THE EFFECT OF CHRONIC PSYCHOLOGICAL STRESS ON THE CUTANEOUS IMMUNE RESPONSE IN THE DEVELOPMENT OF A CONTACT HYPERSENSITIVITY REACTION

THESIS

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CHAPTER I

INTRODUCTION

Psychological stress has been reported to impact various physiological functions in living animals, including alterations to the nervous and endocrine systems. Furthermore, accumulating evidence shows that generation of psychological stress can hinder or exacerbate the immune response, thus affecting disease outcome or disease manifestation [1,2]. The skin represents the largest bodily organ and acts as the first line of defense against daily environmental insults. Additionally, there is interplay between the cutaneous immune response and psychological stress [3,4]. Contact hypersensitivity (CHS) is a model of the widespread skin disease, allergic contact dermatitis (ACD). ACD is responsible for 20% of work-related health diseases and results in 4 million lost work days per year in the United States [5].

CHS has been used to study the effects of stress on the cutaneous immune response; however, most studies to date have focused on the effects of acute psychological stress. The goal of this study was to examine the effects of chronic psychological stress on CHS and the mechanism by which chronic psychological stress exerts its effects on cutaneous immunity during a CHS reaction.

We hypothesize that chronic restraint stress suppresses the cutaneous immune response in a CHS reaction in a persistent manner through dysregulated cell trafficking.

1.1. Conceptual basis for this study and hypothesis

Although it is generally believed that psychological stress has a detrimental and lasting impact on the immune system [2,6], increasing studies show that acute periods of stress and chronic periods of stress can yield opposing effects on the immune response. This phenomenon is evident in skin disease, for which there exists a strong association with psychological stress [3,4]. Acute stress has been shown to enhance cutaneous immunity during a CHS reaction [7-9] whereas studies concerning chronic psychological stress and CHS have generated conflicting results. Fewer studies have been performed to examine the mechanism behind the impact of chronic psychological stress on CHS. This study was designed to study the effects of chronic restraint stress in female BALB/c mice in the elicitation of a CHS reaction and the mechanism in which cutaneous immunity is altered by chronic psychological stress.

Hypothesis: Chronic psychological stress suppresses the cutaneous immune response in a CHS reaction in a persistent manner through dysregulated cell trafficking.

1.2. Significance

Increasing evidence supports the concept that the presence of psychological stress can suppress or intensify the immune response [1,2]. For example, female patients with post-traumatic stress disorder (PTSD) have been shown to have enhanced cell-mediated immune function creating an increased risk for asthma, auto-immune disease, inflammatory bowel syndrome, and atopic diseases [10]. Additionally, there exists an interplay between the immune response in the skin and psychological distress [4,11]. Psychological stress is known to exacerbate several skin disorders including ACD, atopic dermatitis, rosacea, psoriasis, and acne.

Highlighting the link between psychological status and skin disease, Griesemer has developed the skin index which ranks the impact of emotional distress on various skin disorders [12].

In the clinical setting, depression has been shown to be independently linked with a heightened risk of psoriasis in female nurses in the United States [13] and likewise, those with psoriasis have been shown to have the mental functioning similar to individuals suffering from depression [14]. In fact, it was discovered that psychological stress following the 1995 Great Hansin earthquake in Japan was the root cause of exacerbated atopic dermatitis in the area [15]. Furthermore, 30% of psychiatric patients have associated dermatological diseases and 10% of individuals in a dermatology clinic were shown to possess co-occuring psychiatric disorders [12]. In addition, many psychotropic medications can lead to severe skin reactions, and many dermatological medications have been shown to have adverse psychiatric effects [16]. It has also been shown that the treatment of primary psychosomatic diseases and therapies focusing on countering and relieving stress have very promising results on associated dermatological disorders [12].

Like many skin disease, CHS is influenced by psychiatric distress mediated by the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) [17]. CHS is a model of the skin disease, ACD. ACD is responsible for 20% of work-related health diseases and a total of 4 million lost work days per year. This results in an annual cost of $400 million in the United States [5]. In fact, ACD is responsible for one of the highest rates of work-related skin disorders [18], and 15-20% of the general public suffers ACD to at least one chemical [19]. In the research field, CHS is often used as a model to study the impact of psychological stress on the cutaneous immune response and dermatological disease outcome due to its simplicity and convenience [20-22].

1.3. Overview of the physiological activation of the stress response

Psychological stress is perceived by the central nervous system (CNS) and influences the immune system through two major pathways, the HPA axis and the SNS [1,23,24]. A schematic of these systems and the resulting effects on the cutaneous immune response is shown in **Figure 1**.

The HPA axis initiates with the paraventricular nucleus (PVN) of the hypothalamus receiving signals that correspond to perceived dangers or stress. This results in the secretion of hormones including corticotropin-releasing hormone (CRH) from the hypothalamus, and CRH induces the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) [25,26]. ACTH then induces the secretion of glucocortiocoids like cortisol from the adrenal cortex [27]. Cortisol has several physiological functions including negative feedback and inhibition of the anterior pituitary and hypothalamus to reduce further secretion of CRH and ACTH. Additionally, cortisol stimulates the adrenal medulla to secrete catecholamines including epinephrine and norepinephrine [28]. Cortisol and catecholamines released by activation of the HPA axis have been shown at high concentrations to be immunosuppressive and low concentrations to be pro-inflammatory in reference to cutaneous immunity [8].

Neurogenic inflammation is mediated through the stimulation of the norepinephrine cells (NE) of the locus ceoruleus (LC) in the brain [23]. SNS activation results in the production of neuropeptide products such as substance P (SP), calcitonin-gene related protein (CGRP), and nerve growth factor (NGF). These neuronal mediators act on vascular endothelial cells producing vasodilation and increased permeability of vasculature, allowing for recruitment of circulating leukocytes [29-31]. In addition, CGRP and SP have been shown to bias dendritic cell (DC) differentiation of T helper (Th) cells to Th2 and Th17, respectively [32]. NGF has also

been shown to be released from inflammatory leukocytes to act upon peripheral sensory neurons, resulting in a positive feedback loop [32]. Mast cells play a large role in neurogenic inflammation through degranulation and production of IL-6, and have been implicated as major down-stream effector cells of SNS activation during restraint stress [33,34]. In addition, IL-6 and pro-inflammatory mediators like TNF-α and IL-1 have been shown to activate both the HPA axis and the SNS [23]. Furthermore, the SNS and the HPA axis have a positive, reverberatory feedback loop wherein activation of one system induces activation of the other [35].

Both the HPA axis and SNS have been shown to impact the innate and adaptive immune systems. Physical restraint is known to activate both the HPA axis and SNS in mice to culminate in a psychological stress response [17,21]. Some studies, however, suggest that the HPA axis may be a larger contributor in CNS modification of the immune response during CHS. Administration of corticosterone to adrenalectomized mice containing an intact SNS but non-existent HPA axis function resulted in the pronounced decrease in blood leukocyte percentages commonly seen in stressed subjects. Additionally, cyanoketone treatment (eliminating the corticosterone stress response) substantially diminishes stress-induced decreases of circulating leukocytes [36]. More studies are needed to fully understand the contributions of both of these pathways to not only the cutaneous immune response, but overall systemic immunity.

1.4. Overview of the immune response during a contact hypersensitivity reaction

CHS is a type IV delayed-type hypersensitivity and is an allergic skin reaction resulting from an exposure to low molecular weight haptens. The CHS reaction occurs in two phases termed the sensitization, or afferent, phase and the later effector, or challenge, phase. Haptens by themselves cannot initiate an inflammatory response, but conjugation to host proteins initiates an

immune response [37]. In the sensitization phase, this activation can occur through release of damage associated molecular pattern (DAMP) molecules like ATP and reactive oxygen species from damaged, haptenized cells. Innate immune activation may also occur through hapten-initiated breakdown of high molecular weight hyaluronic acid to low molecular weight hyaluronic acid, activating pattern recognition receptors [38]. Local DC's that have taken up the hapten-carrier complex or become haptenized themselves then travel to draining lymph nodes to prime a T-cell specific antigen response [39]. Memory and effector T cells then proliferate and enter circulation.

In the challenge phase, re-exposure to the chemical hapten elicits a delayed inflammatory response characterized by induration (hardening) and erythema (reddening) within 1-3 days following exposure [40]. During CHS and other cutaneous allergic responses, it has been shown that this re-exposure elicits a dual-phase T cell response. Early inflammatory responses are initiated by Th1 and IFN-γ producing $CD8^+$ cytotoxic T cells [41]. In one study examining CHS driven T cell populations in lymph node cultures, IFN-γ production from hapten stimulated T cells was limited to CD8 cytotoxic T cells, whereas $CD4^+$ T cells produced IL-4 and IL-10 with no observable IFN-γ production. This indicates that $CD8^+$ cytotoxic T cells are the major effector cells in the development of a CHS reaction, suggesting Th1 cells may not play a large effector role during CHS [42,43]. Effector cells, in particular $CD8^+$ T cells, infiltrate prior to observable inflammation and initiate keratinocyte apoptosis and skin damage [44]. The importance of $CD8^+$ cytotoxic T cells during a CHS response is evidenced by a complete elimination of inflammation in $CD8^+$ T cell depleted mice [45-47]. Furthermore, IFN-$\gamma R^{-/-}$ mice show reduced inflammation in response to hapten challenge and administration of IFN-γ in WT mice increased inflammation [7]. A role for IL-17 producing $CD8^+$ cytotoxic T cells during

CHS is beginning to emerge [19,37,48]. This cell type, in addition to IFN-γ production, has been shown to be a major contributor to inflammation induced during CHS and further cell recruitment [49].

At the peak of inflammation and decline of CHS, IL-4 (Th2) and IL-10 (T regulatory) producing $CD4^+$ cell infiltration increases [8]. These cells suppress and regulate CHS [42]. In fact, $CD4^+$ T cell depleted mice show increased inflammation during a CHS response [46,47]. A basic representation of CHS responses through the sensitization and effector phase is depicted in **Figure 2**.

Other tissue-resident immune cells or recruited cells also have important roles during CHS responses. Skin resident dendritic cells consist of dermal dendritic cells (dDC) and Langerhans cells (LC) in the epidermis. Both subsets are known to contribute to the initiation of CHS and T cell activation, but LC have been shown to have suppressive roles during CHS [50], specifically in the effector phase [51]. Other groups, however, have shown that specific depletion of LC in mice reduces CHS-driven ear inflammation but does not completely ablate the ear swelling response [52]. This suggests LC play a small pro-inflammatory role during CHS initiation, but dDC are still able to amount a significant inflammatory response in the absence of LCs. These conflicting studies may be linked to use of different contact sensitizers [50]. Other tissue resident antigen-presenting cells include dermal macrophages, and although they have been shown to traffic to lymph nodes during hapten application, their role during CHS is less understood [53]. However, ablation of $Gr\text{-}1^+$ cells (targeting monocytes and neutrophils) decreases inflammation due to CHS, highlighting the important role of either macrophages, neutrophils, or the contribution of both cell subsets. Another less studied contributor to skin inflammation and CHS is dermal γδ T cells which have been shown to activate mast cells and

induce neutrophil recruitment through IL-17 production in mice and humans [19,54]. Keratinocytes themselves have also been shown to have pro-inflammatory functions and the ability to secrete cytokines, including IL-1α, IL-1β, and TNF-α, also contributing to CHS [37,55]. Tissue damage in cutaneous inflammation has also been linked to the development of autoreactive Th17 cells [56].

Recently, it has been shown that *Rag2*$^{-/-}$ mice (lacking T and B cells) develop hapten-specific CHS mediated through natural killer (NK) cells that participate in adaptive immunity to contact sensitizing agents [57,58]. Although NK cell infiltration is observed at challenged sites in humans and WT mice, it has yet been shown that antigen-specific NK cell responses occur in human CHS [19]. In addition, inflammation mediated by NK cells in *Rag2*$^{-/-}$ mice differs from traditional T cell-mediated CHS by absence of cellular infiltrate in ear tissue and lack of upregulated markers of cytotoxicity and inflammation in the skin. Furthermore, the memory response of NK cells is transient compared to traditional $CD8^+$ T cell-induced inflammation upon hapten re-exposure [59]. NK cell involvement in traditional T cell mediated CHS remains unclear [19,60], but abnormal, adverse drug reactions may be attributed to NK cell activity and involvement [59].

Mast cells have also been shown to contribute to cutaneous inflammatory responses. Traditionally, upon cross-linking of the FcεRI, mast cells release pre-formed mediators such as histamine and tryptase upon degranulation and proceed to synthesize leukotrienes and prostaglandins. Specifically, prostaglandin D_2 synthesis has been shown to be inhibited by topical glucocorticoid application potentially disrupting the recovery of the cutaneous barrier in ACD [61]. IL-33 has been recently implicated as a potent mast cell activator through the receptor ST2, and is known to be released by keratinocytes and endothelial cells in psoriatic

lesions and during general skin damage [62,63]. The study of the specific role of mast cells during CHS has generated conflicting data, possibly due to inherent immunological defects in mast cell deficient mice or by compensation of other immune cells like basophils. Some studies have shown that both conditional depletion and constitutive deficiency of mast cells in mice leads to diminished CHS, whereas others indicate mast cells play a suppressive role through IL-10 production [19,60,64].

1.5. Acute stress and the cutaneous immune response

Acute psychological stress has been defined as isolated, single stress events that last only minutes to hours [65]. Acute psychological stress has been shown to enhance the cutaneous immune response in a CHS reaction resulting in increased pinna thickness when sensitizing agent is applied to the abdomen and challenged on the ear [8,9,66,67]. This enhancement has been shown to correlate with a decrease in circulating T cells, B cells, NK cells, and monocytes, but this decrease is not through leukocyte destruction or apoptosis [36,68]. In fact, decreases in circulating leukocytes have been hypothesized to be due to redistribution. The redistribution hypothesis explains that decreases in circulating immune cells correlate with increased extravasation into peripheral tissues like lymph nodes, spleen, and skin resulting in the enhanced inflammatory response observed [65]. Examination of circulating neutrophil percentages in these studies, however, has generated inconsistent and conflicting results [9,69]. Increased levels of serum corticosterone are also found after application of acute restraint stress [68,70].

While enhancement of CHS reactions can last up to nine months after application of acute stress, it is shown that circulating leukocyte number returns to baseline 3 hours after cessation of stress [36]. It has also been shown that IFN-γ, IL-1β, and TNF-α gene expression is

enhanced in acute stressed mice [36] whereas other groups have shown only moderate increases in TNF-α and IFN-γ protein with no modification to IL-1β protein levels [71]. The adhesion molecule CD62L has been implicated to respond to stress hormones including corticosterone, epinephrine, and norepinephrine in acute stress models, resulting in increased extravasation of leukocytes into tissue [65]. CD62L is expressed on different cell subsets, and this study focused on CD62L for cell subset identification rather than as a target for stress-induced alterations to immune cell trafficking.

1.6. Chronic stress and the cutaneous immune response

Chronic stress has been defined as several stress events that last multiple hours per day for a time period of several weeks or months [65]. Chronic stressors are thought to have persistent effects on immunity [6] and can have lasting impacts on other physiological functions like cardiovascular regulation up to three months after stress cessation [72]. Fewer conclusive studies have been conducted on the impact of chronic psychological stress on the cutaneous immune response however, and these studies have yielded conflicting results. Some groups have shown that repeated stress produces enhancement of CHS similar to acute psychological stress [22]. CHS enhancement following chronic stress was correlated with decreased blood lymphocyte and monocyte levels and increased neutrophil percentages [9]. In addition, erythema induced by delayed type hypersensitivities and skin barrier recovery were enhanced in individuals suffering from post-tramatic stress disorder (PTSD) [10].

Conversely, other groups have shown that chronic stress provides a suppressive impact on the cutaneous immune response and the immune response in general [6]. Chronic stress has been shown to decrease the inflammation and swelling causative of CHS in rodents [73]. These

results correlate, again to decreased circulating lymphocyte numbers that was less pronounced than with acutely stressed rodents [20]. Chronic stress-induced suppression of CHS reactions showed elevated levels of serum corticosterone, but attenuated corticosterone increase after several weeks of repeated restraint stress. This is hypothesized to be due to disruption of the circadian rhythm [36]. In addition to CHS, chronic stress has been shown to increase susceptibility to UV-induced squamous cell carcinoma (an immunogenic, non-melanoma skin cancer) in mice. This increased susceptibility correlated with decreased numbers of tumor-infiltrating $CD4^+$ T cells and increased numbers of infiltrating $CD25^+$ suppressor cells and $CD4^+CD25^+$ suppressor T cells in circulation [17]. Another group has shown that acute and chronic stress decrease leukocyte numbers in circulation, but these numbers return to baseline quickly after the stressing period. This group also showed that serum corticosterone returned to baseline within several hours after the chronic stressing period, raising the notion that the time in which measurements are taken may provide some reasoning behind the conflicting reports generated concerning chronic psychological stress [70].

Conflicting results in both acute and chronic stress studies may be due to use of different rodent models. Studies have shown that acute psychological stress and the impact on CHS varies between mouse strain, indicating genetic background may impact the correlation between skin inflammation and psychological stress [67]. This may also account for differences seen between rodent models utilizing rats and those utilizing mice. Additionally, it has been shown that the method of stress induction can have varying impacts on CHS [9]. Different sensitizing agents also induce varying CHS responses [40]. Studies concerning the effects of chronic psychological stress may yield conflicting results due to differing definitions of "chronic stress" [70]. Further

studies are needed to elucidate the impact of chronic stress on the immune response and how each of these variables contributes to this impact.

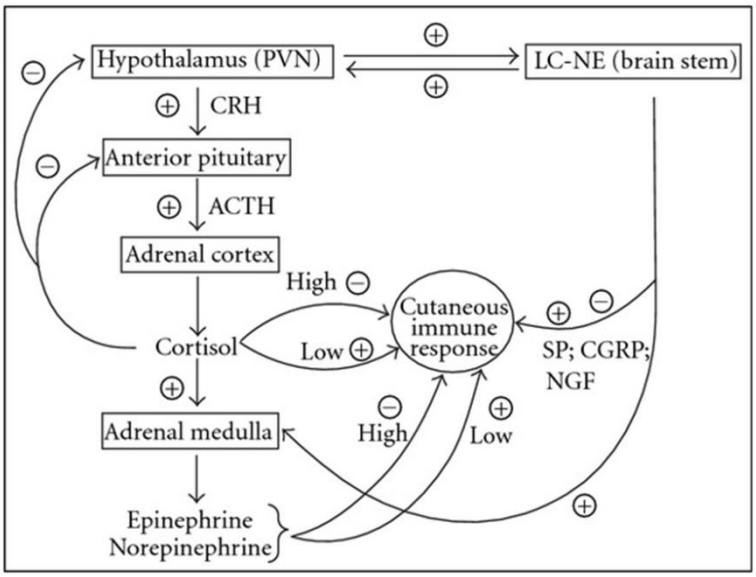

Figure 1. A schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) interaction with the cutaneous immune system. Stress signals induce release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus in the HPA axis. CRH induces adrenocorticotropic hormone (ACTH) release from the anterior pituitary. ACTH regulates glucocorticoid secretion from the adrenal cortex. Cortisol negatively regulates the hypothalamus and anterior pituitary and induces epinephrine and norepinephrine (NE) secretion from the adrenal medulla. Glucocorticoids, like cortisol, as well as epinephrine and norepinephrine enhance cutaneous immune responses at low concentrations and suppress immune responses at high concentrations. Stress signals also stimulate the NE cells of the locus coeruleus (LC) of the SNS. Neuropeptide products of the sympathetic response (substance P (SP), calcitonin gene-related peptide (CGRP), and cutaneous nerve growth factor (NGF)) have been shown to be pro-inflammatory and anti-inflammatory dependent on the immune cell type. There also exists a positive, reverberatory feedback loop between the HPA axis and LC-NE. Results show that the HPA axis and SNS both modify the cutaneous immune response [74].

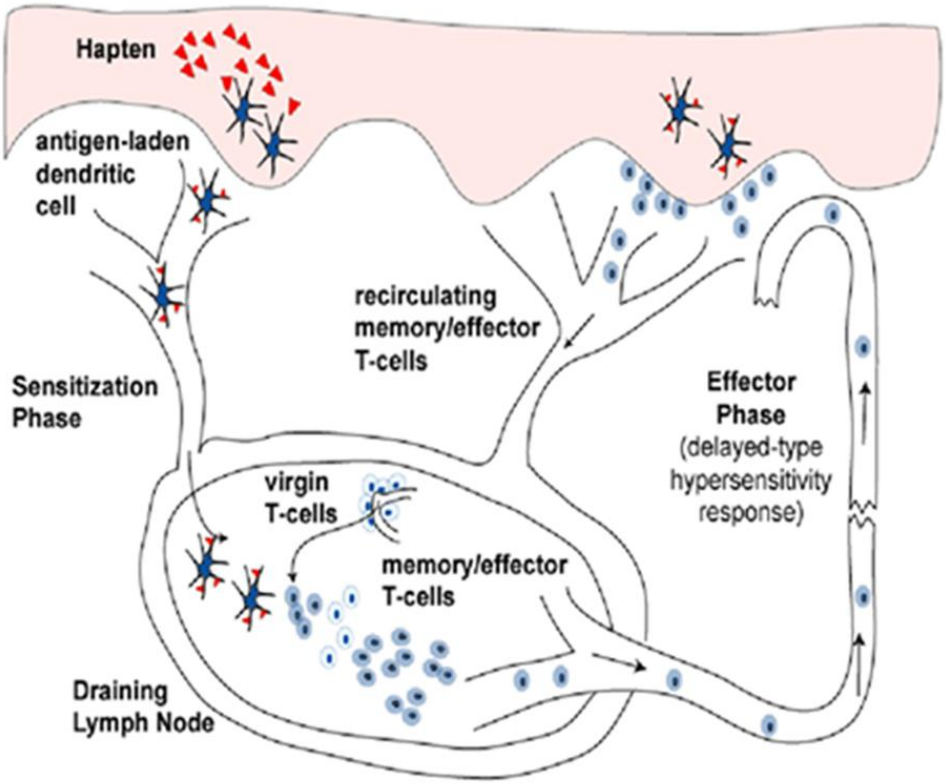

Figure 2. A basic representation of contact hypersensitivity (CHS) reactions. In the sensitization phase, hapten becomes covalently bound to self-protein which is then taken up by dendritic cells. These cells travel to the draining lymph node and present peptides from haptenized protein to naïve T cells which are activated to become memory and effector T cells. These activated T cells leave the draining lymph node and enter circulation. In the challenge or effector phase, re-exposure of hapten initiates an immune response clinically characterized by induration and erythema.

CHAPTER II

MATERIALS AND METHODS

2.1. Animals

Female BALB/c mice (4-6 weeks of age) were utilized for the following experiments. The mice were housed (4 per cage) at the University of North Texas Health Science Center animal facility. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were sacrificed via CO_2 inhalation overdose.

2.2. Chronic stress event

Chronic stress was conducted using a restraint stress model. Restraint stress was performed 2 hours every day for a period of thirty days [9,71]. Briefly, stressed mice were restrained in 50-mL conical tubes with proper ventilation. During the stress period, mice (stressed and control groups) were deprived access to food and water. Outside of the 2-hour stress period, food and water was provided *ad libitum*. Restraint stress was conducted at approximately the same time point each day. Weight gain was evaluated daily.

2.3. Resting period

Mice were allowed to "rest" for a period of 30 days with minimal handling following the restraint stress period. Restraint stress was not performed during this “rest” period. Food and water was provided *ad libitum* during this time.

2.4. Corticosterone measurement

Approximately 100 μL of blood obtained from the lateral tail vein was collected via heparin-coated glass capillary tubes. The blood was allowed to coagulate at 4◦C overnight and serum was isolated by centrifugation at 1000 rcf for 15 minutes. Corticosterone was evaluated by enzyme immunoassay (Enzo Life Sciences) per manufacturer's instructions. Final concentration of corticosterone was normalized to serum protein as determined by Bradford assay. Blood was drawn on day 0, day 20 of restraint stress, and 20 days in to the resting period (day 51).

2.5. Contact hypersensitivity reaction

Oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) (Sigma-Aldrich) was used as the contact sensitizing agent. Oxazolone has been shown to be a strong sensitizing agent [40], producing dramatic swelling responses 24-48 hours after re-exposure [47]. Briefly, an area of 2 x 2 cm was shaved on the abdomen of mice. Via pipette, 100 μL of oxazolone [1.25% (wt/vol) in 3:1 acetone:olive oil vehicle] was applied to the abdomen on day 25. Five days after sensitization (day 30), the mice were challenged on the right pinna. Via pipette, 12.5 μL of oxazolone [0.3% (wt/vol) in 3:1 acetone:olive oil vehicle] was applied to both outer and inner surfaces of the ear. The left ear was treated with 12.5 μL of the 3:1 acetone:olive oil vehicle on

both outer and inner surfaces of the pinna. Ear thickness was measured with calipers 24 hours after challenge (day 31). This process of sensitization and challenge was repeated after a period of rest on day 56 and 61, respectively. Ear thickness was measured on day 62.

2.6. H & E Histology

Both the vehicle and challenged ear from the stressed and control groups were harvested on day 31 and fixed in paraformaldehyde. The tissue was embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H & E) by the DermPrep facilities in Tampa, FL. Cell infiltrate in the dermis was visualized with an Olympus IX71 inverted research microscope. Three representative areas of the dermis in each image were examined and dermal cell infiltrate number was assessed for a total of 10 images per ear.

2.7. **In vitro** *T cell proliferation and glucocorticoid sensitivity*

On the day of sacrifice (day 31), spleens were harvested from all mice. The spleens were suspended in HBSS + 5% FCS and mechanically homogenized. After centrifugation, splenocytes were resuspended in a red blood cell lysis buffer and passed through a Mouse T-cell Enrichment Column Kit (R & D Systems) to isolate $CD3^+$ T cells. A total of 1×10^6 $CD3^+$ T cells per well were counted with Beckman Coulter Counter and incubated with CRPMI culture media + concanavalin A (conA) at 4 μg/mL. In addition, cells were incubated with increasing concentrations of corticosterone at 0.1 μM, 1 μM, and 10 μM corticosterone. After incubation at 37°C for 48 hours, T cell proliferation was assessed via MTS assay utilizing the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

2.8. **In vivo** ***cell proliferation***

On the day of oxazolone challenge (day 30), mice were injected i.p. with 0.1 mg BrdU per gram of body weight [75]. This injection was performed prior to challenge. A second i.p. injection of BrdU was performed one hour prior to sacrifice (day 31). On the day of sacrifice (day 31), draining lymph nodes from the ear (auricular lymph nodes) were collected in HBSS + 5% FCS, mechanically homogenized, and resuspended in HBSS. A total of 500,000 cells were counted with Beckman Coulter Counter and assessed for BrdU incorporation via the BrdU Cell Proliferation Kit (Millipore) according to the manufacturer's instructions.

2.9. T cell activation

Auricular draining lymph nodes were harvested and mechanically homogenized on day 31. Cells were resuspended in DMEM. Approximately 2.5 x 10^6 cells were stained with saturating amounts of the following antibodies: anti-CD4 PE (BD Pharmingen), anti-CD8 PE-TR (Abcam), anti-CD25 FITC (BD Pharmingen), and anti-CD69 PE-Cy7 (Biolegend). Cells were incubated for 15 minutes at 4◦C with antibody and anti-CD16/CD32 Fc receptor block (eBioscience) in 2% FCS plus 0.1% sodium azide in PBS. Detection and quantification was performed utilizing a Beckman Coulter flow cytometer.

2.10. T cell activity

Brachial, axillary, and inguinal lymph nodes were harvested and pooled per mouse on day 31. The lymph nodes were homogenized by mechanical separation. Cells (2.5 x 10^6) were counted with Beckman Coulter Counter and incubated at 37°C with and without anti-CD3 (BD

Pharmingen) (1μg/mL). Cell culture supernatants were collected after 48 hours and analyzed for IFN-γ production via ELISA (eBiosciences) per the manufacturer's instructions.

2.11. Circulating leukocyte assessment

On day 0 and day 31 of restraint stress, 100 μL of blood was drawn from the lateral tail vein via heparin-coated glass capillary tubes. Blood was collected in blood collection tubes containing EDTA to prevent coagulation and assessed utilizing a DrewScientific HEMAVET to examine total WBC and cell populations of lymphocytes, neutrophils, monocytes, eosinophils, and basophils. A mouse Multispecies Hematology Reference control was utilized to calibrate the results obtained.

2.12. Peripheral splenocyte cell populations

On the day of sacrifice (day 31), spleens were harvested and placed in HBSS + 5% FCS. Splenocytes were mechanically homogenized and placed in a red blood cell lysis buffer. Approximately 2 x 10^6 cells were stained with saturating amounts of the following antibodies: anti-CD11b FITC (Biolegend), anti-B220 PE (BD Pharmingen), anti-CD8 PE-TR (AbCam), anti-CD4 PE Cy-7 (Biolegend), and anti-Ly6C AF647 (Biolegend). Cells were incubated for 15 minutes at 4◦C with antibody and anti-CD16/CD32 Fc receptor (eBioscience) block in 2% FCS plus 0.1% sodium azide in PBS. Detection and quantification was performed utilizing a Beckman Coulter flow cytometer.

2.13. Cytokine assessment in inflamed ear

The right challenged ears were collected on day 31. The tissue was flash frozen in liquid nitrogen, mechanically homogenized, and resuspended in PBS + protease inhibitor (Thermo Scientific). After centrifugation, the supernatant was collected. The homogenized ear was analyzed via a Multi-Analyte ELISarray (Qiagen) per manufacturer's instructions. This allowed for relative quantification of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17a, IFN-γ, TNFα, G-CSF, and GM-CSF protein levels in inflamed ear tissue. Values were normalized to total protein as determined by Bradford assay.

2.14. Statistical analysis

Experimental groups were compared with unpaired two-tailed Student's *t* test unless noted otherwise in the figure legends. A *p*-value of less than 0.05 was considered significant in all cases.

CHAPTER III

RESULTS: THE IMPACT AND DURATION OF IMPACT OF CHRONIC RESTRAINT STRESS ON A CONTACT HYPERSENSITIVITY REACTION

3.1. Chronic psychological stress reduces contact hypersensitivity-initiated skin inflammation

The impact of chronic psychological stress on CHS responses was assessed utilizing the model depicted in **Figure 3**. Female BALB/c mice were chosen as this strain and gender have been shown to be particularly susceptible and responsive to psychological stress [67,76]. Restraint stress has been shown to produce the most dramatic alterations in CHS response when compared to forced swim, low temperature, isolation, and stress induced by handling [9]. In addition, restraint stress has been shown to activate both the HPA axis and SNS to culminate in a psychological stress response [17]. Therefore, this model was utilized to induce chronic psychological stress.

To verify that the chronically stressed mice were experiencing differential levels of psychological stress as compared to the control group, weight was measured daily and serum corticosterone was evaluated before and during the restraint stress period. Chronic stressors have been shown to induce metabolic disturbances such as decreased circulating leptin and elevated insulin resistance resulting in alterations in weight gain [77,78]. The mice undergoing daily restraint stress displayed a delay in weight gain as compared to the control animals (Fig. 4 A). Mice undergoing restraint stress also showed a significant elevation in circulating corticosterone

after 20 days of chronic psychological stress as compared to control animals (Fig. 4 B). These results indicate that the restraint stress model is inducing psychological stress, indicated by the delay in weight gain and HPA axis stimulation resulting in increased production of the glucocorticoid, corticosterone [26,79].

To assess the impact of chronic psychological stress on the cutaneous immune response, a CHS reaction was performed. On day 25 of restraint stress, all mice were sensitized on the abdomen with oxazolone and challenged on the ear five days later. Caliper measurements of the inflamed ear show a reduced inflammatory reaction induced by CHS in chronically stressed mice as compared to the control (Fig. 5). These results were substantiated by H & E histology of ear tissue (Fig. 6). These results indicate that chronic psychological stress suppresses the cutaneous immune response in a CHS reaction in female BALB/c mice.

3.2. The effects of chronic psychological stress are not persistent after a period of rest

Psychological stress has been shown to have a lasting impact on the body's physiological processes. The effects of acute stress on CHS has been shown to last several months [36]. Additionally, chronic stress has been shown to result in long-term dysregulation of the circadian rhythm [80] and immunity [6]. Lasting physiological dysregulation due to chronic stress has been hypothesized to result in several secondary health concerns including hypertension, atherosclerosis, and obesity [81]. Therefore, we wanted to examine the longevity of the impact of chronic psychological stress on CHS.

Chronically stressed mice were allowed to rest for a period of 30 days in which restraint stress was discontinued. After this period of rest, circulating corticosterone was no longer elevated in the restraint stressed group (Fig. 7 A). In addition, CHS-induced ear inflammation

was no longer suppressed in the stressed mice (Fig. 7 B). Therefore, these results show that chronic psychological stress does not have a lasting impact on corticosterone secretion or cutaneous immunity after 30 days of rest.

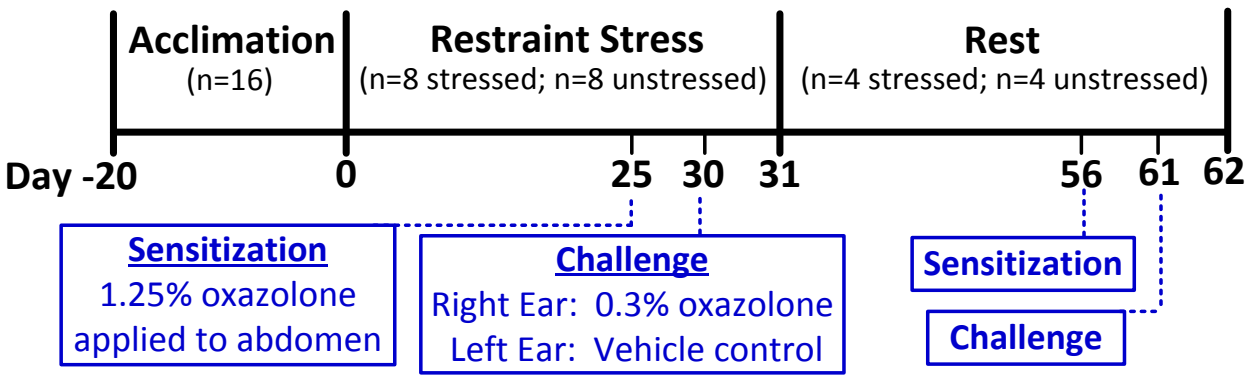

Figure 3. Experimental model for restraint stress and induction of CHS. Female BALB/c mice were acclimated to the environment for 20 days. Chronic restraint stress was performed for 2 hours each day for a total of 30 days. On day 25 of restraint stress, all mice were sensitized on the abdomen with oxazolone in vehicle. On day 30 of restraint stress, all mice were challenged on the right ear with oxazolone in vehicle and on the left ear with vehicle alone. Ear swelling was measured 24 hours after challenge. Restraint stress was discontinued for an additional 30 days. Sensitization and challenge was repeated and the ear swelling response was measured 24 hours later.

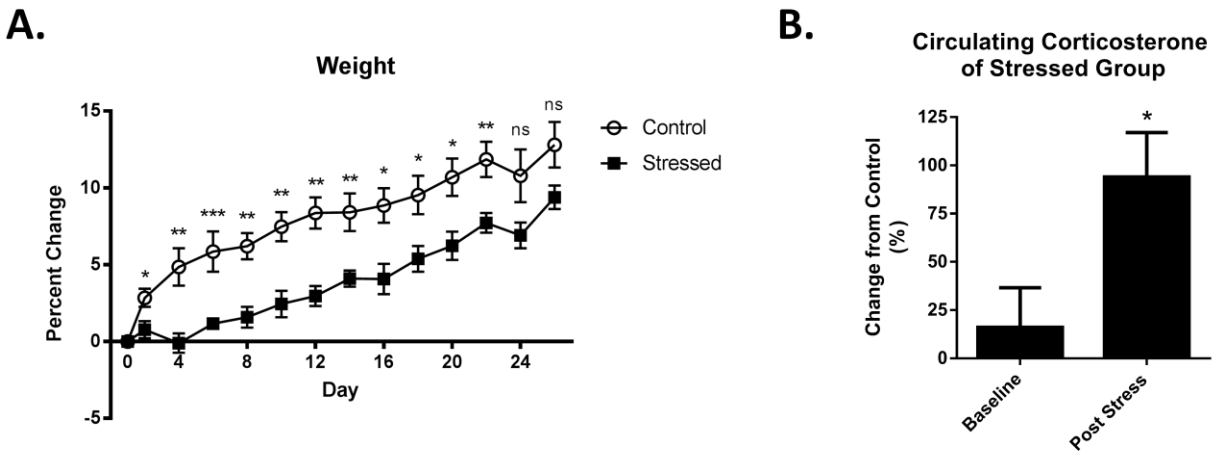

Figure 4. Chronic psychological stress induces a delay in weight gain and an elevation in serum corticosterone. (A) Weight of female BALB/c mice was assessed daily beginning from day 0 of restraint stress through the sensitization phase of the CHS response. Results are expressed as percent change of weight from starting weight at day 0, and measurements are shown every other day for clarity. Data are representative of three independent experiments. Results are expressed as the mean ± SEM (n = 8). (B) Blood attained from the lateral tail vein was assessed for serum corticosterone via EIA before the restraint stress period, designated "baseline", and on day 20 of the restraint stress period, designated "post stress". Corticosterone concentration was normalized to total serum protein. Data are expressed at the percent change of serum corticosterone in the restraint stress group from the control group. Results are expressed as the mean ± SEM and combined from two experiments (n = 16). Statistical analysis was performed by unpaired Student's *t* test. (ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001)

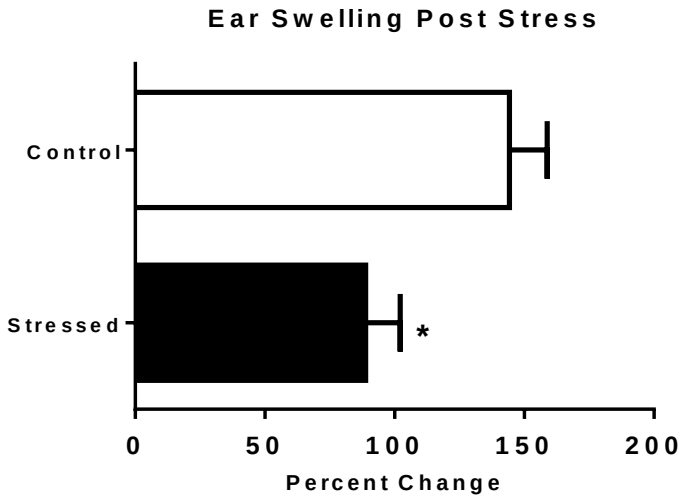

Figure 5. Chronic psychological stress reduces the ear swelling inflammatory response in CHS reactions. On day 25 of restraint stress, all mice were sensitized with 1.25% oxazolone (wt/vol) in 3:1 acetone:olive oil vehicle on the abdomen. On day 30, mice were challenged on the right pinna with 0.3% oxazolone (wt/vol) in 3:1 acetone:olive oil with the left ear receiving the 3:1 acetone:olive oil vehicle. Ear swelling was assessed by caliper measurement 24 hours following challenge. Results are expressed as the percent thickness of the right ear as compared to the left ear vehicle control. Results are representative of three independent experiments. Results are expressed as the mean ± SEM (n = 8). Statistical analysis was performed by unpaired Student's *t* test. (*$p < 0.05$)

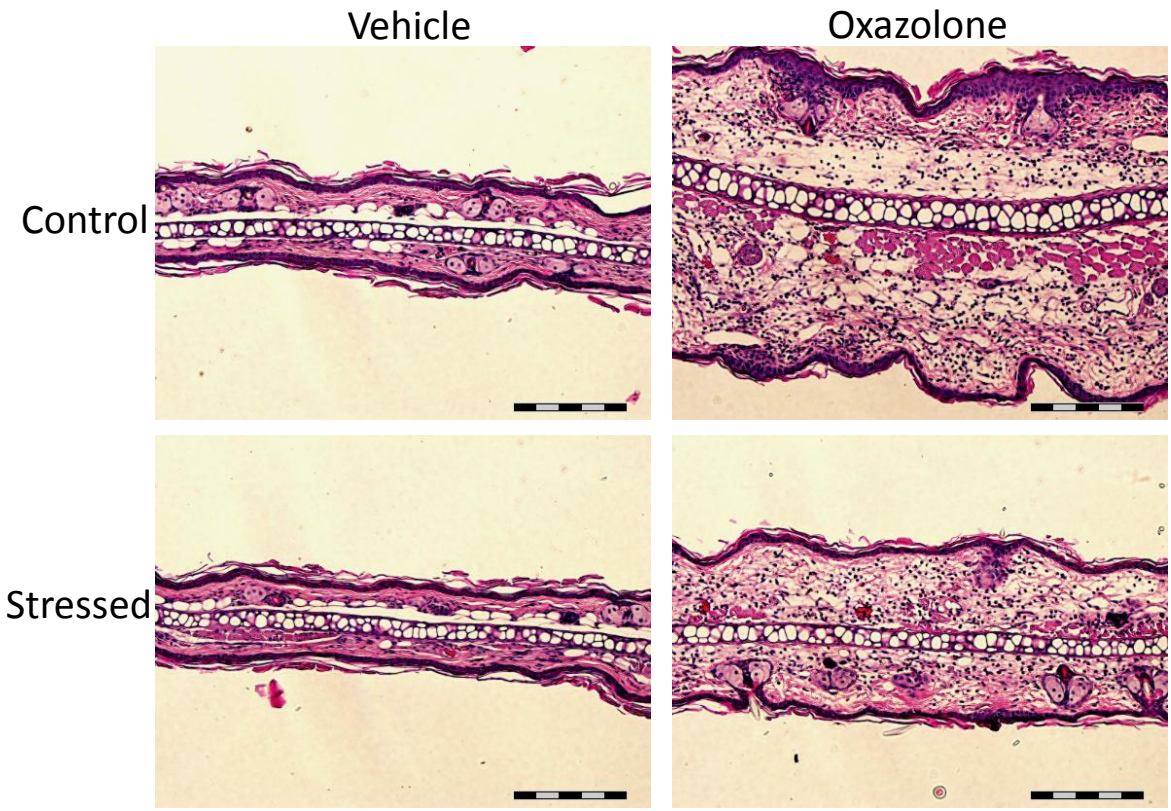

Figure 6. Chronic psychological stress reduces the ear swelling inflammatory response in CHS reactions. Ears were harvested on day 31 of restraint stress, 24 hours after elicitation of CHS. Tissue was paraffin-embedded, sectioned at 5 μm, and stained with H & E. Representative images of the designated ear tissue are shown at 200X. Scale is 200 μm.

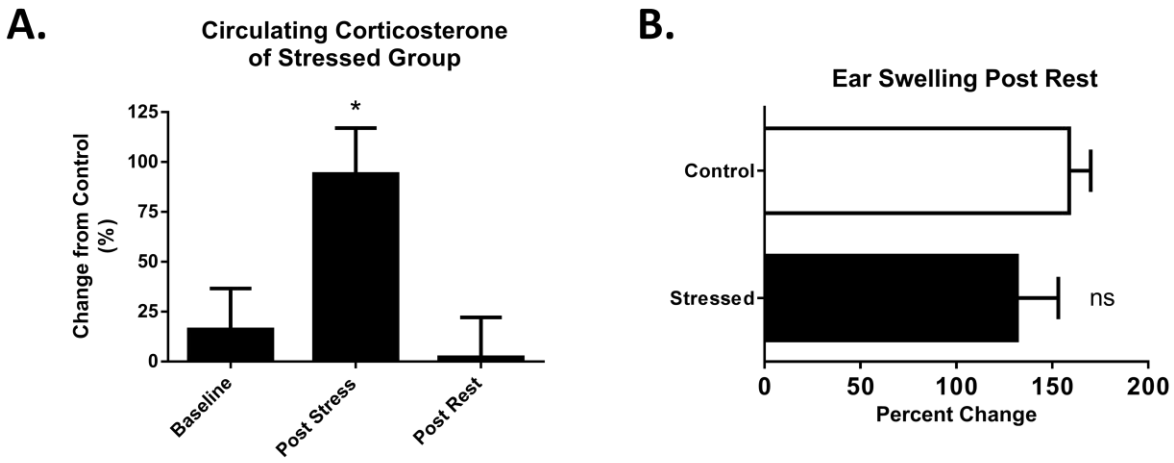

Figure 7. After 30 days of rest, the effects of chronic stress are no longer detected in the restraint stress group. (A) Blood attained from the lateral tail vein was assessed for serum corticosterone via EIA before the restraint stress period (baseline), on day 20 of the restraint stress period (post stress), and 20 days in to the resting period or day 51 (post rest). Corticosterone concentration was normalized to total serum protein. Results are expressed as the percent change of serum corticosterone in the restraint stress group from the control group. Results are combined from two experiments. Statistical analysis was performed via one-way ANOVA (n = 15-16). (B) On day 25 of rest, all mice were sensitized with 1.25% oxazolone (wt/vol) in 3:1 acetone:olive oil vehicle on the abdomen. Five days later, mice were challenged on the right pinna with 0.3% oxazolone (wt/vol) in 3:1 acetone:olive oil with the left ear receiving the 3:1 acetone:olive oil vehicle. Ear swelling was assessed by caliper measurement one day later. Results are expressed as the percent thickness of the right ear as compared to the left ear vehicle control. Statistical analysis was performed with unpaired Student's *t* test (n = 3-4). Results are expressed as the mean ± SEM. (ns, not significant; $*p < 0.05$)

CHAPTER IV

RESULTS: THE CELLULAR MECHANISM OF THE IMPACT OF CHRONIC RESTRAINT STRESS ON A CONTACT HYPERSENSITIVITY REACTION

4.1. Chronic psychological stress does not affect T cell proliferation or sensitivity to glucocorticoids

CHS is a cell-mediated allergic response, largely orchestrated by T cells, including Th1 and $CD8^+$ cytotoxic T cells [60]. Therefore the state of T cells after animal exposure to chronic psychological stress was assessed. Considering the elevated corticosterone in the stressed mice and studies that show that glucocorticoids suppress lymphocyte proliferation [82], T cell proliferation was assessed. Peripheral splenic $CD3^+$ T cells were isolated after chronic restraint stress. Overall mitogen-induced proliferation of T cells did not differ significantly between groups (Fig. 8 A). In the clinical practice, continual application of glucocorticoids has been shown to induce reduced T-cell sensitivity to steroids resulting in diminished inhibitory functions on T cell proliferation [83]. In addition, it has been shown that leukocytes from individuals under periods of prolonged stress have decreased sensitivity to glucocorticoids, possibly through downregulation of cortisol receptors on the cell surface [84]. Considering the chronic state of elevated corticosterone, T cell proliferation was evaluated under increasing concentrations of corticosterone. The results show that 10 μM and 1 μM corticosterone significantly reduces T

cell proliferation in both groups as compared to mitogen-induced proliferation, but this reduction in proliferation was not significantly different between groups. Therefore, we did not observe a change in T cell sensitivity to glucocorticoids (Fig. 8 A).

Due to inherent limitations in this *in vitro* model (i.e. stimulation outside the natural environment and the inability to determine if different rates of proliferation are due to inherent variations in the ability to proliferate or due to altered cell viability), an *in vivo* BrdU injection model was utilized to assess cell proliferation. Injections were performed on the day of oxazolone challenge and one day following challenge. The results support the *in vitro* data and show that there was not a significant change in BrdU incorporation in auricular draining lymph node cells (Fig. 8 B). In summary, the results suggest that chronic psychological stress does not alter induced T cell proliferation in chronically stressed mice nor do T cells become sensitized to increased corticosterone induced by chronic psychological stress.

4.2. Chronic psychological stress does not impact T cell activity or activation but induces a dysregulation in T cell trafficking.

To further study the potential effects of chronic stress on T cells, T cell activity and activation was evaluated. It has been shown previously that stress can alter T cell activity, specifically in response to CD3 T cell receptor stimulation [85]. The results show that INF-γ production of anti-CD3 stimulated T cells from pooled axillary, brachial, and inguinal lymph nodes was not significantly different between the stressed and control group (Fig. 9 A). It has also been hypothesized that stress may impact lymphocyte activation, having downstream effects on the generated immune response [86]. Therefore, auricular draining lymph nodes from the ear were assessed by flow cytometry for CD25 and CD69 activation expression on $CD4^+$ and $CD8^+$ T

cells. A statistical difference was not observed in percentage of activated $CD4^+$ or $CD8^+$ T cells induced by chronic psychological stress (Fig. 9 B). However, there was a significant elevation in the percentages of total $CD4^+$ and $CD8^+$ T cells in the auricular draining lymph nodes of the chronically stressed group (Fig. 9 B). These results suggest that chronic psychological stress impacts T cell trafficking.

In summary, the results suggest that chronic psychological stress does not alter basal level T cell activity or activation induced by a CHS reaction. The results do suggest that chronic psychological stress may be imparting a dysregulation in the trafficking of $CD4^+$ and $CD8^+$ T cells.

4.3. Chronic psychological stress reduces the number of circulating white blood cells

It is known that stress can impact circulating white blood cell numbers [6]. During periods of acute stress, it has been shown that decreased leukocyte circulation leads to an increase in inflammation induced by CHS. This has been hypothesized to be due to an increase in immune cell redistribution to the periphery and to the skin [65].

Hematological analysis before the restraint stress period indicated that both groups had similar total white blood cell (WBC) numbers in addition to discrete blood cell populations (data not shown). After chronic psychological stress and CHS, the stressed group of mice displayed significantly lower percentages of overall WBCs, lymphocytes, and monocytes as compared to the control (Fig. 10). The stressed group and control group displayed an increase in circulating neutrophil number in response to CHS. While the stressed group appeared to not reach the same magnitude in neutrophil increase, this difference was only marginally significant. Total circulating WBC number was reduced in the stressed mice, differing from the moderate elevation

in WBC in the control group. Circulating monocyte number in the stressed group was also decreased in contrast with the elevation of monocyte number in the control group. The stressed group displayed a similar trend in decreased lymphocyte number, however the magnitude of this reduction is significantly greater. There was not a significant difference in circulating basophils or eosinophils. Therefore, the results further indicate chronic psychological stress driven dysregulation of immune cell trafficking during a CHS response.

4.4. Chronic psychological stress does not impact splenic cell profile during a CHS response but does influence dermal infiltrating cells

As mentioned, stress driven decreases in circulating WBC numbers have been hypothesized to be the result of redistribution of these immune cells to peripheral sites like lymph nodes, spleen, and the skin [65]. Considering the data that chronic stress decreased circulating WBC number and resulted in increased T cell percentages in the draining lymph node, the cellular profile in the spleen was assessed to discern similar increases of cells in peripheral tissue.

The results show, however, that there are no significant differences in percentages of B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, or monocytes between the stress and control group in the spleen (Fig. 11). However, analysis of the H & E histology revealed decreased dermal cell infiltrate per area in the chronically stressed group (Fig. 12). These data suggest that chronic stress does not impact global immune cell trafficking and may be influencing trafficking of immune cells specific to the CHS reaction itself.

4.5. Cytokine profile at site of inflammation indicates reduced IFN-γ production

The data thus far indicate a dysregulation in cell trafficking and skin infiltration in response to a CHS reaction. Therefore, the cytokine production at the site of inflammation was examined to assess specific alterations to immune cells activity. For this assessment, a multi-analyte cytokine array was utilized to compare relative concentrations of common inflammatory cytokines in inflamed ear tissue from both groups. There was no discernible difference between relative amounts of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17a, TNF-α, G-CSF, or GM-CSF in inflamed ear tissue (Fig. 13). However, chronically stressed mice displayed decreased amounts of IFN-γ in inflamed ear tissue as compared to the control.

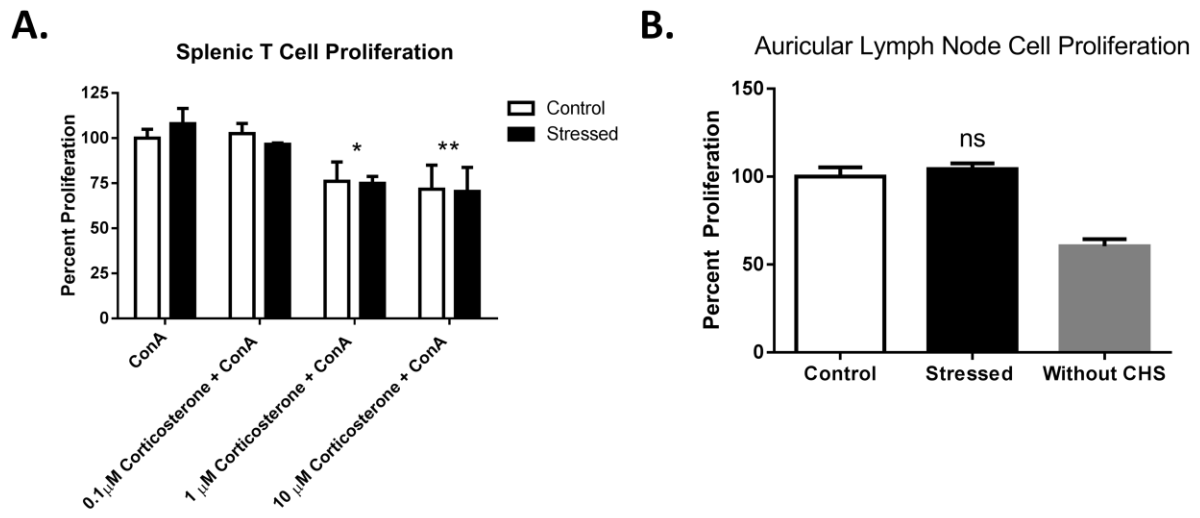

Figure 8. Chronic psychological stress does not affect T cell proliferation or sensitivity to corticosterone. (A) Spleens were harvested on day 31 of restraint stress. $CD3^+$ T cells were isolated and proliferation was induced with concanavalin A (4 μg/mL) in the presence of 0.1 μM, 1 μM, and 10 μM corticosterone. T cell proliferation was assessed via MTS assay. Results are normalized to percent of control cell proliferation with conA in four replicates. Unstimulated cells were subtracted as background. Results are representative of two independent experiments. Statistical analysis was performed by two-way ANOVA with Sidak's multiple comparisons test. Results are expressed as the mean ± SEM (n = 4). (B) BrdU was injected i.p. (0.1 mg BrdU per gram body weight) 24 hours and 1 hour prior to sacrifice. Auricular draining lymph nodes were harvested and homogenized by mechanical separation. BrdU incorporation as an assessment of proliferation was detected via ELISA. Control population without stress or CHS is designated "Without CHS". Results are normalized to percent of control cell proliferation with three replicates. Statistical analysis was performed by unpaired Student's *t* test. Results are expressed as the mean ± SD (n = 4). (ns, not significant; $*p < 0.05$; $**p < 0.01$)

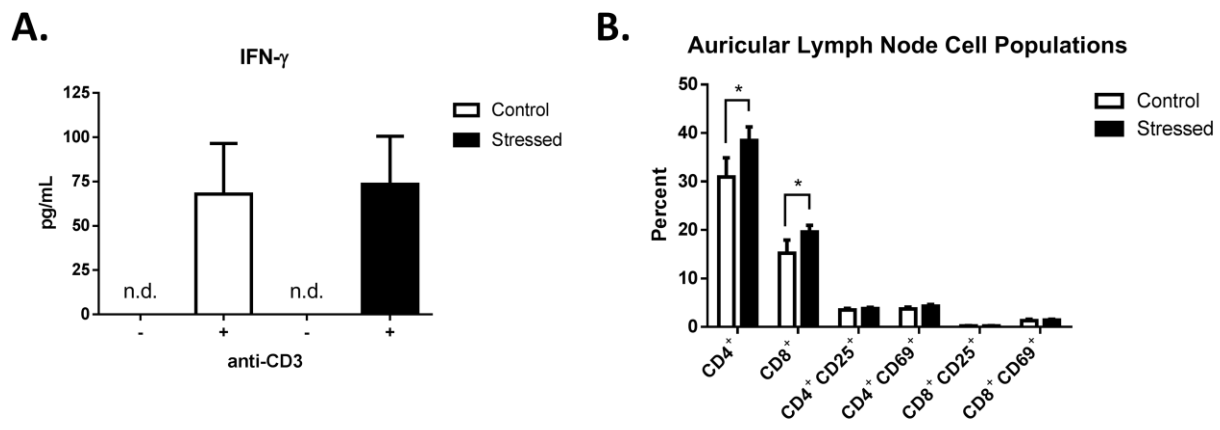

Figure 9. Chronic psychological stress does affect T cell activity or activation but impacts T cell trafficking. (A) Brachial, axillary, and inguinal lymph nodes were pooled per mouse 24 hours after challenge (day 31 of restraint stress). 2.5 x 10^6 cells were plated with and without anti-CD3 (1µg/mL). Cell culture supernatants were analyzed for IFN-γ concentration via ELISA. Results are expressed as the mean ± SD (n = 8). (B) Auricular lymph nodes were harvested 24 hours after challenge (day 31 of restraint stress) and homogenized by mechanical separation. Cells were stained with anti-CD4, anti-CD8, anti-CD69, and anti-CD25 and assessed by flow cytometry. Results are shown as percent of live cell gate. Statistical analysis was performed by unpaired Student's *t* test. Results are expressed as the mean ± SEM (n = 4). (*$p < 0.05$)

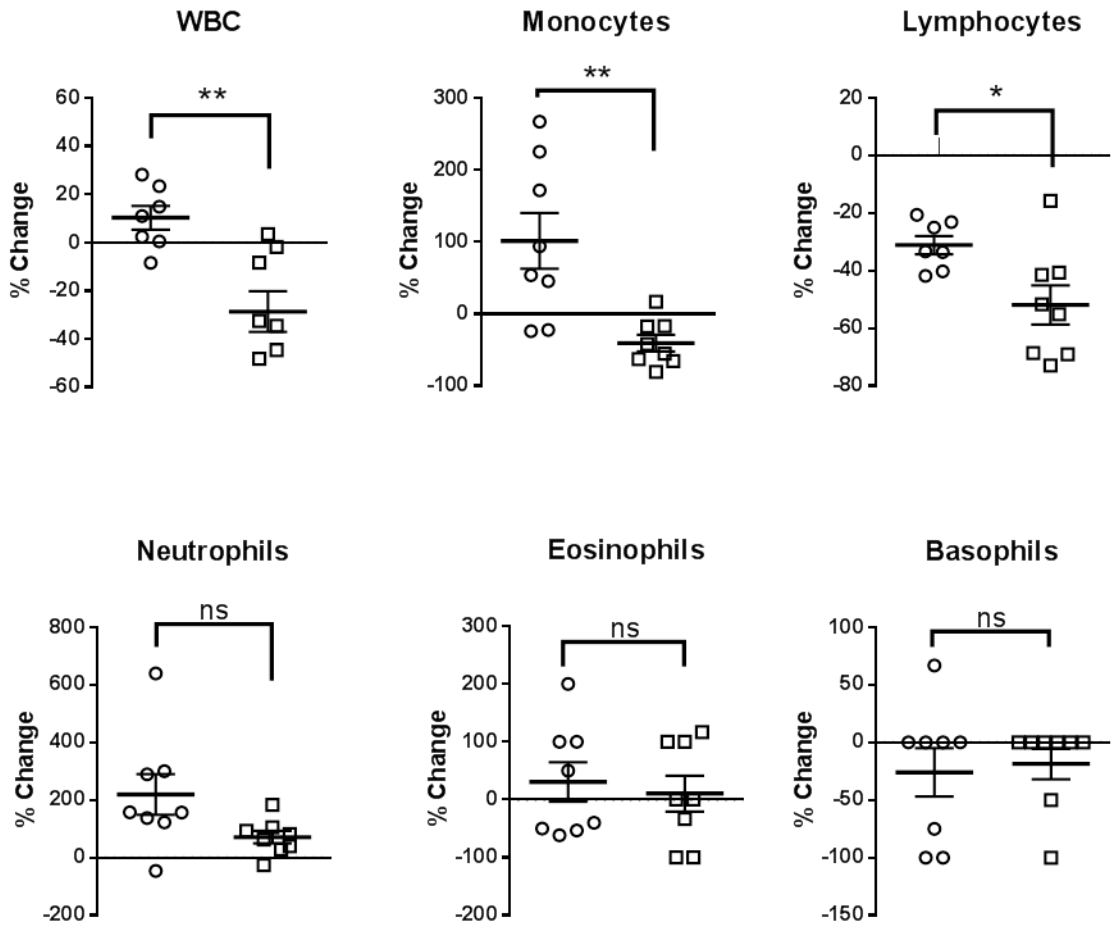

Figure 10. Chronic psychological stress impacts circulating cell trafficking during a CHS reaction. Blood was drawn from the lateral tail vein on day 0 and day 31 of restraint stress and hematological assessment of circulating leukocytes populations was performed. Results are shown as the percent change in leukocyte population number after CHS from baseline (day 0). Statistical analysis was performed by unpaired Student's *t* test (n = 8). Results are expressed as the mean ± SEM. (○: control; □: stressed) (ns, not significant; $*p < 0.05$; $**p < 0.01$)

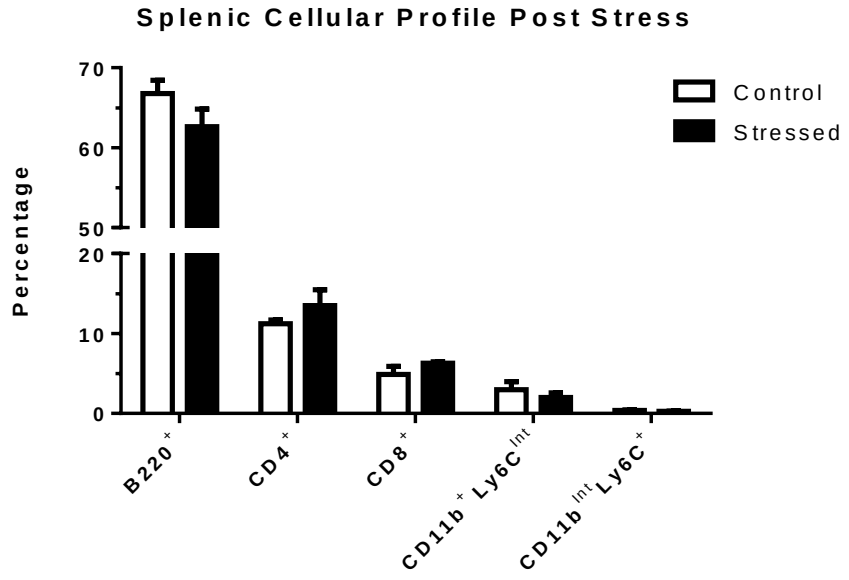

Figure 11. Chronic stress does not impact splenic cell profile. Spleens were harvested after restraint stress and mechanically homogenized. 2 x 10^6 cells were stained with saturating amounts of the following antibodies: anti-CD11b, anti-B220, anti-CD8, anti-CD4, and anti-Ly6C and assessed by flow cytometry. Results are shown as percent of live cell population. Statistical analysis was performed by unpaired Student's *t* test. Results are expressed as the mean ± SD (n = 3).

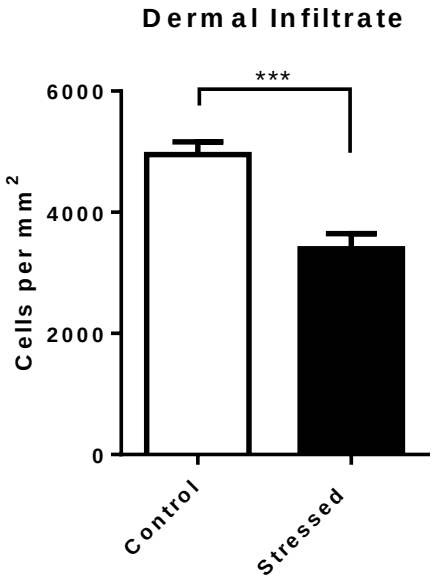

Figure 12. Chronic psychological stress reduces dermal cell infiltrate. Dermal cell infiltrate per area in inflamed ear was determined by manual counting of dermis-infiltrating cells from three representative areas per image for a total of 10 images per ear. Results are expressed as the mean ± SEM. Statistical analysis was performed by unpaired Student's *t* test. (***$p < 0.001$)

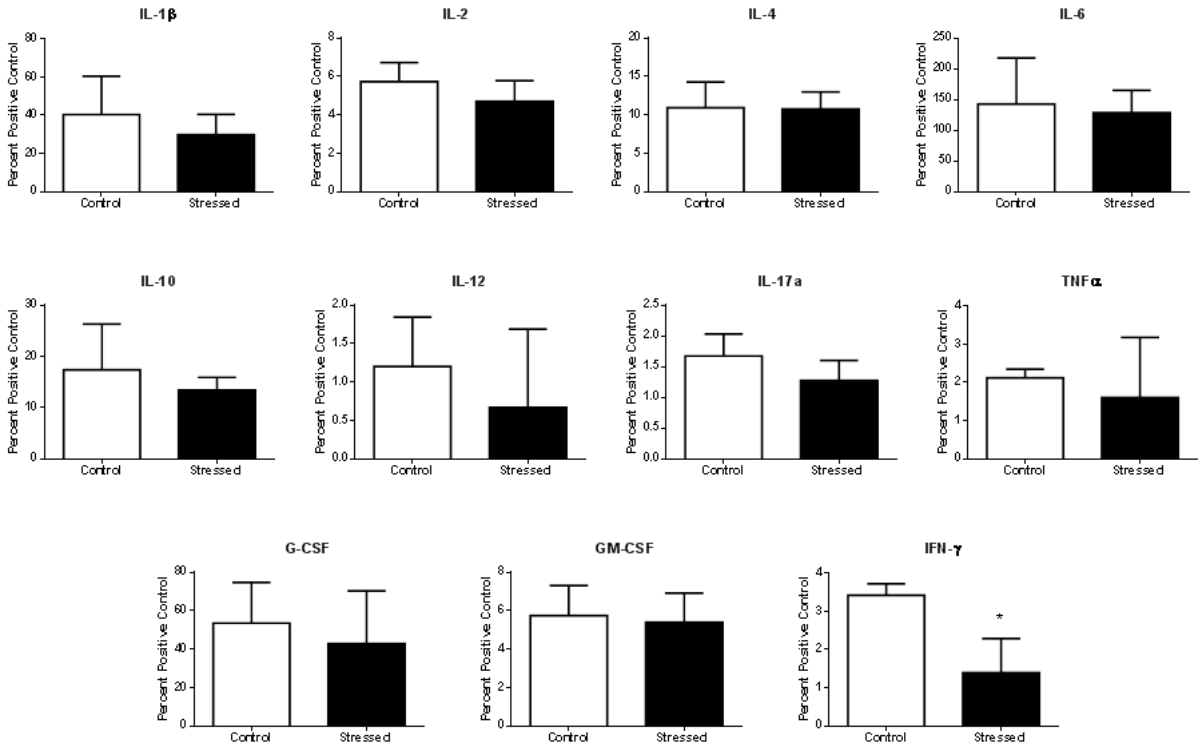

Figure 13. Chronically stressed mice display less IFN-γ production in inflamed ear tissue. The right challenged ears were collected 24 hours after challenge. The ears were homogenized and assessed via ELISarray for the indicated cytokine production at the site of inflammation. Results are shown as percent OD of positive control. Statistical analysis was performed by unpaired Student's *t* test (n = 3) and results are expressed as the mean ± SD. (*$p < 0.05$)

CHAPTER V

DISCUSSION AND CONCLUSION

Psychological stress is a perplexing phenomenon that triggers multiple physiological responses in the nervous, endocrine, and immune systems [23,35]. Since its description almost a century ago by Cannon, in what can be interpreted as one of the first hypotheses of the "flight or fight" response, psychological stress has been shown to achieve more than preparation of an individual for immediate survival. We are beginning to elucidate the complexity of psychological stress and the impact it may have in the exacerbation or suppression of immunity, including dermatological disease. Although the impact of chronic psychological stress on the ear swelling response has been described during CHS, the immunological underpinnings remain to be evaluated. Thus, the purpose of this project was to investigate the effect of chronic psychological stress on the immune response during a CHS reaction.

To study the impact of chronic psychological stress on CHS development, restraint stress was utilized as outlined (Fig. 3). To determine differential levels of psychological stress induced by restraint between groups, weight was measured daily and serum corticosterone was evaluated. Chronic restraint resulted in delayed weight gain (Fig. 4 A) and elevated concentrations of serum corticosterone (Fig. 4 B). We interpret these results to suggest that physical restraint induces psychological stress in mice.

Due to the few, conflicting reports generated concerning the impact of chronic stress on CHS, we sought to evaluate the effect of chronic restraint stress on cutaneous immunity utilizing a CHS ear swelling model. Chronic restraint stress induced a diminished ear swelling response as shown by caliper measurements of pinna thicknesses (Fig. 5) and histology (Fig. 6). We interpret these results to conclude that chronic restraint stress results in suppression of the CHS reaction in BALB/c female mice.

Additionally, it has been hypothesized that both acute and chronic periods of stress can have a lasting impact on not only immunity, but many physiological systems [72,87]. Therefore, we sought to measure the duration of impact of chronic stress. This was assessed by allowing chronically stressed mice to undergo a period of 30 days of rest in which restraint stress was terminated. After this 30 days of rest, circulating concentrations of serum corticosterone were no longer elevated (Fig. 7 A) and the ear swelling response was no longer diminished in the chronically stressed mice as compared to the control (Fig. 7 B). Therefore, we concluded that chronic restraint stress does not have persistent effects on immunity after one month of stress cessation. Future studies will need to be performed to elucidate the length of effect of chronic psychological stress, if any. Proposed studies might include exposing mice to chronic psychological stress and performing CHS one, two, three, and four weeks after stress termination. Continual exposure to CHS reactions have been shown to shift the inflammatory response from a Th1 response to Th2 [88,89], therefore this must be taken into consideration for future experiments by use of separate groups of mice for each indicated time point.

The mechanism of chronic psychological stress driven suppression on cutaneous immunity remains relatively unexplored. T cells play a prominent role in CHS, therefore we investigated the impact of chronic psychological stress on this cell type and their activities *in*

vitro and *in vivo*. We found that T cells proliferated at the same rate in response to mitogen and were similarly inhibited by corticosterone (Fig. 8 A). We also found that draining lymph node cells proliferated at the same rate *in vivo* in response to stimulation by topical oxazolone application (Fig. 8 B). Based on these findings we concluded that chronic psychological stress did not impact the capacity of T cells to proliferate, and T cells did not display altered sensitivity to corticosterone induced by chronic stress. Furthermore, we found that anti-CD3 stimulated T cells isolated from chronically stressed mice had the same capacity to produce IFN-γ as T cells isolated from control animals (Fig. 9 A). Numbers of activated $CD4^+$ T cells ($CD4^+CD25^+$ and $CD4^+CD69^+$) and $CD8^+$ T cells ($CD8^+CD25^+$ and $CD8^+CD69^+$) were not significantly different between groups (Fig. 9 B). We interpreted these results to suggest that chronic psychological stress does not impact T cell activity or activation during a CHS reaction. Due to high expression of CD25 by Treg cells, it is generally accepted that $CD4^+CD25^+$ T cells represent a Treg population [90]. Therefore, our results could be interpreted to suggest that chronic psychological stress does not significantly alter percentages of Treg cells. However, further studies examining FOXP3 transcription factor expression would need to be performed to draw conclusions from these data [91]. On the other hand, percentages of $CD4^+$ T cells and $CD8^+$ T cells were significantly elevated in the draining auricular lymph nodes of the stressed group as compared to the control group (Fig. 9 B). These findings suggested that chronic psychological stress may lead to dysregulation in T cell trafficking during CHS responses.

Acute and chronic psychological stress has been hypothesized to lead to a dysregulation of cell trafficking. This dysregulation stems from a decrease in circulating WBC, leading to an accumulation of immune cells in peripheral sites like lymph nodes, spleen, and skin [65]. Therefore, we examined circulating cells and calculated the change in WBC, neutrophils,

lymphocytes, monocytes, basophils, and eosinophils in response to CHS. Chronic psychological stress led to a decrease in total WBC's as compared with their non-stressed counterparts (Fig. 10). Differences in circulating neutrophils, basophils, and eosinophils between control and stressed mice were insignificant. Circulating lymphocytes and monocytes, however, were significantly decreased in the stressed mice as compared with the control animals in response to CHS. These results further suggest that chronic psychological stress disrupts cellular trafficking during CHS responses.

The results thus far indicated that the observed decrease in circulating WBC would lead to redistribution to peripheral tissues like lymph nodes, spleen, and the skin. The data show increased lymphocyte populations in auricular draining lymph nodes, and therefore splenic cell populations should be increased in chronically stressed mice. However, we did not observe significant difference between splenocyte subsets (Fig. 11). These results indicate the chronic psychological stress does impact cell trafficking, but this impact may be specific to the site of induced inflammation and not a global dysregulation of trafficking cells.

Our results thus far indicate dysregulation of cell trafficking in a skin-specific or CHS-specific manner. The reduction in circulating WBC in response to CHS in the stressed group would lead to a hypothesis that there is more infiltration into the inflamed tissue, and therefore a greater CHS response [65]. However, ear swelling assessed by caliper measurements was reduced in mice undergoing chronic restraint stress (Fig. 5). Therefore, we sought to assess the site of inflammation. Assessment of the cellular infiltrate into the ear showed decreased numbers of cells in the skin of the chronically stressed mice as compared with non-stressed animals (Fig. 12). Interestingly, another study utilizing rats showed that leukocyte infiltration into subcutaneously implanted sponges was decreased during the chronic stressing period and

increased immediately (2-6 hours) upon cessation of stress [70]. Our results, however, show that 24 hours after termination of the restraint stress period, dermal cell infiltrate into inflamed ear tissue was decreased as compared to the control. Additionally, our results indicate that chronic stress may not induce redistribution in the manner that has been previously hypothesized, and the redistribution hypothesis may need some amendment when chronic psychological stress is the effector mechanism.

Thus far, our results indicated that chronic psychological stress is disrupting cell trafficking in a site specific manner as opposed to global cell trafficking dysregulation. This may be due to chronic stress influences on DC trafficking during the sensitization phase. It has been shown that acute stress enhances DC trafficking to lymph nodes, resulting in increased pro-inflammatory $CD8^+$ T cell recruitment to the skin [92]. Future studies utilizing FITC painting would allow for the study of DC trafficking to draining lymph nodes after the sensitization phase of CHS. Additionally, chronic stress may influence memory T cell trafficking. This may be due to altered extravasation of effector memory T cells after the sensitization phase (i.e. resident effector memory T cells during the challenge phase). This may also be due to altered trafficking of central memory T cells during the challenge phase and differentiation into effector T cells upon stimulation in draining lymph nodes [93]. Future studies evaluating the expression of cutaneous lymphocyte antigen (CLA) and CCR4 (both homing to the skin) on T cells in the skin and lymph nodes before and after the challenge phase may help to elucidate chronic stress driven disruption of T cell trafficking [94,95]. In addition, evaluation of CCR7 expression of resident $CD69^+CLA^-CCR4^-$ T cells in lymph nodes and blood will further elucidate central memory T cell recirculation and any alterations resulting from stress [93].

Considering that the results thus far indicate site specific alterations to CHS response, we sought to assess the inflammatory environment of the site of inflammation by evaluating the cytokine profile in the challenged ear. There was not a significant difference in concentration of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17a, TNF-α, G-CSF, or GM-CSF (Fig. 13). It has been shown that CHS is regulated by IL-4 and IL-10 producing Th2 and Treg cells, respectively [42]. Additionally, it has been shown that chronic stress induces T cell polarization from a Th1 response to Th2 [96]. Furthermore, chronic psychological stress has been shown to increase the number of skin-infiltrating $CD25^+$ suppressor T cells during UV-induced squamous cell carcinoma development [17]. Therefore, it is tempting to speculate based on the cytokine data shown, that in our model of chronic psychological stress and CHS, chronic stress does not enhance Th2 or Treg influence or activity supported by similar concentrations of IL-4 and IL-10, respectively [97]. However, further experiments examining IL-4 and IL-10 protein levels in ear tissue must be performed to draw definitive conclusions from these data.

We did observe a significant decrease in IFN-γ concentration at the site of inflammation in chronically stressed mice (Fig. 13). It has been shown that $CD8^+$ T cells are the major IFN-γ producing effector T cells during CHS [42], therefore chronic stress may be altering the trafficking of $CD8^+$ T cells specifically. Another group has shown that initial $CD8^+$ T cell interaction with local endothelial cells facilitates further cell infiltration during CHS [49], therefore decreased $CD8^+$ T cell trafficking may result in decreased cell infiltration to the site of inflammation. Furthermore, IFN-$\gamma R^{-/-}$ mice show reduced inflammation during CHS as well as IFN-γ neutralization in WT mice, highlighting the importance of IFN-γ production to the inflammatory response during a CHS reaction [7]. This decrease in IFN-γ production was slight, however, and these experiments need to be repeated to draw further conclusions from these data.

The psychological nature of this project presents a unique limitation when interpreting the data generated. Although various methods were utilized to determine differential levels of psychological stress between the control and stressed group, it is difficult to obtain a true naïve control group. Handling the mice for weight and blood measurements and the CHS reaction itself may have provided additional stressors for both groups. Assumption of varying levels of hierarchy within the group of mice (i.e., alpha female) may have presented a variation of stress response within the groups themselves. In addition, difficulty in maintaining equivalent levels of psychological stress in a controlled, laboratory setting highlights the complications of applying experimental data generated to the clinical practice. Differences between acute and chronic stressors are not definitive in the clinical or laboratory setting, and multiple stressors may have inconsistent effects on individuals of varying age, sex, race, or state of mind [72,76]. Genetic background and type of stressor have been shown to have dramatic impacts on stress-driven immune modification in the laboratory setting [9,67] in addition to the type of sensitizing agent utilized for CHS [40] or time point in which stress is applied during the CHS reaction [21,98]. These discrepancies in addition to different interpretations of acute and chronic stress may have led to the conflicting data reported in this area of research [70].

Fully understanding the role of psychological stress and its impact to exacerbate or suppress CHS may provide novel treatment methods for not only CHS, but many dermatological disorders in which there exists a psychological contribution. Treatment of primary psychiatric disorders and therapies that focus on relieving and countering stress have shown very promising results with associated skin disorders [12]. The immunomodulatory functions of acute psychological stress also have implications in immunization efficiency [86], autoimmunity [99], and cancer [17,100]. The mechanism of chronic psychological stress on immunity and potential

clinical ramifications still requires further investigation. We have shown that chronic psychological stress disrupts local cell trafficking in a transient manner leading to suppression of cutaneous immunity during a CHS reaction. Disruption of local cell trafficking induced by chronic psychological stress may account for less IFN-γ production at the site of inflammation and the suppressed ear swelling observed in response to CHS. Further studies focusing on the role of chronic psychological stress on immunity need to be performed to elucidate the mechanisms behind the mind-body continuum.

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