflammatory response in studies on CD4, NK cells and B cells in regards to specific inflammatory cytokines (Dhabhar *et al.*, 2012;Dimsdale *et al.*, 1994;Mausbach *et al.*, 2005). Our study used a global gene expression analysis to examine all the possible genes influenced by NE in memory CD8 T cells and found several pro-inflammatory cytokines were increased, while growth-related cytokines were decreased. Limitation in cell number, finances, and time prevented us from extending the global gene expression analysis to other immune cell subsets, as well as conducting epigenetic studies of these cells. Future studies would benefit from our approach of studying isolated immune cell subsets to draw out the impact of NE on these specific cell types without the influence of outside variables. In addition, this study is important in showing that individuals may be at greater risk for previously encountered antigenic challenge-related infections and not new infections since memory cells were more impacted by NE than naïve cells. This may have a particularly important role in the elderly since memory cell populations increase with age while naïve cell populations decrease.

Individuals with high levels of NE in their serum mimic in vitro results of a pro-inflammatory state

In Chapter 3, we found individuals with high levels of NE in their serum mimicked the changes we saw *in vitro* in memory CD8 T cells compared to the low NE level group. High levels of NE in the serum were associated with memory CD8 T cell increase in pro-inflammatory cytokines and a decrease in growth-related cytokines. Compared to our findings *in vitro*, these changes seen in the group with high levels of NE were influenced by multiple factors, aside from just NE. Still, it is impressive to find some inflammatory-related cytokines and chemokines in

adults with high levels of NE are also elevated in memory CD8 T cells compared to the low NE group, as we saw *in vitro*.

As noted by previous literature, it is important to address the likelihood that changes seen *in vivo* could be the result of a combined influence of hormones and neurotransmitters, including NE, that directly or indirectly alter immune function (Straub *et al.*, 2000;Straub *et al.*, 1998). The bidirectional communication between the immune and nervous system may modulate the effects seen *in vivo* that were not seen in an *in vitro* investigation. In addition, immune cells are influenced by autocrine and paracrine factors *in vivo* that ultimately influence their function, kinetics, and cytokine production. It is difficult to separate the influence of neuroendocrine factors and immune cell functions *in vivo.* Further work needs to be done to better understand the role of NE in moderating CD8 T cell subsets' function *in vivo*. Due to a laboratory error of mislabeled samples, we were unable to utilize all forty-one samples from our study, limiting sample size and hindering statistical significance. Unfortunately some of the NE samples were mislabeled by the laboratory staff, making it impossible to tell whether the sample belonged to a caregiver or non-caregiver. We had to eliminate donors for the NE measurement analysis that had mislabeled NE measures for one or more time points. Larger investigations with humans need to be conducted to see if our observations could be repeated.

Family caregivers of stem cell transplant patients have altered T cell function compared to matched controls

In Chapter 4 of this dissertation, we investigated differences between family caregivers of stem cell transplant patients and matched controls in terms of immunophenotype and CD8 T cell subsets (naïve and memory) expression of cytokines and intracellular cytokines. We did not find a significant difference in immune cell composition of caregivers compared to controls; however caregivers had increased expression of pro-inflammatory cytokines (TNF) and decreased expression of activation induced proliferative cytokines (IL2, IFNG). Intracellular level of TNF was

also increased at the protein level, though the other changes observed in gene expression were not seen in the intracellular cytokine measures.

Our findings concur with previous literature examining caregivers and matched control subjects to find caregivers' illnesses last longer, they had lower levels of IL-2 from CD4 T cells as well as an increase in IL-6 production (Kiecolt-Glaser *et al.*, 1987;Kiecolt-Glaser *et al.*, 1995;Kiecolt-Glaser *et al.*, 2003). Another study found caregivers of Alzheimer's patients had an altered immune composition with a 60% reduction in CD62L CD8 T cells (Mills et al., 1999); we did not see any significant change in immune composition in our caregiver population. In combination, these findings show that caregivers are at risk for a pro-inflammatory state which mimics that seen in aging (Gouin *et al.*, 2008;Weng *et al.*, 2012). Especially since these changes are seen in memory cells, caregivers may be at greater risk for opportunistic infections or infections with previously encountered antigens rather than new antigenic challenges that would be addressed by naïve cells. However, more work needs to be done with larger populations of caregivers and age- and gender-matched controls to verify these findings.

Limitations

There were a few limitations to this work. One of the first challenges we faced was how we conceptualized chronic stress in an *in vitro* and *in vivo* setting; it is difficult to mimic a chronic stress environment in culture since in real life we can examine months or years of exposure to a stressor as a chronic experience. In culture, cell viability and limitations of cell survival limit our ability to induce a truly 'chronic stress' experience. While we attempted to induce a prolonged stress experience in our cells by treating them with norepinephrine for 16 hours, we were limited in the dosage and time of exposure to be able to see changes in the cells, but not cause extensive cell death by over treatment. Future studies like ours should identify the conceptualization of chronic stress and realize the limitations of conducting *in vitro* experiments. Other studies may benefit from a similar model of our work by studying stress-induced changes both in a culture and real life environment.

While we were able to detect transcriptional, translational and functional changes in CD8 T cells after treatment with norepinephrine, we encountered challenges identifying the molecular changes, or epigenetic changes, behind these alterations in gene expression. Epigenetic methodology including chromatin immunoprecipitation assay (ChIP) requires significant amount of blood for isolation of CD8 T cell subsets to conduct the ChIP assay and is a challenging protocol in itself. Challenges with financial cost, protocol difficulty, adequate cell number and access to human donors limited our ability to conduct this experiment. Further work should be conducted in this area, though it is challenging to conduct, expensive and a time consuming process.

We were able to conduct a global gene expression analysis via microarray of central memory CD8 T cells and confirmation with RT-qPCR for the other CD8 T cell subsets (naïve and effector memory). While we would have liked to have done a microarray for the naïve and effector memory subsets as well, the cost of conducting a microarray and time-consuming nature of the assay and analysis prevented us from doing so. It may be worth in the future examining global gene expression changes in other immune cell subsets as well.

The study took place at the National Institutes of Health Clinical Center and Apheresis Unit at the National Institute on Aging, providing a diverse sample of participants. However, donors for the *in vitro* CD8 T cell work were a particularly healthy population, as selected for numerous studies at the NIH; thus, they may not be a representative sample of the general population, and particularly older adults since the NIA participants tend to be very healthy older adults. In addition, although we were able to detect statistical significance with our sample size for a few of the cytokines measured, we were limited in the number of donors from the caregiver and matched controls since this was a secondary study utilizing donors from a completed primary investigation at the NIH CC. We also were unable to salvage the majority of norepinephrine measures from these donors due to laboratory error, limiting our sample size further. In addition with our caregiver samples, we were only able to study our population by combining the blood samples from the pre- and post-transplantation collection time points in order to achieve

adequate cell number for experiments. It was impossible for us to get a baseline measure of individuals before they become caregivers; future studies may benefit from conducting longitudinal examinations of caregivers though this is a logistically challenging task. Still, longitudinal studies of individuals before, during and after they become family caregivers could provide valuable insight into the effects of chronic stress on immune outcome changes over time.

Future Research

This dissertation adds to the preliminary literature about chronic stress effects via norepinephrine or caregiving on CD8 T cell subsets (naïve and memory) function in humans. There are a variety of avenues for future research resulting from the major findings of our studies, which are outlined below.

Histone modifications and T cell alterations in chronically stressed populations

We have a very limited understanding of the mechanisms behind T cell alterations in chronically stressed populations. As discussed in Chapter 2, epigenetic modifications may serve as a mechanism and mediator of stress-induced changes in T cells, as well as other immune cell types. We cited previous studies that examined similar factors seen in family caregivers such as dietary alterations and traumatizing stress experiences that led to alterations in the epigenome and subsequent alterations in health outcomes. In order to understand if epigenetics plays a role in mediating the effects of chronic stress in family caregivers, studies must be completed with large populations of family caregivers and matched controls with isolated immune cell subsets, like CD8 T cells, to examine potential histone methylation markers and other epigenetic mechanisms that may moderate the pro-inflammatory response seen in this population.

Future research should also incorporate the large data analysis techniques that are now available. Technologies such as global gene expression analysis by microarray, as we conducted in Chapter 3, provide an opportunity to guide epigenetic work by knowing which genes are being over or under transcribed in individuals. The current methodologies for epigenetic studies require

a large number of cells, which poses a significant obstacle in obtaining a sufficient amount of blood from study participants. Technologies such as ChIP-Seq make it possible to analyze epigenetic modifications at a genome-wide level (Northrup *et al.*, 2011;Sharov, Dudekula, and Ko, 2005). While a significant amount of data will need to be analyzed by experts, these technologies can provide researchers insight into potential avenues to focus on in understanding stress-induced alterations in immune function as mediated by epigenetic changes.

Mechanisms behind stress induced changes in immune cells

Mechanisms behind altered immune responses can be direct by biological pathways or indirect, where stress leads to altered behaviors that in turn impacts immune responses. Our work focused primarily on the biological pathways that lead to immune dysfunction. To our knowledge, the examination of norepinephrine induced changes in gene expression, protein level and function in CD8 T cell subsets (naïve, central memory, effector memory) is the first of its kind. As described in detail in Chapter 2, we found memory CD8 T cells were more susceptible to the effects of norepinephrine than naïve cells due in part to their higher expression of the beta 2 adrenergic receptor. We also found individuals with high serum levels of norepinephrine mimicked some of the changes we saw in culture with a pro-inflammatory state. We contributed to the literature in illustrating how norepinephrine, one of the important hormones released during a stress response, can mediate immune function.

Other studies have examined how stress can lead to dysregulation of the immune response by studying the role of cortisol as a mediator between stress and vaccination responses. One study found an inverse relationship between cortisol levels and antibody responses to influenza vaccination in caregivers (Vedhara, Cox, Wilcock, Perks, Hunt, Anderson, Lightman, and Shanks, 1999), however they were unable to replicate this finding (Vedhara, Miles, Bennett, Plummer, Tallon, Brooks, Gale, Munnoch, Schreiber-Kounine, Fowler, Lightman, Sammon, Rayter, and Farndon, 2003). In another study children with the greatest cortisol increase had the lowest antibody responses to pneumococcal vaccination (Boyce, Adams,

Tschann, Cohen, Wara, and Gunnar, 1995). More studies with larger sample sizes need to be conducted to clarify if cortisol is a mechanism behind stress-induced altered immune function.

A few studies have focused on potential mediating mechanisms within the cell. For example, some focus has been on plasma and intracellular levels of cyclic AMP (cAMP) which is a low molecular weight substance that plays an important role in many control systems including acting as a second messenger after the binding of hormones to receptors on immune cells (Bonneau et al., 1990). cAMP has been shown to be able to inhibit lymphocyte proliferation, cytotoxicity, antibody production and cell migration. There have been studies on the increase of levels of cAMP in acute stress situations by beta adrenergic stimulation (Coffey and Hadden, 1985;Okada, Tokumitsu, Honma, and Ui, 1991); however we do not know the impact of long term stress. In addition, more work needs to be done on the exact role of cyclic nucleotides, like cAMP, in modulating immune function.

Coping moderates chronic stress-induced alterations in immune outcomes

Despite the reported poor health outcomes of family caregivers and other chronically stressed populations, there is little research on interventions to help moderate the stress-induced negative health alterations. For HSCT caregiver in particular, they are often most distressed at the time of transplant; thus, an intervention offering stress and coping skills may influence caregivers distress and improve their feelings of control (Laudenslager, 2014). Caregiver behavior is likely to benefit from interventions though the impact of coping interventions on processes like inflammation need to be elucidated (Laudenslager, 2014).

Furthermore, some caregivers may be ‘resilient’ or take on the responsibility of caregiving with less negative effects on their health compared to others; still, some caregivers are likely to require greater intervention since they may be a more vulnerable subset of caregivers compared to others (Brown, Smith, Schulz, Kabeto, Ubel, Poulin, Yi, Kim, and Langa, 2009;Kim, Schulz, and Carver, 2007). Further study needs to be done to examine different subsets of caregivers and the role of coping interventions on these groups. In addition, future work should examine the

effects of coping interventions for caregivers or the type of caregiver (vulnerable or resilient) on the patient's health outcomes. The patient-caregiver dyad is an important relationship to examine, particularly in examining interventions to assist caregivers in better coping with their role (Bevans et al., 2011). One would assume a healthy and prepared caregiver would perform their duties better compared to a stressed caregiver; however this has rarely been examined in measurable physiological outcomes in caregivers or patients.

Notably, much of the work on caregivers have focused on their response to vaccination; many of these studies have been observational in nature and provided information on how stressed individuals respond to vaccination compared to non-stressed individuals (Glaser *et al.*, 1998;Gouin *et al.*, 2008;Kiecolt-Glaser *et al.*, 1996;Vedhara *et al.*, 1999). A few studies examined the impact of coping interventions on vaccination including an emotional disclosure intervention on responses to Hepatitis B vaccination and found participants who completed the intervention had higher levels of Hepatitis B antibodies than controls 4 to 6 months later, though no immediate difference was seen post-vaccination (Pennebaker and Beall, 1986). In another study, caregivers of dementia patients underwent a stress-management intervention and found caregiver groups reported higher levels of distress before and after the intervention, however significantly more of the intervention caregivers generated a clinically appropriate response to the influenza vaccine compared to caregivers who did not undergo the intervention, and a fourfold increase in levels that exceeded the non-caregiver control group (Vedhara *et al.*, 1999;Vedhara *et al.*, 2003). Finally, a study examined the impact of a meditation intervention on influenza virus vaccination and found individuals who participated in the meditation had greater influenza virus antibodies in two follow-up periods compared to those who did not receive the intervention (Davidson, Kabat-Zinn, Schumacher, Rosenkranz, Muller, Santorelli, Urbanowski, Harrington, Bonus, and Sheridan, 2003). However more studies need to be done to determine the efficacy of these types of interventions since it has been suggested that the observed improvement in vaccination response may not be unique to the coping interventions (Smith, 2004). Still, these types of studies are

crucial since they can provide information that may aid in improving the effectiveness of vaccination programs, particularly for stressed populations.

Conclusion

In summary, this dissertation explored the impact of stress on gene expression, protein levels and function of CD8 T cell subsets by examining the effect of a stress released hormone, norepinephrine, and effect of caregiving of family caregivers of hematopoietic stem cell transplant recipients. We performed a review of the literature on epigenetic mechanisms as potential mediators of stress-induced changes in T cells, particularly in family caregivers, and discussed potential challenges and new technologies that are important for researchers conducting this type of research, as well as a proposed framework illustrating the role of epigenetic changes that had not been present in existing models of stress and caregiving and immune function. We discovered that not all immune cells respond similarly to the stress hormone, norepinephrine: memory CD8 T cells were more susceptible to the effects of norepinephrine and expressed more of the receptors for norepinephrine compared to naïve cells. We were the first to conduct a global gene expression analysis via microarray of norepinephrine treated cells and found norepinephrine induced a pro-inflammatory state before and after activation, as well as decreased expression of growth-related cytokines and chemokines after activation. We found individuals with high levels of norepinephrine in their serum mimicked the changes we saw in culture with an increase in pro-inflammatory cytokines and chemokines. Finally, we found family caregivers of hematopoietic stem cell transplant recipients were prone to an increased pro-inflammatory cytokine production by memory CD8 T cells.

This dissertation builds on previous research examining the impact of stress on immune health with a specific focus on CD8 T cell subsets. Future research should explore how different subsets of immune cells may respond differently to stress or stress-related hormones, how different stress hormones impact immune cells, and the long term consequences of chronic

stress on immune health outcomes for a better understanding of how exposure to stress can modulate immune function in adults.

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