Psychosocial Distress Mediates Immune Dysregulation Through Alterations in Global Epigenetic Patterns and Chromatin Remodeling Proteins

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PSYCHOSOCIAL DISTRESS MEDIATES IMMUNE DYSREGULATION THROUGH
ALTERATIONS IN GLOBAL EPGENETIC PATTERNS AND CHROMATIN
REMODELING PROTEINS

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<td>GCs</td>
<td>glucocorticoids</td>
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<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenocortical</td>
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<td>NK</td>
<td>Natural Killer</td>
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<td>GRs</td>
<td>glucocorticoid receptors</td>
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<td>IFN gamma</td>
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<td>TNF alpha</td>
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<td>IL</td>
<td>interleukin</td>
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<td>TSA</td>
<td>Trichostatin A</td>
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<tr>
<td>NcoR</td>
<td>nuclear receptor co-repressor</td>
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<td>SMRT</td>
<td>silencing mediator for retinoic acid and thyroid hormone receptor</td>
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<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>LU</td>
<td>lytic units</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
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<tr>
<td>H3K9-Ac</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin gamma</td>
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<tr>
<td>LAC</td>
<td>leukocyte activation cocktail</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MFI</td>
<td>mean fluorescent intensities</td>
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<tr>
<td>NKp30</td>
<td>Natural Killer cell activation receptor; anti-CD337</td>
<td></td>
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<td>NKp46</td>
<td>Natural Killer cell activation receptor; anti-CD335</td>
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<tr>
<td>LFA-1</td>
<td>lymphocyte adhesion molecule; CD11a</td>
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<td>MSK-1</td>
<td>mitogen stress kinase-1</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate, succinimidyl ester</td>
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<tr>
<td>RPMs</td>
<td>revolutions per minute</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>PSS</td>
<td>Perceived Stress Scale</td>
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<tr>
<td>CES-D</td>
<td>Center for Epidemiologic Studies–Depression</td>
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<tr>
<td>NT</td>
<td>No Treatment</td>
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<tr>
<td>HDAC2-p</td>
<td>phosphorylated histone deacetylase 2</td>
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<td>HDACi</td>
<td>histone deacetylase inhibitor</td>
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<td>GRE</td>
<td>glucocorticoid response element</td>
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<tr>
<td>CD4+ T lymphocytes</td>
<td>T helper lymphocytes</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CK2alpha1</td>
<td>Casein kinase 2 alpha 1</td>
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<tr>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
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<tr>
<td>HiCK</td>
<td>Human intracellular cytokine</td>
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</tr>
<tr>
<td>CAR</td>
<td>Cortisol awakening rise</td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>histone 3</td>
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<tr>
<td>H4</td>
<td>histone</td>
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ABSTRACT

Psychosocial distress, characterized by increased perceived stress, anxiety and mood disturbance, is a common response of women to a diagnosis of breast cancer (Northouse, 1992; Pettingale et al., 1988; Stark and House, 2000; Witek-Janusek et al., 2007). This psychosocial distress leads to activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and increased circulating glucocorticoids (GCs) (Chrousos, 2000; Chrousos and Gold, 1992; Schoneveld and Cidlowski, 2007). Increased psychosocial distress and increased HPA activation can lead to immune dysregulation consisting of reduced natural killer (NK) cell activity (NKCA) (Biondi, 2001; Kiecolt-Glaser et al., 1987; Kiecolt-Glaser et al., 2002; Witek-Janusek et al., 2008; Witek-Janusek et al., 2007). This is of particular relevance to women with breast cancer as altered immune cell function is important for tumor control (Curcio et al., 2003; D'Anello et al., 2010; Diefenbach and Raulet, 2002; Hartman et al., 2011; Kishimoto, 2005; Knupfer and Preiss, 2007; Street et al., 2001; van den Broek et al., 1996). The psychological distress in response to a diagnosis of breast cancer diagnosis has relevance for women with breast cancer, as it may contribute to poor cancer outcome. Yet little is known about the molecular mechanism(s) by which psychosocial distress results in NK cell dysregulation. The overall purpose of this project was to evaluate a potential mechanism posited to underlie altered NK cell function observed in women with breast cancer. Increased psychosocial distress increases GCs like cortisol which can
impact NK cell function; therefore cortisol levels were measured. Morning cortisol rise inversely correlated with NKCA, such that women with decreased NKCA exhibited an elevation in the morning cortisol rise. Additionally, in vitro treatment of a human NK cell line, NK92 cells, treated with a synthetic GC (dexamethasone) resulted in decreased NKCA. Together these results suggest that cortisol altered lytic function of NK cells. As GCs have been shown to alter cell function by altering epigenetic patterns, global histone modifications were investigated. In both ex vivo analysis of NK cells derived from peripheral blood of women with breast cancer and NK92 cells treated with GCs significant reductions in global acetylation of histone 4 lysine 8 (H4-K8-Ac) were observed, when compared to the age-matched Control women or untreated NK92 cells, respectively. These reductions in H4-K8-Ac correlated with NK cell lytic function as measured by NKCA and intracellular perforin levels in NK cells. Further others show GCs alter epigenetic patterns through recruitment of HDACs, thus this was investigated. Findings show decreased global H4-K8-Ac patterns in NK cells were associated with increased nuclear localization of HDAC2, suggesting that GCs recruit HDAC2 into the nucleus causing decreased global H4-K8-Ac and NKCA. These data identify decreased global H4-K8-Ac and increased nuclear localization of HDAC2 as potential markers of decreased NK cell lytic activity in women experiencing psychosocial distress. This mechanistic insight advances the field of psychoneuroimmunology by identifying both an epigenetic modification (H4-K8-Ac) and a chromatin remodeling protein (HDAC2) as indicators of GC mediated immune dysregulation in NK cells of women experiencing psychosocial distress.
CHAPTER 1
INTRODUCTION

It is well-established that neuro-chemical pathways link the brain and the immune system and provide biological pathways whereby one’s emotions influence immune function. The effect of psychological stress on immune function has been, in part, attributed to elevations in glucocorticoids (GCs) produced by activation of the hypothalamic-pituitary-adrenocortical (HPA) axis [1-3]. GCs can suppress Natural Killer (NK) cell lytic activity [4-6] and alter cytokine balance [7-12].

Previous work has shown that the diagnosis of breast cancer can result in increased psychological stress, HPA activation and altered NK cell function [5, 13-17]. For early stage breast cancer patients, a tumor is removed and prognosis is good, but the disease can recur after apparent successful eradication of the tumor. Recurrence occurs in some women but not in others [18]. The reasons are unclear and certainly are multiple, but alterations in NK cell function could be a contributing factor.

However, the molecular mechanism linking psychological stress and GCs to reductions in NK cell function remain poorly understood. It is known, however, that GCs affect target cells through alteration of gene transcription and this may result from epigenetic modification of histone residues [19]. One unexplored and promising possibility is that GCs recruit chromatin remodeling proteins leading to global epigenetic modifications resulting in NK cell dysregulation. Understanding the alterations in epigenetic modifications and chromatin remodeling proteins associated with these
modifications will allow for a mechanistic understanding of the effects of stress upon the immune system and is the focus of this dissertation work.
CHAPTER 2
BACKGROUND

Breast Cancer and Psychological Consequences:

Cancer affects a large number of Americans each year, with an estimate of over 1.5 million Americans diagnosed with cancer in 2011 [20]. Of these cancer cases, over 230,000 were breast cancer, making breast cancer the most frequently diagnosed malignancy in the Western world [20, 21]. In American women, it is the second leading cause of cancer death, with over 40,000 American women dying of breast cancer yearly [20]. There is clear evidence that a diagnosis of breast cancer can have short- as well as long-term psychological consequences [22], characterized by increased perceived stress, depressive mood and anxiety [5, 13-17]. Increased mood disturbance and increased fatigue also accompany a psychological stressor, such as the diagnosis of breast cancer [15, 23-25]. These psychological consequences, termed psychosocial distress, are independent of disease state, as women with early stage breast cancer, as well as women with metastatic disease both report increased psychosocial distress [15, 26, 27]. Additionally, previous work from this laboratory found that women who underwent breast biopsy had increased perceived stress regardless of biopsy outcome [15]. These results demonstrated that breast cancer diagnosis resulted in a psychological and emotional response, termed psychosocial distress in this dissertation work.
The dynamics of psychosocial distress are influenced by the duration of the stressor (i.e. either acute or chronic). Acute stressors last for minutes to hours, whereas chronic stressors last months to years [28, 29]. Acute stressors can redistribute immune cell trafficking and even increased NK cell function [30-32], whereas prolonged chronic stress can dysregulate immune function and undermine health [33-36]. Further, depending upon the condition of the stressor or the nature of the neuroendocrine response to the stressor, the immune system can be reduced or alternatively activated in its function [37, 38]. The impact of psychosocial distress on immune function is further discussed below (See Psychosocial Distress and Immune function). Additionally, there are variations in both the intensity and duration of psychosocial distress reported in individuals with breast cancer [39]. For some individuals the diagnosis of breast cancer activates appropriate allostatic responses. Allostasis is defined as “the active process of responding to a challenge to the body by triggering chemical mediators of adaptation” [40]. Thus, in some women the diagnosis of breast cancer causes an initial, appropriate emotional and biological response, which subsides allowing the body to both adapt to the stressor and to return to allostatics. However for others the psychosocial distress is too great resulting in increased allostatic load. Allostatic load is the “wear and tear on the body and brain that results from chronic dysregulation (over activity or inactivity) of mediators of allostatics” [40]. Approximately 80% of women with breast cancer report some psychosocial distress experienced during the initial treatment phases [41].
Additionally, an association between increased allostatic load due to early life adversity and breast cancer occurrence has been identified [42]. Individual variation makes understanding the psychosocial distress that accompanies breast cancer diagnosis even more complex. For some individuals psychosocial distress subsides after the initial cancer diagnosis, for others it continues beyond treatment and recovery from the disease [43, 44].

*Glucocorticoids: Mediators of Psychosocial Distress:*

Psychosocial distress leads to activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and the secretion of glucocorticoids (GCs) [1, 2]. GCs, such as cortisol, have been shown to affect a number of biological systems, including the immune system. GCs are postulated to alter thousands of different cellular genes [45], including many immune effector genes. GCs exert effects on immune cells by binding to glucocorticoid receptors (GRs), which are present on all immune cells [33, 34, 46]. GR is a ligand activated transcription factor, which is a member of the nuclear receptor super family of proteins. It predominantly exists within the cytoplasm, but when ligand activated, GR translocates to the nucleus [47]. Once in the nucleus activated GR can alter gene transcription through recruitment of transcription factors, co-regulators and chromatin remodeling proteins [48-51]. These interactions between activated GR and other cellular proteins are discussed later (*See HDACs, HATs and Glucocorticoids*). Under normal conditions GCs are regulated by
both the nervous and endocrine systems maintaining homeostasis resulting in proper immune cell function. Individuals experiencing psychological distress exhibit HPA activation and elevations in cortisol. If the stress perception is chronic, disruption of the diurnal cortisol rhythm is observed [52, 53], resulting in elevated circulating GCs [33, 34, 46].

A typical diurnal cortisol rhythm is characterized by an initial spike approximately 15-45 minutes after waking, this initial spike it termed the cortisol awakening rise (CAR) [54]. Cortisol levels then gradually subside throughout the course of the day, reaching a minimal level in the evening, just prior to bedtime. Increases in psychosocial distress have been linked to alterations in diurnal cortisol rhythms, which can lead to immune cell dysregulation [52, 53]. However, studies have found differential interplay between cortisol and psychosocial distress. Some groups found increased CAR with increased activation of the HPA [55, 56] and increased psychological stress and depressive mood [57]. Another found relationships between increased evening cortisol levels and increased mood disturbance in breast cancer survivors [58]. In these studies increased cortisol levels were related to increased psychosocial distress.

However, other studies have measured decreased or blunted cortisol responses in relationship to psychosocial distress. Analysis of total cortisol levels measured throughout a diurnal rhythm is termed area under the curve. Women with metastatic breast cancer, who report increased perceived stress, have decreased area under the
Similar findings were found in caregivers, with increased depressive mood relating to a decreased cortisol response, throughout the day and CAR[62].

**Psychosocial Distress and Immune Cell Function:**

A large number of studies have shown that psychosocial distress and GCs negatively impact immune function [63-67]. Psychological stress has been shown to; alter cytokine production [14, 15, 64, 68, 69], decrease proliferative response to mitogens [70-72], reduce specific antibody responses, reduce T-lymphocyte mediated cellular responses [73], and decrease synthesis of interferon gamma (IFN gamma) [15, 74, 75]. In these studies multiple different psychological stressors were related to altered immune cell function. Such stressors included caregiving stress (individuals caring for patients with Alzheimer’s disease), post-traumatic stress disorder, exam stress and the diagnosis of breast cancer. Such observations coupled with the well-established immunosuppressive effect of GC [76], suggest an explanation for the reduced immune function associated with increased psychosocial distress.

However, even though GCs are known to inhibit aspects of immune function under some conditions, psychosocial distress and GCs under other conditions can promote immune responsiveness [77-81]. Psychological stress has been shown to increase circulating levels of tumor necrosis factor alpha (TNF alpha) and interleukin one beta (IL-1 beta) levels in humans [82] and in rodents to increase circulating IL-1
beta and IL-6 [83, 84]. Moreover, GCs have been shown to synergistically enhance the induction of acute-phase proteins by IL-1 beta and IL-6 [85] and the biological effects of IL-2, IFN gamma, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and oncostatin M [86]. These immune modulating effects of GC are concentration and time dependent [87, 88] and it has been clear for many years that the effect of the nervous and endocrine system on the immune system are not necessarily suppressive. Substantial evidence demonstrates GC concentrations support the activity of the immune system in a permissive and preparative manner [89, 90].

Natural Killer Cells and Psychosocial Distress:

NK cells are multi-functional lymphocytes that do not require a pre-activation process and are not restricted to antigen presentation by major histocompatibility complex molecules [91, 92]. Their rapid action places NK cells as a front line of defense against cancer and infectious agents [93-95]. Considerable attention has been paid to the direct anti-tumor activities of NK cells [96] and evidence has existed for some time for their role in tumor protection. Moreover, direct evidence has shown these cells to be involved in immune surveillance, controlling the initiation and growth of tumors [97-104]. NK cells produce their effects against tumor cells by first engaging the tumor target, followed by exocytosis of NK cytolytic granule constituents against the tumor cell, disengagement of the tumor cell by the NK cell and lysis of the tumor targets [91, 92].
Reciprocal neuro-chemical pathways and shared receptor systems link the brain and the immune system, including NK cells. In particular, NK cell activity (NKCA) is especially responsive to the impact of psychosocial distress and ample evidence demonstrates that psychological stress or negative affective states reduce NKCA [15, 63, 105] and result in a poorer NK cell response to cytokines [71, 106]. Specifically in individuals experiencing the diagnosis of breast cancer, increases in perceived stress were predictive of decreased NK cell lytic activity [71, 72]. In this same study NK cells were also less responsive to known NK stimulators, such as IFN gamma. An altered cortisol rhythm predicted NKCA in women with breast cancer [53], with the levels of distress correlating inversely with NKCA [107]. As these women recovered from regional breast cancer (8-18 months after diagnosis) distress subsided and NKCA improved [107]. This same group also investigated if NKCA dysregulation was related to altered expression of NK cell inhibitory receptors [108]. Although, differential inhibitory molecule expression was measured, relationships between the expression of receptors and psychosocial distress mediated reductions in NKCA were not found. Additionally, optimal NK cell function is especially important to women with breast cancer, as NK cell function has been linked to increased survival in individuals with metastatic disease [109-115]. Hence, the elevations in psychological distress in response to a diagnosis of breast cancer and subsequent reduction in NK cell function may compromise cancer outcomes and/or increase risk of cancer reoccurrence. Despite these numerous links between psychosocial distress, GCs and NK cell function, molecular mechanisms of NK cell dysregulation in
response to psychological distress remain poorly understood. Understanding molecular mechanism(s) contributing to alterations in NK cell function in woman experiencing psychosocial distress can lead to the development of approaches to enhance NK response; thereby improving disease prognosis.

**Epigenetics: Histone Modifications:**

One potential mediator of NK cell dysregulation in response to psychosocial distress is epigenetic alterations. The term epigenetic was first introduced in the 1940s. A current working definition of epigenetic is “molecular or cellular alterations, which influence gene expression, and by extension physiology and behavior without causing alterations to the DNA sequence itself” [116, 117]. The epigenetic code can now be divided into three major parts; DNA methylation, histone modifications and non-coding RNAs [117]. All of these epigenetic modifications can regulate gene transcription without altering DNA sequence. The following will focus on a discussion of histone modifications.

Histone proteins H2A, H2B, H3 and H4 dimerize to form an octomeric histone core protein [118]. Approximately 147 base pairs of DNA wrap around the histone core proteins forming the nucleosome particle. The histone proteins, possess highly basic amino-terminal tail domains located outside of the core nucleosome particle, which are accessible to multiple post-translational covalent modifications [119]. These modifications include acetylation, phosphorylation, methylation, sumoylation and
ubiquitination [120]. Depending on the modification, these can either lead to activation or repression of genes. Generally, acetylation has been shown to be a mark of activation, whereas methylation is a mark of repression [121, 122]. Hyperacetylated genes are actively transcribed, whereas hypermethylated genes are repressed. Reversible acetylation of the epsilon amino group of lysine is a dynamic process that regulates chromatin structure [123]. Lysine acetylation neutralizes histone proteins, whereas deacetylation restores the positive charge of lysines. Following deacetylation, interactions between positively charged lysines and the negatively charged DNA phosphodiester backbone, stabilize the DNA/histone complex, restricting nucleosome mobility on the DNA, and hindering accessibility of the promoter to transcription machinery [124]. Thus, deacetylated lysine residues restrict access of transcription factors for regulatory regions of immune effector genes. By repressing a downstream target of transcription factor activation (chromatin accessibility), GCs reduce gene expression irrespective of the exact transcription factor requirement for an individual gene.

Histone acetylation patterns are altered throughout immune cell differentiation and this is best characterized in the T lymphocyte populations. One study found that alterations in cytokine profiles of T lymphocytes related to altered histone 3 (H3) and histone 4 (H4) acetylation patterns [125]. Specifically differentiation of naïve CD4+ T lymphocytes into either Th1 or Th2 lymphocytes resulted in a dynamic interplay between acetylation patterns of H3 and H4. In Th1 lymphocytes increased acetylation of the IFN gamma loci occurred, while in Th 2 lymphocytes increased H3 and H4 acetylation of the
IL-4 loci occurred. In CD8+ T lymphocytes connections between H3 acetylation and cytotoxicity have been demonstrated. One study provided evidence that H3-K9-Ac contributed to production of cytotoxic molecules such as granzyme B and perforin [126]. Increases in H3-K9-Ac were related to increased promoter accessibility and increased protein production of granzyme B and perforin.

Associations between NK cells and histone acetylation patterns are less well characterized. There is evidence that H4 acetylation affects cell surface molecules, such as inhibitory or activating molecules [127]. This effect is most dramatic with selective deacetylation at histone 4 lysine position 8 (H4-K8), resulting in suppressed gene transcription [48, 127-129]. Additionally, alterations in H4-K8-Ac have also been linked to altered chromatin accessibility [130]. The effects of histone acetylation patterns on chromatin structure and on immune response genes are reversible with histone deacetylase (HDAC) inhibitors, such as Trichostatin A (TSA) [131-133]. Examination of H3 and H4 acetylation patterns in relationship to NK cells cytotoxicity and pro-inflammatory cytokines remains to be determined.

**HDACs, HATs and Glucocorticoids:**

Two classes of enzymes are responsible for acetylation patterns of histones; HDACs and histone acetylases (HATS). HDAC activity, either directly or indirectly, affects expression of at least 1,120 genes [134]. HDACs are enzymes that remove acetyl
groups from the epsilon amino group of lysines on the N-terminal tails of histone proteins [135]. HATs transfer acetyl groups onto lysine residues of histone tails [136, 137].

HDACs are divided into four major classes, Class I – Class IV, based on their homology with yeast orthologs. Class I HDACs include HDAC 1, 2, 3, and 8. While HDAC 1, 2, and 3 are ubiquitously expressed, HDAC8’s activity is limited to smooth muscle [138, 139]. Class II-IV are less well characterized with regard to transcriptional repression and in some cases are more restricted in their expression. Additionally, HDACs 1, 2 and 3 can be recruited by GR [49-51] leading to transcriptional repression [140]. Work by Hong et al. found that cochaperone, Bcl-2 associated athanogene 1 (Bag-1) interaction with GR led to transcriptional down regulation by recruitment of nuclear receptor co-repressor (NcoR) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), along with HDAC3. This complex is critical for down regulation of known GC response genes. Work from this laboratory has shown that GC treatment of an NK cell line resulted in HDAC1 and SMRT co-localization with GR in NK cells with decreased lytic activity [49]. Others found a new Class I specific HDAC inhibitor which measured increased NK cell lytic activity of human PBMCs at low nanomolar concentrations, suggesting that HDAC1, 2 and 3 are involved in regulation of NK cell lytic activity [141]. These data suggest that alterations in Class I HDACs can lead to altered NK cell lytic activity. However, connections between class I HDACs and global histone acetylation patterns in NK cells remain to be determined.
There are limited connections between HDACs and psychological stress with most investigation in experimental animals. One study in mice did find evidence that early life stress alters class I HDACs and histone acetylation [142]. In that study, mice which experienced infant maternal separation had decreased expression of class I HDACs. This decrease in expression of class I HDACs related to increased acetylation of H4 in the brain of those mice that experienced early life stress when compared to mice that did not. Acetylation status of H4 in the immune cells of these mice was not investigated. Nevertheless this study did suggest that alterations in class I HDACs can lead to altered H4 acetylation. Investigation into GCs, HDACs and psychosocial distress in humans remains to be determined.

Other data suggest that GCs can up-regulate gene expression. This can occur by displacing resident HDACs from gene promoter regions, allowing for histone acetylation and transcriptional activation. One such study showed that 24 hour treatment with Dex reduced nuclear expression of HDAC3 [143]. Additionally, interaction of the GR with target genes can lead to transcriptional activation via recruit of co-activators and HATs [144-146]. HATs are usually divided between type A and type B depending on cellular localization [147]. Enzymes such as p300, CPB and pCAF have intrinsic HAT activity. Additionally, p300 has been shown to interact with nuclear factors (NF kappa B) [146, 148], which regulate NK cell lytic activity genes (perforin and granzyme B). Additionally, in vitro treatment with a synthetic GC (dexamethasone) increased p300 HAT activity [143]. These data demonstrate clear evidence of interaction between
HDACs and GCs. However, how these factors interact in NK cells of women experiencing psychosocial distress is still unknown. Further understanding of global acetylation patterns and chromatin remodeling protein patterns in women experiencing psychosocial distress will allow for a critical assessment of epigenetic mechanisms that may mediate immune dysregulation.
CHAPTER 3

SIGNIFICANCE

Increased psychosocial distress is well documented in women with breast cancer. These increases in psychosocial distress lead to HPA activation, which can alter immune cell function, with NK cell function found to be especially responsive to these alterations. Investigation into potential molecular mechanisms (epigenetic modifications and chromatin remodeling proteins) of NK cell dysregulation will provide an important step in understanding the effects of psychosocial distress on the immune dysregulation that accompanies a diagnosis of breast cancer. Further understanding of global epigenetic modifications and chromatin remodeling proteins could serve as a model for development of methods for immune enhancement of individuals during periods of psychological stress.
CHAPTER 4
AIMS AND HYPOTHESIS

Overall Aim: Determine whether global NK cell epigenetic pattern is associated with the immune dysregulation that accompanies the psychosocial distress of breast cancer diagnosis.

Specific Aim 1. Characterize an in vitro system to identify effects of GCs treatment on global epigenetic pattern and immune cell function.

Hypothesis 1a. Dexamethasone treatment of NK92 cells will alter NK cell function; lytic activity and cytokine production.

Hypothesis 1b. Dexamethasone treatment of NK92 cells will alter global epigenetic patterns.

Specific Aim 2. Characterize the chromatin remodeling proteins associated with global epigenetic patterns observed in NK92 cells treated with the glucocorticoid, dexamethasone.

Hypothesis 2a. Global epigenetic patterns of NK92 cells will be associated with the cellular localization of chromatin remodeling proteins that repress transcription (HDACs).
Hypothesis 2b. Global epigenetic patterns of NK92 cells will be associated with the cellular localization of chromatin remodeling proteins that activate transcription (HATs).

Specific Aim 3. Characterize the global epigenetic patterns and the intracellular localization of chromatin remodeling proteins of the NK cells of women as they respond to and recover from breast cancer diagnosis.

Hypothesis 3a. Global NK cell epigenetic patterns will differ among women who exhibit psychosocial distress mediated immune dysregulation and those who do not.

Hypothesis 3b. Global epigenetic patterns will relate (correlate) to the immune functional activity of the NK cells of women as they respond to and recover from breast cancer diagnosis.

Hypothesis 3c. The cellular localization of chromatin remodeling proteins will differ among the NK cells of women who exhibit psychosocial distress mediated immune dysregulation and those who do not.
CHAPTER 5
MATERIALS AND METHODS

Natural Killer Cell Assay (NKCA):

NK cell function was assessed by NKCA. NK cell lytic activity against tumor targets was assessed and analyzed using a standard chromium release assay, as previously described [15]. K562 tumor cells were radioactively labeled with 100 uCi of \(^{51}\text{Cr}\) (New England Nuclear, Boston, MA). Radiolabeled K562 cells were incubated for 4 hour with peripheral blood mononuclear cells (PBMCs) or 3 hours with NK92 cells. Following incubation, the supernatants were removed using a Skatron harvesting press (Skatron Inc., Sterling, VA) and the associated radioactivity was determined. NK92 cell effector to target ratios for NKCA was 3, 2, 1 and 0.5:1. PBMCs effector to target ratios for NKCA were 50, 30, 20 and 10:1.

Results were expressed as % cytotoxicity and calculated by the formula:

\[
\% \text{ Cytotoxicity} = \frac{(\text{experimental DPM}^*) - (\text{minimum DPM})}{(\text{maximum DPM}) - (\text{minimum DPM})} \times 100.
\]

All experimental means were calculated from triplicate values. Lytic units (LU) were calculated by a program written by David Coggins, FCRC, Frederick, MD and represents the number of cells per \(10^7\) effectors required to achieve 20% lysis of the targets.

*DPM=disintegrations per minute.
**Cytokine Production (ELISA):**

NK92 (2.5 x 10^5 cells/ml) cells were incubated in 24 well plates for 24 hours at 37°C. All molecules were measured using quantitative sandwich enzyme immunoassay techniques (Quantikine kits, R & D Systems, Minneapolis, MN). Sensitivities for cytokines were; (interleukin-6 (IL-6) <0.7 pg/ml, interferon gamma (IFN gamma) <3 pg/ml, tumor necrosis factor alpha (TNF alpha) <1.6 pg/ml, and Perforin<40 pg/ml). The coefficient of variation ranged between 2.6 – 8.1% for the individually assessed molecules.

PBMCs were unstimulated or stimulated for 48 hours with phorbol 12-myristate 13-acetate (PMA) and phytoheamagglutin (PHA) (Sigma Aldrich, St. Louis, MO). PMA is a phorbol ester that is a polyclonal activator of PBMCs through activation of protein kinase C which leads to protein production. PHA is able to crosslink proteins expressed on the surface of PBMCs causing stimulation. After 48 hours, supernatants were collected and an ELISA was performed.

**Intracellular Staining by Flow Cytometry:**

**PBMCs:**

PBMCs were aliquoted into fluorescent activated cell sorting (FACS) tubes (500,000 cells/tube). Surface staining antibodies were added for 30 minutes on ice, agitated every 15 minutes to identify PBMC sub populations. Dilutions for all antibodies
(surface, intracellular and intranuclear) when appropriate was recorded in Supplemental Table 3, antibodies not recorded in table were added neat. Surface antibodies used included anti-CD4 (Pacific Blue conjugated), anti-CD8 (PerCP-Cy5.5 conjugated), anti-CD56 (APC conjugated), anti-CD56 (PE conjugated), anti-CD45RO (APC conjugated), anti-CD3 (APC-Cy7 conjugated) and anti-CD14 (APC conjugated) (BD Pharmingen, San Jose, CA). PBMC sub populations were identified as: CD4+ T lymphocytes (CD4+, CD8-, CD56-), CD8+ T lymphocytes (CD8+, CD4-, CD56-), Natural Killer (NK) cells (CD56+, CD4-, CD8-) and CD14+ Monocytes (CD14+). Following surface antibody staining, the cells were washed twice with 0.1% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in phosphate buffer solution (PBS) (Gibco, Grand Island, NY). PBMCs were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Jose, CA) for 20 min at 4°C. The cells were then washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA) and probed with antibodies specific for intracellular molecules of interest for 1 hour at 4°C.

For histone residues, anti-acetylated (Ac)-histone lysine 8 (H4-K8) (Millipore, Temecula, CA) (unconjugated), anti- H3K9-Ac (Alexa 488 conjugated) (Cell Signalling Beverly, MA), anti-trimethylated (me3)-H3K4 (unconjugated ) (Cell Signalling Beverly, MA), anti-phosphorylated (P)-H3S10 (Alexa 488 conjugated) (Cell Signalling Beverly, MA) were added to cells in FACS tubes. The cells were washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA), after which secondary anti-immunoglobulin gamma (IgG) (FITC conjugated) (Millipore, Temecula, CA) was added for 30 min at 4°C
to the H4-K8-Ac and H3-K4-me3 tubes. Following this treatment, the cells were washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA) and resuspended in 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY).

For intracellular cytokine analysis, cells were permeabilized and then incubated with antibodies specific for intracellular proteins for 1 hour at 4°C. Antibodies for intracellular staining used included anti-IFN gamma (PE conjugated) (BD Biosciences, San Jose, CA), anti-granzyme B (Alexa Fluor 647 conjugated) (BD Biosciences, San Jose, CA), anti-TNF alpha (PE-conjugated) (BD Biosciences, San Jose, CA), anti-TNF alpha (Alexa 488-conjugated) (BD Biosciences, San Jose, CA), anti-IL-6 (PE conjugated) (BD Biosciences, San Jose, CA) and anti-perforin (PE conjugated) (BD Biosciences, San Jose, CA). For intracellular cytokine analysis of IFN gamma and TNF alpha, cells were incubated in leukocyte activation cocktail (LAC) (BD Pharmingen, San Jose, CA) at 37°C for 4 hours prior to permeabilization and antibody staining. LAC contains PMA, ionomycin and brefeldin A. PMA is a phorbol ester which is a polyclonal activator of PBMCs, ionomycin is a calcium ionophore which increases calcium levels in the cell resulting in PBMC stimulation. Brefeldin A is a protein transport inhibitor which allowed for intracellular assessment of cytokines. For intracellular cytokine analysis of IL-6, cells were incubated in leukocyte activation cocktail (BD Pharmingen, San Jose, CA) and 100 ng of sonicated lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO) at 37°C for 4 hours prior to permeabilization and antibody staining.
For chromatin remodeling protein analysis, cells were permeabilized and then incubated with antibodies specific for chromatin remodeling protein for 1 hour at 4°C. For chromatin remodeling proteins, cells were probed with antibodies specific for histone deacetylase 1 (HDAC1) (Alexa-647-conjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HDAC2 (unconjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated-HDAC2 (unconjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HDAC3 (unconjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p300 (Alexa 647-conjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary anti-IgG (FITC conjugated) (Millipore, Temecula, CA) was added for 30 min at 4°C to unconjugated primary antibodies.

For all PBMCs, cytokine and histone mean fluorescent intensities (MFIs) were standardized to cytokine and histone MFIs of HiCK Control Cells (BD Pharmingen, San Jose, CA). Surface antibodies were not added to HiCK Control Cells; intracellular and histone antibodies were added as described above. HiCK Control Cells were thawed and aliquoted into individual microcentrifuge tubes before storage at -80 °C. On the day of PBMC staining, HiCK Control Cells were thawed at 37°C for 2 minutes and then aliquoted into FACS tubes for staining. HiCK Control Cells were stained concurrently with PBMCs, therefore staining times, incubations and washes were identical to PBMCs. No change in HiCK cell staining was observed over time.

For MFI calculations, the following formula was used:
MFI = Cytokine or Histone MFI of sample/Cytokine or Histone MFI of HiCK cell.

After staining, samples were analyzed by flow cytometry with a FACS CantoII Fluorescence-Activated Cell Sorter or a LSR Fortessa using FACS Diva software for data acquisition [149-151]. 10,000-30,000 events were recorded and analyzed with FlowJo v8.4.1.

**NK92 cells:**

NK92 cells were aliquoted into FACS tubes (0.25-1 x 10^5 cells/tube). Staining protocols, lengths of incubations and antibodies used as previously described for PBMCs. Additional antibodies used for NK92 cell staining but not PBMC staining are listed below:

Surface staining antibodies included; anti-CD337 (NKp30) (Alexa Fluor 647 conjugated) (BD Biosciences, San Jose, CA), anti-CD335 (NKp46) (PE conjugated) (BD Biosciences, San Jose, CA) and anti-LFA-1 (CD11a) (FITC conjugated) (BD Biosciences, San Jose, CA). For chromatin remodeling proteins, antibodies used were anti-glucocorticoid receptor (GR) (unconjugated) (Abcam, Cambridge, MA), and anti-mitogen stress kinase-1 (MSK-1)-phosphorylated (unconjugated) (Cell Signaling, Boston, MA). Secondary anti-IgG (FITC conjugated) (Millipore, Temecula, CA) was added for 30 min at 4°C to unconjugated primary antibodies.
Treatment MFI = \( \frac{\text{Treatment MFI}}{\text{Average MFI of No Treatment}} \) x 100

After staining, samples were analyzed by flow cytometry with a FACS CantoII Fluorescence-Activated Cell Sorter or a LSR Fortessa using FACS Diva software for data acquisition [149-151]. For NK92 cells 1,000-10,000 events were recorded and analyzed with FlowJo v8.4.1.

Cellular Culture:

The human erythroleukemic like cell line, K562, was obtained from the American Type Culture Collection, Rockville, MD. K562 cells were maintained in suspension cultures in vitro in Corning 75 cm\(^2\) tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) low LPS; (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100\(\mu\)g/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY). K562 cells were passaged every 48 hours with media.

NK92 cells (established from a patient with non-Hodgkin’s lymphoma with the capacity to lyse a broad range of leukemia, lymphoma and myeloma cell lines at low effector to target ratio in vitro) were obtained from the American Type Culture Collection, Rockville, MD and maintained in alpha MEM with 12.5% horse serum.
(Gibco Laboratories, Grand Island, NY), 12.5% fetal bovine serum (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100ug/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.2 mM inositol (Sigma Aldrich, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Gibco Laboratories, Grand Island, NY) and 0.02 mM folic acid (Sigma Aldrich, St. Louis, MO). NK92 cell cultures were also supplemented with interleukin-2 (IL-2) (100 units/ml). NK92 cells were passaged every 48 hours with media and IL-2. NK92 cell viability is dependent on addition of IL-2.

**NK92 Cellular Treatment Systems:**

- **Short-Term, High Dose (Dexamethasone 10^{-7} M for 24 hours):**

  NK92s, cultured at 2.5 x 10^5 cells/ml, were treated with dexamethasone (Dex) (Sigma Aldrich, St. Louis, MO) (10^{-7} M), for 24 hours in the absence of IL-2. NK92s were washed with media and resuspended to 1 x 10^6 cells/ml for assay. In a similar manner, NK92s were treated with Dex (10^{-7} M) for 24 hours and then Trichostatin A (Cell Signaling Technology, Danvers, MA) (100 nM) (TSA) was added to cultures for 1 to 6 hours. 1 hour TSA treatments were used for NKCA, histone analysis (western and flow cytometric) and 6 hour TSA treatments were used for intracellular IFN gamma assays. NK92s were washed and resuspended with media to 1 x 10^6 cells/ml. Cell number and viability were determined by exclusion using 0.1% Trypan blue.

- **Chronic Treatment (Dex 10^{-10} M for 5 days):**
For chronic Dex treatments, NK92s, cultured at $2.5 \times 10^5$ cells/ml, were treated with Dex (Sigma Aldrich, St. Louis, MO) ($10^{-10}$M) for 5 days. For the first 4 days of treatment, cells were cultured in the presence of IL-2. For the final 24 hours cells were cultured without IL-2. Cells were counted and resuspended every 48 hours with fresh media and Dex. NK92s were washed with media and resuspended to $1 \times 10^6$ cells/ml for assay. Cell number and viability was determined by exclusion using 0.1% Trypan blue.

**Cell Conjugation Assay:**

K562 cells were suspended in 1 ml of 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) at a final concentration of $5 \times 10^6$ cells/ml, then labeled for 10 minutes at 37 °C with carboxyfluorescein diacetate, succinimidyl ester (CFSE) (2 uM, Sigma Aldrich, St. Louis, MO). The cells were washed 3 times with ice-cold 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY). NK92 cells were labeled with anti-CD56 (APC-conjugated) (BD Biosciences, San Jose, CA) for 30 minutes on ice and agitated after 15 minutes. Following antibody staining, the cells were washed twice with 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) and resuspended in alpha-MEM media (Gibco Laboratories, Grand Island, NY). NK92 cells at a concentration of $5 \times 10^6$/ml were mixed with $5 \times 10^6$/ml CFSE-labeled K562 target cells at an effector to target ratio of 1:1, allowing the formation of effector-target conjugates. The NK92-K562 cell mixture was centrifuged at 1,000 revolutions per minute (rpms) for 2 seconds and incubated at 37 °C for 10 minutes. Then
the cell mixture was gently resuspended in 0.1% BSA in PBS and fixed with 1% paraformaldehyde (PFA) in PBS (Sigma Aldrich, St. Louis, MO). The conjugation ratio was calculated as the portion of CSFE/APC double-positive events [152].

**Western Blot:**

For Western Blot analysis, nuclei of NK92 cells were extracted from 1-5 x 10⁶ cells via the Fermentas ProteoJET Cytoplasmic and Nuclear Protein Extraction protocol (Fermentas, Burlington, ON). Nuclei were lysed using Nuclear Lysis Buffer (Fermentas, Burlington, ON) and resuspended in Laemmleis SDS-Sample Buffer (4x) (Boston Bioproducts, Boston, MA). Samples were boiled for 10 minutes and proteins separated by electrophoresis with a 15% agarose gel and transferred to a nitrocellulose membrane for blotting. Proteins were visualized with anti- H4-K8 Ac antibodies or anti-H3 antibodies (Millipore, Temecula, CA), horseradish peroxidase (HRP) conjugated anti-IgG secondary antibody (Millipore, Temecula, CA) and chemiluminescence reagent (ThermoScientific, Rockford, IL). Blots were standardized to total H3 protein (Cell Signaling, Danvers, MA) and blot density quantified using Image J software.

**Immunofluorescent Microscopy:**

**NK92 cells on glass slides:**

Glass coverslips (Fisher Scientific) were sterilized with 100% ethanol (Sigma Aldrich, St. Louis, MO). Coverslips were treated with Cell Tak poly-L-lysine (BD
Biosciences, Bedford, MA) for 10 minutes to aid adherence of NK92s to coverslips. Dex treated and untreated NK92 cells (500,000 cells/coverslip) were added to the coverslips for 10 minutes. Coverslips were treated with methanol-free formaldehyde (Polysciences Inc., Warrington, PA) diluted in saponin buffer (Sigma Aldrich, St. Louis, MO) for 10 minutes. This fixed cells to coverslips while maintaining 3D structure of cells. Coverslips were washed with PBS. Cells were permeabilized with 0.2% Triton-X (Sigma Aldrich, St. Louis, MO) for 12 minutes. Coverslips and cells were blocked for 15-60 minutes with 0.1% BSA in PBS. Next, antibodies specific for chromatin remodeling proteins were added for 1 hour at room temperature. Primary antibodies were added at a 2.5:100 dilution in 0.1% BSA in PBS. Antibodies used included: anti-HDAC1 (Cell Signaling, Danvers, MA) (Abcam, Cambridge, MA), anti-HDAC2 (Cell Signaling, Danvers, MA), anti-HDAC3 (Cell Signaling, Danvers, MA) (Abcam, Cambridge, MA) and anti-GR (Abcam, Cambridge, MA). Coverslips were washed with 0.1% BSA in PBS. After primary antibodies, secondary antibodies were added for 20-30 minutes at room temperature. Secondary antibodies were added at a 1:500 dilution. Secondary antibodies to be used were: donkey-anti-mouse IgG (Cy3-conjugated) (Jackson Immunoresearch, West Grove, PA) and donkey-anti-rabbit IgG (Alexa 488-conjugated) (Jackson Immunoresearch, West Grove, PA). Coverslips were washed with 0.1% BSA in PBS. After washing coverslips were fixed to slides with Prolong Gold (Invitrogen, Carlsbad, CA) containing DAPI stain for nuclear identification. Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP
HQ; Photometrics), using a 1.4-numerical aperture 100× objective lens, and were recorded with SoftWoRx software (Applied Precision). Images were assessed and analyzed with Imaris Software.

**NK92 Cells- Image Stream:**

Cellular permeabilization, reagents and antibody staining procedures as described above in *Intracellular Staining by Flow Cytometry Section.* After staining, samples were analyzed by immunofluorescent microscopy with an Amnis Image Stream for data acquisition. For NK92 cells 500-3,000 events were recorded and analyzed with IDEAS Software.

**Recruitment/Enrollment:**

Women 43-75 years of age, with early stage, breast cancer, treated with breast conserving surgery were enrolled. Some women also underwent radiotherapy and/or hormonal therapy. Women were excluded if they were treated with systemic chemotherapy, had recurrent breast cancer or other cancers (within the last 5 years), immune-based disease (e.g. multiple sclerosis, HIV), psychoses, cognitive dysfunction; were not fluent in English, abused controlled substances, were under psychotherapy, took anxiolytics, hypnotics, psychotropics, cortico-steroids, or immune-altering drugs. At each time point subjects were screened for infections. These criteria applied equally to a Comparison Group (Control).
Missing data were minimal for both psychological and immune data. Reasons for
missing immune data were inadequate specimen blood volume, inability to perform
venipuncture or frank refusal of venipuncture.

Study Outline:

For all women enrolled psychological instruments were administered, salivary
cortisol was collected and peripheral blood was obtained. Women were assessed as they
responded to and recovered from breast cancer diagnosis at four time points (See Figure
13). Data were collected in the period of initial cancer diagnosis (T1), 10-12 days post
breast surgery and before any adjuvant therapy was initiated (T2). An interval of 10-12
days after surgery provided ample time for surgical or anesthesia-induced effects to
dissipate [110, 153-162]. Hence, this window of time permitted analysis of variables
without confounds of surgery or adjuvant therapy. The third time point (T3) was two
months following radiation treatment completion (for those receiving radiation treatment)
or approximately four months after diagnosis (for those not receiving radiation treatment)
and the fourth time point (T4) was six months after radiation treatment completion (for
those receiving radiation treatment) or approximately 8 months after diagnosis (for those
not receiving radiation treatment). PBMCs were isolated from blood specimens obtained
from these women by a ficol histopaque gradient density separation, as described
previously [151].
Psychological Assessments:

Women completed the Perceived Stress Scale (PSS), which provided a general measure of stress (i.e., controllability of life events). Women with breast cancer may exhibit depressive symptoms, so the Center for Epidemiologic Studies–Depression (CES-D) was administered, which captured depressive symptoms, as well as monitored for risk of depression. PSS is a 10-item scale that assesses the degree to which a person finds their lives as unpredictable, uncontrollable or overloaded (i.e., exceeding their adaptive capacities). It is a measure of global stress appraisal, as opposed to a specific event which evokes a stress response [163]. Reliability (stability) was 0.85 and Cronbach alphas range from 0.75-0.86 [164]. The PSS is widely used in studies of stress on immunity, including studies of the stress-immune response of women with breast cancer [15, 107]. PSS was shown to be strongly related to trajectories of change in NKCA observed in women as they adapted to a diagnosis of breast cancer[107]. CES-D provided a measure of depressive symptoms. CES-D assesses frequency and duration of depressive symptoms [165]. Scores range from 0-60 and ≥16 suggests depression risk. CES-D is widely used in studies of women with breast cancer [166, 167]. It has good construct validity, good test-retest reliability [165]. In cancer patients and healthy controls CES-D showed good internal consistency (alpha=0.87 and alpha=0.89), respectively [168].

Selection Criteria- Cancer Pre, Cancer Post, Control:
The response to breast cancer diagnosis as assessed by the PSS is quite individual and varied for the cohort evaluated, as part of this dissertation. For example, 28% of the Cancer group reported PSS scores that did not change from T1 and T2. Whereas, PSS scores decreased from T1-T2 for 40% of the Cancer group while 32% of the Cancer group reported an increase in PSS from T1-T2. These variations in PSS overtime are common in individuals with cancer [39] as individuals vary in their perception of stress, coping effectiveness, and supportive networks. In order to capture results during the peak of perceived stress for these women at T1 and T2, a selection technique was used to identify a pre radiation time point at which an individual woman expressed the greatest level of perceived stress and in addition also demonstrated reduced NKCA. The rationale for inclusion of reduced NKCA as additional criteria is that NKCA is highly sensitive to the effects of perceived stress [5, 109-114]. For each woman the time point (either T1 or T2) where increased PSS and decreased NKCA were measured was selected for the Cancer Pre group. In some Cancer women decreased stress and/or increased NKCA was observed at T1 and T2, for these women the time point where the higher PSS and lower NKCA was used (see Supplemental Table 7). This resulted in 27 women selected for T1 or T2 time periods out of the total 29 women. The remaining 2 women were not included in this evaluation. This selection technique was used by other groups as well to assess women with increased PSS and decreased NKCA [108, 169]. Limited numbers of women enrolled in the Cancer group prevented assessment of a separate group of patients exhibiting low PSS and low NKCA.
The Cancer Post group assessed Cancer women following radiation treatment. For the Cancer Post group either the T3 or T4 time points were chosen that reflected decreased PSS. Limited sample size prevented selection based on NKCA. This resulted in 20 Cancer women selected from T3 and T4 time period out of a total of 22 women. The remaining 2 women were not evaluated. The Cancer women used for the Cancer Pre group were the same Cancer women used for the Cancer Post group, simply following therapeutic treatment. For each woman either the T3 or T4 was included. Additionally, availability of psychological and immune data also determined which time point was assessed. For the Control group psychological assessments remained consistent throughout the study and thus one group was created (Control).

_Salivary Cortisol:_

Women collected saliva for measurement of cortisol two days prior to a blood draw at each time point. On each of the two collection days, saliva was obtained at awakening (“when your eyes open and you are ready to get up”), at 30 minutes after wakening, noon, 5 PM and at bedtime (“right before getting into bed”). Women recorded collection times on saliva vials. They did not brush their teeth for at least 15 minutes prior to collection and abstained from smoking at least 1 hour before sampling. Using salivettes (Salimetrics™), subjects gently chewed (60-90 seconds) on a soft swab that fits into a holder resting in a centrifuge tube. The morning sample was obtained within 15 minutes of each person’s wake time [170, 171], while compliance for the later samples
was ± 60 min around the sampling time. Women who woke after 11 AM and night shift workers were excluded[172]. Centrifuged samples were frozen (-20°C) and assayed in duplicate within 6 months using immunoassay kits (Salimetrics™). Intra-assay coefficient of variation was 3.35-3.65%. Inter-assay coefficient of variation was 3.75-6.41%. The minimal detectable cortisol concentration was < 0.003 µg/dL. The cortisol awakening response (the difference between the sample collected at 30 minutes and the wakeup sample) was determined.

The waking rise was calculated with the following formula:

\[ \text{Waking Rise} = \frac{[(\text{Salivary Cortisol 30 minutes post wakening (ng/ul)})-(\text{Salivary Cortisol at awakening 9ng/ul})]/[(\text{Time 30 minutes post wakening (minutes)})-(\text{Time Wakening(minutes)})]} \]

**Statistical Analysis:**

**PBMCs:**

Missing data were treated as such and no imputation techniques were used. Independent student t test was performed to analyze differences between the Cancer group and Control group at each time point (e.g. Cancer T1 vs. Control T1). A two-sided alpha of 0.05 was set for statistical significance. Correlations were examined by Pearson’s correlation coefficient with an alpha of p< 0.05 set for statistical significance. When data sets were not normally distributed or skewed log transformations were
performed on raw data prior to analysis. Skewness was tested by the Shapiro-Wilks test. One-way ANOVAs with Least Significant Difference (LSD) and Bonferroni post hoc analysis were used for statistical assessment of selected groups (Cancer Pre, Control and Cancer Post). Statistical package for Social Sciences (SPSS: version 15.0) was used for data analysis.

NK92 cells:

Data were expressed as means with the standard error of the mean (SEM). Main study variables were analyzed using independent Student’s t test when two groups were compared and one-way ANOVA followed by Tukey post hoc analysis when three groups were compared. Pearson’s correlation coefficients were calculated for correlation analysis. A two-sided alpha of 0.05 was set for significance, except as noted. The Statistical Package for Social Sciences (SPSS: version 15.0) was used for data analysis.
CHAPTER 6

RESULTS

Specific Aim 1. Characterize an in vitro system to identify effects of GCs treatment on global epigenetic pattern and immune cell function.

It is well-established that psychosocial distress reduces natural killer cell activity (NKCA) [14, 15, 63, 105, 173] and dysregulates cytokine balance mediated by stress-induced release of glucocorticoids (GCs) [7-9]. This aim sought to identify whether GCs (dexamethasone) reduce NK functional activity and alter cytokine production through global epigenetic modification of histone residues. A human NK cell line (NK92 cells) was used to identify those functional activities of NK cells that are affected by dexamethasone (Dex). As human samples were limited, an in vitro model system provided a screening tool for determining which global epigenetic modifications were altered by Dex and also related to NK cell function.

Short-Term, High Dose Dex Treatment (Dex $10^{-7}$ M for 24 hours) of NK92 cells:

Effect of Dex on NKCA:

The ability of Dex to reduce NK92 cell lysis of tumor cell targets was evaluated. Treatment of NK92 cells for 24 hours produced a dose-dependent reduction in NK92
lytic activity (**Figure 1**). A concentration of \((10^{-7}\text{M})\) Dex produced an approximate 50% reduction in NKCA with no impact on cell viability, as judged by Trypan blue exclusion (**Supplemental Table 2**).

**Effect of Dex on NK92 Adhesion when Engaged by Tumor Cells:**

NKCA requires first the adhesion of NK cells to a tumor cell target, followed by the exocytosis of lytic granules, which destroy the tumor cell target. The ability of NK92 cells to form conjugates with target cells after Dex treatment was analyzed by flow cytometry. NK92 cells were labeled with APC-conjugated antibodies specific for the NK cell surface molecule CD56. K562 tumor targets were labeled with CFSE. NK92-K562 conjugates were measured as double positive (CD56+, CFSE+) populations. The ability of NK92 cells to form conjugates with K562 cells was significantly \((p=0.001)\) decreased in response to Dex \((10^{-7}\text{M})\), as compared to untreated NK92 cells, **Table 1**. These results indicate that Dex reduced NKCA, in part, by reducing the capacity of NK92 cells to bind to K562 target cells.

**Effect of Dex on Production of IFN gamma and Perforin:**

Experiments were conducted to assess the effect of Dex on the capacity of NK92 cells to produce effector molecules: IFN gamma and perforin. The effect of Dex on the NK92 production of IFN-gamma and perforin production was assessed by ELISA. A dose dependent effect was observed and Dex at \(10^{-7}\text{M}\) significantly inhibited constitutive
IFN-gamma secretion (30.6 ± 1.1 pg/ml vs. 52.5 ± 1.9 pg/ml; p<0.001) and perforin (17.0 ng/ml vs. 20.5 ng/ml), Figure 2.

Following cellular activation, Dex also reduced NK92 production of intracellular IFN-gamma. As shown in Figure 3 and Table 2, Dex (10⁻⁷ M) decreased not only the detectable level of the cytokines on a cell-to-cell basis (32.4 ± 2.9% reduction, p<0.0001 for IFN gamma) but also the percentage of cytokine positive cells (30.9 ± 1.5% reduction, p<0.001 for IFN gamma). In Table 2 the MFI of these detected intracellular cytokines are presented and demonstrate a significant reduction in detectable cytokine by Dex treatment followed by cellular activation.

Effect of Dex on NK92 Surface and Intracellular Proteins:

Since Dex treatment of NK92 cells decreased conjugate formation the effect of Dex on the capacity of NK92 cells to produce NK adhesion and effector molecules was next assessed by flow cytometry. Adhesion molecules allowed for proper conjugate formation resulting in lysis of target cells. Once inside target cells the effector molecule, granzyme B, initiates apoptotic pathways resulting in target cell death. There was a significant reduction in the expression of the lymphocyte adhesion molecule, LFA-1 (p=0.0001), which is known to mediate binding of NK92 cells to K562 and the granule constituent, granzyme B (p=0.022). In Table 2 MFIs of these surface markers and granule constituent are presented. All NK92 cells stained positive for these molecules, therefore no changes in percent positive cells were measured. Interestingly, these effects
were not universal in that the adhesion molecule, CD2 (also known to mediate binding of NK92 cells to K562) [174, 175] expression was not decreased. See Table 2.

**Effect of Dex on H4-K8 Acetylation:**

To assess the epigenetic effect of Dex, western blot analysis and flow cytometric analysis were performed on nuclear extracts derived from NK92 cells, Figure 4, or on permeabilized NK92 cells, Table 3. The blots and permeabilized cells were probed with antibodies specific for H4-K8 Ac. NK92 cells treated with Dex had decreased global acetylation of H4-K8 (~50% reduction) when compared to untreated cells as measured by western blot. In Figure 4a, a representative western blot is presented and shows the visual reduction in H4-K8 acetylation. Figure 4b illustrates the quantification of western blots for the 4 groups of NK92 cells: No Treatment, Dex treated, TSA treated and Dex plus TSA. ANOVA demonstrated a significant difference among the 4 groups, [F(3,12)=99.526, p<0.0001], such that there was a significant reduction in acetylation of H4-K8 with Dex, which was restored by treatment with TSA, a histone deacetylase (HDAC) inhibitor. Dex also reduced acetylation of H4-K8 as judged by flow cytometric analysis (~20%) Table 3. ANOVA demonstrated a significant difference among the 4 groups, H4-K8 MFI [F(3,11)=33.104, p<0.0001] and H4-K8 % NT [F(3,24)=12.918 p<0.0001] such that there was a significant reduction in acetylation of H4-K8 with Dex which was reversed by treatment with TSA, a HDAC inhibitor. These effects of Dex were not observed with HDAC inhibitor treatment. Western blot analysis was performed
on nuclear extracts from NK92 cells and standardized to total nuclear histone 3 (H3) proteins. Flow cytometric analysis evaluated nuclear acetylation of H4-K8 and was standardized to cell number, rather than cellular H3 protein. These differences likely account for differential reductions in H4-K8 Ac.

These global changes in acetylation suggest that the effect of Dex on NK92s was a possible consequence of epigenetic modification to promoter regions associated with genes essential to NK cell lytic effector function (e.g. perforin and granzyme B). If this was true, then short-term, high dose Dex treatment would reduce accessibility for the promoter regions of these genes, perforin and granzyme B. These epigenetic effects were assessed by, chromatin immunoprecipitation (ChIP) with anti-H4-K8 Ac, followed by quantitative real time PCR, was used to calculate the fold change in accessibility after short-term, high dose Dex treatment. Short-term, high dose Dex treatment reduced H4-K8-Ac at both the perforin and granzyme B promoter regions [6].

*Effect of Histone Deacetylase Inhibitor (TSA) on Interferon gamma Production and NKCA by Dex Treated NK92 Cells:*

These data suggest that the alteration in NK cell functional activity after short-term, high dose Dex treatment may result from promoter deacetylation at immune relevant genes. If so, then treatment with TSA would be expected to modify the effects of Dex on the functional activity of the NK cells. NK92 cells were treated with Dex, followed by incubation with TSA. TSA modified the effects of Dex not only for the
intracellular production of IFN gamma, Table 4, but also for NK lytic activity against tumor targets, Table 5. ANOVA analysis showed significant differences among the 4 treatment groups [No Treatment (NT), Dexamethasone (Dex), Trichostatin A (TSA) and Dexamethasone plus TSA] for IFN gamma MFI [F(3,15)=46.515, p<0.001] and IFN gamma % NT [F(3,15)=49.973, p<0.0001] Table 4. Dex significantly reduced detectable levels of IFN gamma, but with the addition of TSA, these levels returned to that of the untreated cell population. Likewise for NKCA, Dex significantly reduced lytic activity of NK92 cells for tumor targets and treatment with TSA returned NKCA to a level produced by untreated NK92 cells, Table 5. ANOVA analysis showed significant differences among the 4 treatment groups [No Treatment (NT), Dexamethasone (Dex), Trichostatin A (TSA) and Dexamethasone plus TSA] NKCA lytic units [F(3,27)=30.770, p<0.0001] and NKCA % NT [F(3,36)=44.110, p<0.0001].

Brief Discussion of Short-Term, High-Dose Dex Treatment of NK92 Cells:

Short-term, high-dose Dex treatment (10^{-7} M for 24 hrs) reduced NK cell function and global H4-K8-Ac in NK92 cells. Although effects of short-term, high-dose Dex treatment were dramatic and impacted both global and gene-specific acetylation changes [6] immune functional changes do not completely reflect patterns observed in breast cancer patients experiencing psychosocial distress and thus a second in vitro system was investigated. Investigation into dose and duration of Dex exposure on NK92 cells was
explored. Development of a chronic, five day treatment system allowed for increased understanding of GCs induced epigenetic and immunologic changes in NK cells.

**Chronic Dex Treatment (Dex 10^{-10} M for 5 days) of NK92 cells:**

*Effect of Chronic Dex Treatment on NKCA:*

The ability of Dex to reduce NK92 cell lysis of tumor cell targets following chronic Dex treatment was evaluated. Chronic Dex treatment of NK92 cells for 5 days produced a reduction in NK92 lytic activity, *Figure 5*. A concentration of (10^{-10} M) Dex reduced cytotoxicity at each effector:target ratio (p<0.05) with no impact on cell viability, as judged by Trypan blue exclusion (*Supplemental Table 2*).

*Effect of Chronic Dex Treatment on Intracellular IFN gamma:*

Experiments were conducted to assess the effect of Dex on the capacity of NK92 cells to produce the pro-inflammatory cytokine, IFN gamma. Following cellular activation, chronic Dex treatment increased NK92 production of intracellular IFN-gamma. As shown in *Figure 6*, Dex (10^{-10} M) increased the detectable level of intracellular IFN-gamma on a cell-to-cell basis (p<0.05).

*Effect of Chronic Dex Treatment on Global H4-K8 Acetylation:*
To assess the epigenetic effect of chronic Dex treatment, flow cytometric analysis was performed on permeabilized NK92 cells. The permeabilized cells were probed with antibodies specific for H4-K8 Ac. NK92 cells treated with Dex had decreased global acetylation of H4-K8 (~20% reduction) when compared to untreated NK92 cells. In Figure 7a, a representative flow cytometric blot is presented and shows the visual reduction in global H4-K8 acetylation. In Figure 7b, the graph shows the quantification of 3 independent experiments measuring global H4-K8-Ac by flow cytometry. Chronic Dex treatment of NK92 cells global H4-K8-Ac MFI is standardized to untreated NK92 cells.
Figure 1 Short Term, High Dose Dexamethasone (Dex) treatment- NKCA.
The effect of short term, high dose Dex treatment on Natural Killer Cell Lytic Activity is illustrated. NK92 cells were treated with varying amounts of Dex for 24 hours. Percent (%) inhibition was calculated by: [(Lytic Units No Treatment)-(Lytic Units Dex Treatment)]/(Lytic Units No Treatment) X 100. Values are presented as the mean and standard error of the mean (SEM). N = at least three independent experiments. *= p< 0.05, Treatment with Dex vs. No Treatment (NT).
Figure 2 Short Term, High Dose Dexamethasone (Dex) treatment-Produced IFN gamma and Perforin.

The effect of Dex on constitutive production of cytokines and perforin is illustrated. NK92 cells were treated for 24 hour with dexamethasone (10^{-7} M). Supernatants were collected and assayed for (a) IFN-gamma and (b) perforin by ELISA. Results are expressed as mean ± SEM; N = at least three independent experiments. NT=No Treatment, p values represent statistically significant difference, No Treatment (NT) vs. Dex at indicated concentration,*=p<0.05; **=p<0.01; ***=p<0.001.
Figure 3 Short Term, High Dose Dexamethasone (Dex) treatment- Intracellular IFN gamma.
The effect of short term, high dose Dex treatment on induced production of intracellular cytokines is illustrated. NK92 cells were treated with Dex (10^{-7} M) for 24 hour and then stimulated with leukocyte activation cocktail for 4 hrs. Intracellular cytokine staining was performed using anti-human IFN-gamma antibodies and assessed by flow cytometry. Representative results are depicted. NT=No Treatment.
Figure 4 Short Term, High Dose Dexamethasone (Dex) treatment - Global H4-K8 Acetylation Analysis.

The effect of short term, high dose Dex treatment and the histone deacetylase inhibitor (TSA) on acetylation status of H4-K8. (a) Representative western Blot analysis of nuclear extracts of NK92 cells probed with antibodies specific for the acetylated form of H4-K8. (b) Blots (n= 3) were standardized to total H3 protein (blots not shown) and blot density quantified using Image J software. NT = No Treatment, Dex = Dexamethasone, TSA = Trichostatin A (100 nM). % Change= [(Density Dex or Dex +TSA)/(Density NT)] X 100. Results are expressed as mean ± SEM (n = 3). Statistically significant difference, *** = p<0.001. No Treatment (NT) vs. Dex (10⁻⁷), +++ = p <0.001, Dexamethasone vs. Dexamethasone + TSA.
Figure 5 Chronic Dexamethasone (Dex) Treatment- NKCA.
The percent cytotoxicity of NK92 cells against target K562 cells is plotted at 4 effector to target ratios. Effector: target ratios: 2:1, 1:1, 0.5:1, and 0.25:1. The graph is the average of 3 independent experiments, n=9. *p<0.05, Untreated v. Dex -10 M. Untreated = No Treatment.
**Figure 6 Chronic Dexamethasone (Dex) Treatment: Intracellular IFN gamma.**

The effect of chronic Dex treatment on induced production of intracellular IFN gamma is illustrated. NK92 cells were treated with Dex$10^{-10}$ M for 5 days and then stimulated with leukocyte activation cocktail for 4 hrs. IFN gamma MFI is relative to No Treatment IFN gamma MFI average. n=3. *p<0.05, No Treatment v. Dex -10 M. NT=No Treatment.
Figure 7 Chronic Dexamethasone (Dex) Treatment- Global H4-K8-Acetylation Analysis.

Analysis of posttranslational histone tail modifications by flow cytometry following chronic Dex treatment. The representative histogram (a) shows the mean fluorescence intensity (MFI) of H4-K8-Ac between treated (10^{-10} M) in the grey dashed line and untreated (black line) cells from one of three independent experiments. The bar graph (b) representing the percent changes in MFI of histone modifications investigated. Graph is the average of 5 independent experiments, n=3. *** =p<0.001, No Treatment v. Dex -10 M. NT=No Treatment.
Table 1

Effect of dexamethasone and trichostatin A on conjugate formation of NK92 with target cells by flow cytometric analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Conjugates</th>
<th>P value (compared to NT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment (NT)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Dex (10^{-7} M)</td>
<td>73.3 ± 3.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>TSA (100 nM)</td>
<td>97.32 ± 6.0</td>
<td>0.975</td>
</tr>
<tr>
<td>Dex (10^{-7} M) + TSA (100 nM)</td>
<td>72.23 ± 6.11</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M., n>7. Dex= dexamethasone. TSA= Trichostatin A. NT= No Treatment. Isotype control MFI was always < 150. % Conjugates = [(No Treatment (NT) - Dex or Dex + TSA)/(No Treatment (NT))] X 100. Statistically significant difference, NT vs. Dex, NT vs. Dex + TSA, Dex vs. Dex + TSA.
Table 2

Effect of dexamethasone on surface and intracellular proteins of NK92 by flow cytometric analysis.

<table>
<thead>
<tr>
<th>Cellular Protein</th>
<th>No Treatment</th>
<th>Dexamethasone Treatment (10^{-7} M)</th>
<th>P value</th>
<th>% Change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN gamma</td>
<td>376 ± 17</td>
<td>272 ± 4.7</td>
<td>0.004</td>
<td>-31.6 ± 2.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>LFA-1</td>
<td>2,722 ± 50</td>
<td>2,377 ± 28</td>
<td>0.0001</td>
<td>-13.1 ± 0.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>CD-2</td>
<td>783 ± 12</td>
<td>884 ± 9.0</td>
<td>0.0001</td>
<td>12.8 ± 1.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>63,156 ± 1178</td>
<td>57,522 ± 1706</td>
<td>0.022</td>
<td>-11.3 ± 2.7</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are mean fluorescent intensity (MFI) of surface and intracellular proteins ± S.E.M., N= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI No Treatment - MFI Dexamethasone)/(MFI No Treatment) X 100. Statistically significant difference, No Treatment vs. Dexamethasone Treatment.
Table 3
Effect of dexamethasone and trichostatin A on H4-K8 nuclear acetylation of NK92 by flow cytometric analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H4K8-Ac MFI</th>
<th>$P$ value (compared to NT)</th>
<th>% NT (compared to NT)</th>
<th>$P$ value (compared to NT)</th>
<th>H4K8-Ac MFI</th>
<th>$P$ value (compared to Dex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment (NT)</td>
<td>2,437 ± 18</td>
<td>0.002</td>
<td>79.8 ± 3.9</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex ($10^{-7}$ M)</td>
<td>2,054 ± 71</td>
<td>0.002</td>
<td>79.8 ± 3.9</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA (100 nM)</td>
<td>2,701 ± 34</td>
<td>0.018</td>
<td>119.7 ± 8.1</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex ($10^{-7}$ M) + TSA (100 nM)</td>
<td>2,506 ± 47</td>
<td>0.735</td>
<td>106.9 ± 2.8</td>
<td>0.694</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M., n = 7. Dex = dexamethasone. TSA = Trichostatin A. NT = No Treatment. Isotype control MFI was always < 150. % of NT = [(No Treatment (NT) - Dex or Dex + TSA) / (No Treatment (NT))] X 100. Statistically significant difference, NT vs. Dex, NT vs. Dex + TSA, Dex vs. Dex + TSA.
Table 4

Effect of dexamethasone and trichostatin A on intracellular interferon gamma of NK92 by flow cytometric analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN gamma MFI</th>
<th>( P ) value (compared to NT)</th>
<th>% NT (compared to NT)</th>
<th>( P ) value (compared to NT)</th>
<th>IFN gamma MFI</th>
<th>( P ) value (compared to Dex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>753 ± 22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex (10(^{-7}) M)</td>
<td>563 ± 12</td>
<td>0.039</td>
<td>74.8 ± 1.6</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA (100 nM)</td>
<td>1255 ± 25</td>
<td>0.0001</td>
<td>166 ± 3.3</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex (10(^{-7}) M) + TSA (100 nM)</td>
<td>933 ± 78</td>
<td>0.051</td>
<td>123 ± 10</td>
<td>0.043</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. N=4. Dex= dexamethasone. TSA= Trichostatin A. NT= No Treatment. Isotype control MFI was always < 150. % of NT and statistically significant difference calculated as in Table 4.
Table 5  
Effect of dexamethasone and trichostatin A on NKCA of NK92

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lytic Units</th>
<th>P value (compared to NT)</th>
<th>% NT</th>
<th>P value (compared to NT)</th>
<th>Lytic Units</th>
<th>P value (compared to Dex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>1202.24 ± 75</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex (10^{-7} M)</td>
<td>655.97 ± 35</td>
<td>0.0001</td>
<td>51.51 ± 6.8</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA (100 nM)</td>
<td>1584.32 ± 95</td>
<td>0.003</td>
<td>133.5 ± 11.0</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex (10^{-7} M) + TSA (100 nM)</td>
<td>1205.44 ± 53</td>
<td>1.0</td>
<td>101.5 ± 8.2</td>
<td>0.998</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. N=7. Dex= dexamethasone. TSA= Trichostatin A. NT= No Treatment. % of NT and statistically significant difference calculated as in Table 4.
**Supplement Table 1.**

*Additional Statistical Data for Table 3*

<table>
<thead>
<tr>
<th>Protein</th>
<th>For MFI</th>
<th>df</th>
<th>For % Change</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN gamma</td>
<td>5.052</td>
<td>5</td>
<td>11.023</td>
<td>7</td>
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<tr>
<td>TNF alpha</td>
<td>12.505</td>
<td>4</td>
<td>24.832</td>
<td>2</td>
</tr>
<tr>
<td>LFA-1</td>
<td>5.941</td>
<td>10</td>
<td>18.307</td>
<td>12</td>
</tr>
<tr>
<td>CD-2</td>
<td>-6.541</td>
<td>10</td>
<td>-10.663</td>
<td>5</td>
</tr>
<tr>
<td>Granzyme</td>
<td>2.716</td>
<td>10</td>
<td>4.207</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKp30</td>
<td>18.967</td>
<td>4</td>
<td>72.729</td>
<td>2</td>
</tr>
<tr>
<td>NKp46</td>
<td>-14.704</td>
<td>4.511</td>
<td>-16.584</td>
<td>3</td>
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</table>

Values are mean fluorescent intensity (MFI) of surface and intracellular proteins ± S.E.M., N= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI No Treatment - MFI Dexamethasone)/(MFI No Treatment) X 100. Statistically significant difference, No Treatment vs. Dexamethasone Treatment.
### Supplemental Table 2
Percent viability as determined by trypan blue staining

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment- 24 hours</td>
<td>86.6 ± 1.1</td>
</tr>
<tr>
<td>No Treatment- 5 Days</td>
<td>93.9 ± 0.8</td>
</tr>
<tr>
<td>Dex (10^{-7} M)- 24 hours</td>
<td>87.2 ± 1.3</td>
</tr>
<tr>
<td>Dex (10^{-10} M)-5 Days</td>
<td>91.7 ± 1.1</td>
</tr>
<tr>
<td>TSA (100 nM)</td>
<td>86.3 ± 1.5</td>
</tr>
<tr>
<td>Dex (10^{-7} M) + TSA (100 nM)</td>
<td>84.7 ± 0.8</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. N=7. Dex= dexamethasone. TSA= Trichostatin A. NT= No Treatment. Viability determined by: [(Number of living NK92 cells)/(total number of NK92 cells)]*100
**Supplemental Table 3.**

Flow cytometric and Immunofluorescent Antibody Dilutions

<table>
<thead>
<tr>
<th>Antibodies Specificity-Conjugate</th>
<th>PBMCs</th>
<th>NK92 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8-PerCP-Cy5.5</td>
<td>1:25</td>
<td>n/a</td>
</tr>
<tr>
<td>CD4-Pacific Blue</td>
<td>1:25</td>
<td>n/a</td>
</tr>
<tr>
<td>H4-K8-Ac-unconjugated</td>
<td>1:50</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K9-Ac-Alexa 488</td>
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Values are dilution factors for antibodies. n/a = not applicable.
Specific Aim 2. Characterize the chromatin remodeling proteins associated with global epigenetic patterns observed in NK92 cells treated with the glucocorticoid, dexamethasone.

During psychosocial distress immune effector genes are differentially impacted with subsets being up-regulated (pro-inflammatory cytokines) [7-9] and others down regulated (lytic activity) ) [14, 15, 63, 105, 173]. GCs have been shown to mediate immune dysregulation during psychosocial distress by altering global epigenetic patterns [5]. However, the mechanism of how GCs impact global epigenetic patterns during psychosocial distress remains unknown. In vitro analysis has shown that GCs can alter global epigenetic patterns by interacting with chromatin remodeling proteins [49, 144-146]. However, chromatin remodeling proteins have not been linked to immune dysregulation during psychosocial distress. Therefore exploratory analysis of chromatin remodeling protein cellular localization patterns during chronic GCs treatment would advance understanding of molecular mechanisms of immune dysregulation. Identification of chromatin remodeling proteins altered during psychosocial distress may allow for better understanding of these GCs induced immune effects.

**Chromatin Remodeling Protein Levels and Cellular Localization at Day 5:**

GCs can alter global epigenetic patterns by interacting with chromatin remodeling proteins such as HDACs and histone acetylases (HATs). Previous work has shown that
GCs interact with HDAC1, HDAC2, HDAC3 and p300 [49, 143]. Flow cytometry was used to determine intracellular total protein levels for Class I HDACs, HAT p300 and glucocorticoid receptor (GR) at Day 5. **Figure 8a** shows the quantification of three independent experiments measuring HDAC1, HDAC2, HDAC3, p300 and GR at Day 5. Chronic Dex treated NK92 cells chromatin remodeling protein MFI is standardized to untreated NK92 cells chromatin remodeling protein MFI (*See Materials and Methods*). No change in total HDAC1, HDAC2, p300 and GR levels were measured at Day 5. Significant reductions in HDAC3 levels were measured. (p<0.05, **Figure 8a**). A representative FACs plot of total HDAC3 reductions is depicted in **Figure 8b**. The black line depicts the total HDAC3 levels in untreated NK92 cells, which the dashed grey line depicts the reduced total HDAC3 levels in chronic Dex treated NK92 cells.

Cellular localization of chromatin remodeling proteins of NK92 cells was measured by immunofluorescent microscopy on the Amnis Image Stream X (**Figure 9**). Basal cellular localization patterns varied between chromatin remodeling proteins, with some proteins measured primarily nuclear (HDAC1, p300) localization, while others were distributed into both the cytoplasm and the nucleus (HDAC2, HDAC3 and GR). Regardless of cellular treatment, ~75% of NK92 cells followed these staining patterns. The remaining ~25% was due to doublet staining or cells improperly imaged. **Figure 9** depicts a representative image of untreated (**Figure 9a**) and chronic Dex treated (**Figure 9b**) NK92 cells stained for DAPI (nuclear stain), anti-HDAC2 (FITC) and anti-HDAC1 (APC). The chronic Dex treated image shows increased nuclear staining of HDAC2.
(Figure 9). Quantification of nuclear localization was determined by IDEAS nuclear localization wizard software, allowing for measurement of nuclear localization for 1000 images per treatment. Increased nuclear localization of HDAC2 was measured at Day 5 in the chronic Dex treated NK92 cells when compared with the untreated cells (Figure 10).

Chromatin Remodeling Protein Levels and Cellular Localization at Day 2:

Slight decreases in intracellular perforin and granzyme B began at Day 2 (Supplemental Figure 1) therefore chromatin remodeling protein levels were assessed at day 2. Flow cytometry was used to determine intracellular protein levels for Class I HDACs, HAT p300 and GR at Day 2. Figure 11 shows the quantification of 3 independent experiments measuring HDAC1, HDAC2, HDAC3, p300 and GR at Day 2. Chromatin remodeling protein levels (MFIs) of chronic Dex treated NK92 cells are standardized to protein levels (MFIs) of untreated NK92 for each experiment (n=3). No change in total HDAC1, HDAC2, HDAC3 p300 and GR levels were measured at Day 2 (Figure 11).

Phosphorylated Chromatin Remodeling Protein Levels at Day 2:

Chromatin remodeling protein cellular localization at Day 2 was assessed by flow cytometry. Phosphorylation of chromatin remodeling proteins can be a marker for enzymatic activity and nuclear localization [176]. Flow cytometry was used to determine
intracellular phosphorylated protein levels and Figure 12 shows the quantification of 3 independent experiments measuring phosphorylated HDAC2 and phosphorylated MSK-1. Chromatin remodeling protein levels (MFIs) of chronic Dex treated NK92 cells are standardized to protein levels (MFIs) of untreated NK92 for each experiment (n=3). Significant increases in HDAC2-p were measured at Day 2 in chronic Dex treated NK92 cells (Figure 12, p<0.05). No change in MSK-1-p was measured at Day 2 (Figure 12). Increases in HDAC2-p at Day 2 suggest that translocation into the nucleus begins as early as Day 2, with increased nuclear localization remaining at Day 5 (Figure 10). Additionally, increases in HDAC2-p were measured at Day 5, corresponding with increased HDAC2 nuclear localization.
Figure 8 Chronic Dexamethasone (Dex) Treatment- Chromatin Remodeling Proteins.
Analysis of chromatin remodeling proteins by flow cytometry following chronic Dex treatment at Day 5. The bar graph representing the percent changes in MFI of chromatin remodeling proteins investigated at Day 5 (a) Graph is the average of 3 independent experiments. The representative histogram shows the mean fluorescence intensity (MFI) of HDAC3 (b), between treated (10^{-10} M) in grey dashed line and untreated (black line) cells from one of three independent experiments at Day 5. NT=No Treatment.
Figure 9 Chronic Dexamethasone (Dex) Treatment-HDAC1 and HDAC2 Images. Cellular localization of HDAC1 and HDAC2 was determined by immunofluorescent microscopy by Image Stream (40x magnification). Immunofluorescence of NK92 cells stained with anti-HDAC1 (red color), HDAC2 (green color) and DAPI (purple/blue color). (a) NK92 cell Untreated representative image (b) NK92 cell chronic Dex treatment representative image. Merged images for HDAC2/DAPI, HDAC1/DAPI and HDAC1/HDAC2 shown. 500-1000 images taken per treatment.
Figure 10 Chronic Dexamethasone (Dex) Treatment- HDAC2 Quantification.
Quantification of nuclear localization of HDAC2 was determined using merged images from Amnis Image Stream X and IDEAS quantification software, nuclear localization wizard. Graph is the average of 500-1000 images for each treatment. NT=No Treatment. The y axis is the quantification of merged staining between nuclear staining (DAPI) and HDAC2 (FITC) within each treatment; amount of staining similarity (similarity dilate) for each imaged, focused-single cell. For each treatment approximately 75% of the cells stained as presented in the figure.
Figure 11 Chronic Dexamethasone (Dex) Treatment- Chromatin Remodeling Proteins Day 2.
Analysis of total chromatin remodeling proteins within the NK92 cells by flow cytometry following chronic Dex treatment at Day 2. The bar graph representing the percent changes in MFI of chromatin remodeling proteins investigated at Day 2. Graphs are the average of 3 independent experiments. NT=No Treatment.
Figure 12 Chronic Dexamethasone (Dex) Treatment- Phosphorylated Proteins at Day 2. Analysis of phosphorylated proteins by flow cytometry following chronic Dex treatment at Day 2. The bar graph representing the percent changes in MFI of phosphorylated proteins investigated at Day 2. Graphs are the average of 3 independent experiments. *p<0.05, No Treatment vs. Dex -10 M. NT=No Treatment.
Supplemental Figure 1 Intracellular Perforin and Granzyme B Levels in Chronic Dexamethasone (Dex) Treated NK92 Cells at Day 2.

Intracellular perforin and granzyme B was determined by flow cytometry. The effect of chronic Dex treatment on intracellular perforin and granzyme B at Day 2 is illustrated. NK92 cells were treated with Dex $10^{-10}$ M for 2 days. Granule constituent of Dex treated NK92 cells is standardized to untreated NK92 cells. n=3. *=p<0.05. No Treatment v. Dex -10 M. NT=No Treatment.
Specific Aim 3. Characterize the global epigenetic patterns and the intracellular localization of chromatin remodeling proteins of the NK cells of women as they respond to and recover from breast cancer diagnosis.

GCs, which are altered during the psychosocial distress of breast cancer diagnosis [1-3], are known to impact epigenetic patterns [48, 128, 129]. Changes in epigenetic patterns have been linked to changes in gene transcription. It is possible that the mechanism mediating immune dysregulation during psychosocial distress is an epigenetic mechanism. The global epigenetic patterns of women as they respond to and recover from breast cancer were analyzed in order to understand whether epigenetic mechanisms contribute to psychosocial distress mediated immune dysregulation.

Descriptive Characteristic of Participants:

Twenty-nine women were enrolled in the Cancer group and 27 women were enrolled in the Control group. These women were enrolled and evaluated longitudinally at 4 times (T1-T4, Figure 13). Demographics, disease and treatment characteristics of participants are summarized in Table 6. Women were predominantly Caucasian in both the Cancer (77.4%) and Control groups (70.4%). The majority of the women were married in both the Cancer group (74.2%) and Control group (51.9%). The majority of Cancer women had Stage 0 breast cancer (79.2%) and underwent a combination of
surgery, radiation and hormonal treatment (54.2%). The majority of surgeries were breast conserving lumpectomies (70.4%).

**Psychological Assessment:**

Psychological assessments administered to the Cancer and Control groups were the Perceived Stress Scale (PSS), which measured general appraisal of stress, and the Centers for Epidemiologic Studies-Depression (CES-D), which measured depressive mood. Women in the Cancer group had increased PSS (Figure 14) and CES-D (Figure 15) when compared to the Control group at cancer diagnosis (T1; p<0.01). Increases in perceived stress persisted at T2 (p<0.01), T3 (p<0.05) and T4 (p<0.05) in the Cancer group when compared to the Control group (Figure 14). Depressive mood levels of the Cancer group returned to levels comparable to Control group at T2, T3 and T4 (Figure 15).

**Natural Killer Cell Activity:**

Increased PSS have been linked to alterations in NK cell function. The ability of NK cells to lyse tumor cell targets was evaluated for the NK cells of the Cancer and Control groups by measurement of NKCA. NKCA was significantly reduced in the Cancer group at T1 when compared with the Control group at T4 (Figure 16, p<0.05). Analysis of circulating NK cell percentage showed no difference between Cancer vs. Control groups. Data not shown.
**IFN gamma Production:**

Increases in PSS have also been associated with alterations in pro-inflammatory cytokine production [7-9]. The ability of total peripheral blood mononuclear cells (PBMCs) to produce IFN gamma after 48 hour stimulation was measured. Significant increases in produced IFN gamma levels were measured in the Cancer group when compared with the Control group (Figure 17) at T3 (p<0.05) and T4 (p<0.05). IFN gamma was not detected in plasma of Cancer or Control groups.

**Plasma IL-6:**

Circulating interleukin-6 (IL-6) levels in the plasma were measured by ELISA. Significant increased plasma IL-6 levels were observed in the Cancer group when compared with the Control group at T2 (Figure 18, p<0.05).

**Psychological, Immunologic and Epigenetic Analysis of Selected Groups of Women:**

For immunologic and epigenetic analysis a selection technique (described in Materials and Methods) was used to assess women at peak stress time periods. This selection technique created three groups, Cancer Pre, Control and Cancer Post. Cancer Pre women reported increased perceived stress and decreased NKCA prior to radiation treatment when compared to the Control group.
Psychological Assessment of Cancer Pre, Control and Cancer Post Groups:

Perceived stress and depressive mood were measured in selected groups (Cancer Pre, Control and Cancer Post. The Cancer Pre group had increased PSS (Figure 19a) when compared to the Control group (p<0.001) and Cancer Post Group (p<0.05) as measured by ANOVA (F_{2,61}=11.7, p=0.0001). The Cancer Pre group had increased CES-D (Figure 19b) when compared to the Control group (p<0.001) and Cancer Post Group (p<0.05) as measured by ANOVA (F_{2,62}=9.9, p=0.0001).

Natural Killer Cell Activity of Cancer Pre, Control and Cancer Post Groups:

As NK cell are especially sensitive to alterations in psychosocial distress the ability of NK cells to lyse tumor cell targets was evaluated in the Cancer Pre, Control, and Cancer Post groups. NKCA was significantly reduced in the Cancer Pre group when compared with both the Control group (Figure 20; p<0.05) and Cancer Post group (Figure 20; p<0.05) as measured by ANOVA (F_{2,64}=3.159, p=0.049). Analysis of circulating NK cell percentages demonstrated no differences among Cancer Pre, Cancer Post and Control groups (Figure 21a).

Intracellular IFN gamma of PBMCs of Cancer Pre, Control and Cancer Post Groups:

NK cells can lyse tumor cells and produce pro-inflammatory cytokines. PBMCs intracellular IFN gamma levels following 4 hour stimulation with leukocyte activation cocktail were measured by flow cytometry. In the Cancer Pre group, intracellular IFN
gamma MFI of NK cells was numerically increased when compared with the Control group (Figure 22; p<0.05) and Cancer Post group (Figure 22; p<0.05) and as measured by ANOVA (F_{2,56}=8.8, p=0.001). Increases in intracellular IFN gamma of NK cell inversely correlated with NKCA (Table 7).

Increases in intracellular IFN gamma levels were not limited to the NK cell population. In the Cancer Pre group intracellular IFN gamma MFI of CD4+ T helper lymphocytes was numerically increased when compared with the Control group (Figure 23; p<0.05) and Cancer Post group (Figure 23; p<0.05) as measured by ANOVA (F_{2,54}=7.0, p=0.002). No differences in circulating CD4+ T helper lymphocyte percentages were observed among Cancer Pre, Control and Cancer Post (Figure 21b). No differences in intracellular IFN gamma levels were measured in CD8+ cytotoxic T lymphocytes when comparing the Cancer Pre, Control and Cancer Post groups. **Data not shown.**

**Salivary Cortisol of Cancer Pre, Control and Cancer Post Groups:**

Hypothalamic-pituitary-adrenocortical (HPA) axis activation produces a cascade of neuroendocrine products that result in elevated circulating adrenal derived GCs, which is cortisol in humans. Salivary cortisol was collected 5 times per day for 2 days prior to phlebotomy. Salivary cortisol levels were measured by ELISA and diurnal cortisol rhythms were determined. Cortisol waking rise has been shown to be altered in women with breast cancer [59, 60, 177]. No significant differences in cortisol parameters were
Relationships between cortisol waking rise and immunologic function were investigated. Significant negative correlations were measured between salivary cortisol and NKCA (Table 7, p<0.05) for Cancer Pre and Control groups combined. Additionally, significant positive correlations were measured between cortisol waking rise and intracellular IFN gamma of NK (Table 7, p<0.01) and CD4+ T helper lymphocytes (Table 7, p<0.01) for Cancer Pre and Control groups combined. These data suggest that alterations in salivary cortisol are associated with immunologic alterations observed in women.

Intracellular Perforin Levels in NK Cells of Cancer Pre, Control and Cancer Post Groups:

NK cells produce their effects against tumor cells by first engaging the tumor target, followed by exocytosis of NK cytolytic granule constituents, such as perforin, leading to tumor cell lysis. Intracellular perforin levels in NK cells and cytotoxic T lymphocytes were measured by flow cytometry. No differences were observed in perforin levels in cytotoxic T lymphocytes between Cancer Pre (62.1±10), Control (59.3±8) and Cancer Post (76.2±15) groups. Significant positive correlations were found between NKCA and perforin levels in NK cells (Table 8, p<0.05). Relationships between CD56 staining intensity and cytotoxic phenotypes of NK cells have been shown previously [91, 92]. Therefore staining patterns between CD56 versus perforin were analyzed by flow cytometry. The CD56-dim population stained positively for
intracellular perforin (Figure 24). Representative FACS plots for a Cancer Pre and Control woman are shown with the CD56-dim population noted in the box; this represents ~90% of the NK cells which is consistent with previous findings [91, 92]. Additionally, ~90% of the NK cells in the Cancer Pre group also stained positive for intracellular IFN gamma (Supplemental Figure 2). The representative flow histogram depicted in Supplemental Figure 2 shows the typical intracellular IFN gamma staining pattern for the Cancer Pre group with the majority (89.9%) of the NK cells staining positively for intracellular IFN gamma. These cells had differential CD56 staining, with both bright and dim CD56 cells producing IFN gamma. The same NK cells derived from women with breast cancer stained positively for intracellular perforin as were able to produce IFN gamma upon stimulation. These flow cytometric data identify a dichotomous phenotype within the NK cells of the Cancer Pre women, with NK cells exhibiting both decreased NK cell lytic activity (as measured by NKCA and intracellular perforin) and increased intracellular IFN gamma.

*Epigenetic Analysis of Cancer Pre, Control and Cancer Post Groups:*

GCs enter cells by passive diffusion and bind to the intracellular GRs, activating GR. Once activated, GR translocates into the nucleus and can lead to gene repression or transcriptional activation by recruiting chromatin remodeling proteins and altering epigenetic modifications, such as acetylation. Increases in acetylation are associated with transactivation, while decreased acetylation results in gene repression [6, 121, 122, 129].
Global H4-K8-Ac and H3-K9-Ac in NK cells was measured by flow cytometry. Global H4-K8-Ac in NK cells positively correlated with NKCA (p<0.05) and intracellular perforin levels (p<0.01) in NK cells (Table 8). No relationships with global H3-K9-Ac and NKCA (Pearson r =0.326, p=0.328) or intracellular perforin (Pearson r=-0.397, p=0.227) were found. These data suggest that global H4-K8-Ac alterations result in altered NK cell activity in the Cancer Pre women. To investigate if decreased global H4-K8-Ac patterns were measured in women with high perceived stress a median split was performed on the Cancer Pre group and the Control groups, creating sub groups with individuals with very high perceived stress (PSS mean=25±1.2) and very low perceived stress (PSS mean=6±0.7). Global H4-K8-Ac in NK cells was assessed by flow cytometry within these sub groups. Decreased global H4-K8-Ac in CD56+ NK cells for the Cancer Pre, high PSS group was demonstrated when compared to the Control group, low PSS (Figure 25; p<0.05). No significant difference was measured among groups for global H3-K9-Ac groups (Figure 27). CD56 staining patterns did correspond with NK cell lytic activity (CD56 dim v. perforin, Figure 24) and intracellular perforin correlated with global H4-K8-Ac (Table 8). Therefore, sub populations of H4-K8-Ac levels relative to CD56 were investigated to determine if H4-K8-Ac stained brighter in CD56 dim populations. Staining patterns between CD56 v. H4K8-Ac were investigated by flow cytometry. Representative FACS plots depicted in Figure 26 show uniform staining patterns for both Cancer Pre and Control groups with no H4-K8-Ac sub populations identified, thus H4-K8-Ac did not stain relative to CD56 staining.
To investigate if changes in global histone acetylation patterns were specific to NK cell lytic activity relationships between global histone acetylation patterns and CD8+ T lymphocytes were also investigated. CD8+ T lymphocytes have been shown to lyse target cells; by first adhering to the cell, followed by granule release of perforin and granzymes leading to apoptosis of the target cell. No relationships were measured between global H4-K8-Ac and intracellular perforin in CD8+ T lymphocytes (Pearson r=0.252, p=0.121). Relationships between H3-K9-Ac and intracellular perforin levels remain to be determined.

Chromatin Remodeling Proteins Levels of Cancer Pre, Control and Cancer Post Groups:

Activation of the HPA axis by psychosocial distress releases GCs. GCs can alter global epigenetic patterns by interacting with chromatin remodeling proteins such as HDACs. Significant inverse correlations were measured between cortisol awakening rise and NKCA suggesting that GCs were impacting NK cell lytic function (Table 7). These reductions in NKCA also significantly correlated with decreases in global H4-K8-Ac levels in NK cells of the Cancer Pre group. HDAC levels in NK cells were investigated by flow cytometry. Class I HDAC levels and cellular localization patterns were altered in *in vitro* analysis (Figure 9, 10, 12) therefore HDAC1, HDAC2 and HDAC3 were analyzed in the NK cells of the Cancer Pre and Control groups. Exploratory analysis on a subset of Cancer Pre women found increased phosphorylated HDAC2 (HDAC2-p) in the NK cells of women with increased PSS (Figure 28). Phosphorylation of HDAC2 is a
marker of nuclear localization and enzymatic activity [176]. No relationships were found with HDAC1 or HDAC3. Due to limited cell numbers, immunofluorescence microscopy was not performed.
Figure 13 Subject Enrollment Time Line.
Each arrow denotes a time period for psychological assessment, immunologic and epigenetic analysis. Salivary cortisol assessment occurred 2 days prior to blood draw (not shown).
Figure 14 Psychological Assessments of Perceived Stress.
Global appraisal of stress as measured by the perceived stress scale (PSS) at T1-T4 for Cancer and Control groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ◦ = outlier. * = p<0.05, ** = p<0.01, Cancer v. Control. Cancer n = T1=28, T2=23, T3=17, T4=12. Control n = T1=27, T2=27, T3=26, T4=18.
Figure 15 Psychological Assessments of Depressive Mood.
Depressive Mood (DM) as measured by Centers for Epidemiologic Studies-Depression (CES-D) at T1-T4 for Cancer and Control groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ○ = outlier; ⊘ = extreme outlier. *= p<0.05, Cancer v. Control. Cancer n T1=29, T2=23, T3=17, T4=12. Control n= T1=28, T2=27, T3=26, T4=18.
Figure 16 Natural Killer Cell Lytic Activity.
Natural Killer Cell Activity (NKCA), expressed as lytic units at 20%, is shown for Cancer and Control groups at T1-T4. Peripheral blood was collected and NKCA was measured using K562 tumor cells as the target. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ○ = outlier; ⊙ = extreme outlier. *= p<0.05. Cancer T1 v. Control T4. Cancer n T1= 28, T2=24, T3=16, T4=13. Control n= T1=28, T2=28, T3=26, T4=19.
Figure 17 Produced IFN gamma by PBMCs of Cancer and Control groups.
Peripheral blood mononuclear cells (PBMCs) production of IFN gamma is depicted at T1-T4 for Cancer and Control groups. PBMCs were collected and activated with PMA/PHA for 48 hrs after which supernatants were collected. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ○ = outlier. * = p<0.05, Cancer v. Control. Cancer n T1 = 25, T2 = 20, T3 = 17, T4 = 14. Control n = T1 = 26, T2 = 25, T3 = 20, T4 = 19.
Figure 18 Plasma IL-6 levels for Cancer and Control groups. Plasma IL-6 is depicted for Cancer and Control groups at T1-T4. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ∘ = outlier; ⊙ = extreme outlier. * = p<0.05, Cancer T2 v. Control T2. Cancer n T1=27, T2=22, T3=14, T4=11. Control n T1=29, T2=25, T3=19, T4=19.
Figure 19 Psychological Assessments of Perceived Stress and Depressive Mood of Selected Groups.

Perceived stress (PSS) and Depressive Mood (CES-D) for selected Cancer and Control groups. PSS (a) and CES-D (b) for Cancer Pre, Control, and Cancer Post are depicted. Cancer Pre group were women with breast cancer prior to radiation treatment with increased PSS and decreased NK cell lytic activity. Control group was age-matched women with decreased PSS and increased NK cell lytic activity. Cancer Post group were women with breast cancer following radiation treatment with decreased PSS. See Materials and Methods for full selection criteria. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. • = outlier; Θ = extreme outlier. * = p<0.05, Cancer Pre v. Cancer Post, *** = p<0.001, Cancer Pre v. Control. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 20 Natural Killer Cell Lytic Activity of Selected Groups.
Natural Killer Cell Activity (NKCA), expressed as lytic units at 20%, is shown for Cancer Pre, Control and Cancer Post groups. Peripheral blood mononuclear cells (PBMCs) were collected and NKCA was measured using K562 tumor cells as the target. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ○ = outlier. * = p<0.05, Cancer Pre v. Control, * = p<0.05, Cancer Pre v. Cancer Post. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 21 Percentage of NK cells and T helper Lymphocytes for Selected Groups. Percentage of NK cells (CD56+, CD4-, CD8-) and T helper Lymphocytes (CD4+, CD8-, CD56-) of total PBMCs is depicted. Values for Cancer Pre, Control, Cancer Post are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ✓ = outlier. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 22 Intracellular IFN gamma for NK cells for Selected Groups.

Intracellular IFN gamma for CD56+ NK cells is depicted for Cancer Pre, Control, Cancer Post groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. * = p<0.05, Cancer Pre v. Control, * = p<0.05, Cancer Pre v. Cancer Post. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 23 Intracellular IFN gamma in T helper Lymphocytes for Selected Groups. Intracellular IFN gamma in CD4+ T helper lymphocytes is depicted for Cancer Pre, Control, Cancer Post groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. *= p<0.05, Cancer Pre v. Control, *= p<0.05, Cancer Pre v. Cancer Post. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 24 Flow Cytometric plots of Intracellular Perforin in NK cells for Selected Groups. Representative flow cytometric plots for intracellular perforin levels in NK cells is depicted for Cancer Pre (a) and Control (b) women. Cells are gated on NK cells (CD56+, CD4+, CD8-) only. Plots are CD56-APC vs. Perforin-PE. In the box are the CD56-dim cells, which represents about ~90% of the NK cells.
Figure 25 Global H4-K8-Acetylation of NK cells for Selected Groups.
Analysis of posttranslational histone tail modifications: histone 4-lysine 8-Ac (H4-K8-Ac) of NK cells by flow cytometry. NK cells were identified by CD56+, CD4-, CD8- surface molecules. Global H4-K8-Ac of NK cells are standardized to H4-K8-Ac of Hick Control cells. Values for Cancer Pre, High PSS and Control. Low PSS are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. ⊘ = outlier; ⊙ = extreme outlier. *= p<0.05. Cancer Pre, High perceived stress v. Control, Low perceived stress. Cancer Pre, high PSS n= 15, Control, low PSS n=12.
Figure 26 Flow cytometric plots of Global H4-K8-Ac in NK cells for Selected Groups.
Representative flow cytometric plots for intracellular perforin levels in NK cells is depicted for Cancer Pre (a) and Control (b) women. Cells are gated on NK cells (CD56+, CD4-, CD8-) only. Plots are H4-K8-Ac-FITC vs. Perforin-PE. In the box are the CD56-bright cells.
Figure 27 Global H3-K9-Acetylation of NK cells for Selected Groups.
Analysis of posttranslational histone tail modifications: histone 3-lysine 9-Ac (H3-K9-Ac) of NK cells by flow cytometry. NK cells were identified by CD56+, CD4-, CD8- surface molecules. Global H3-K9-Ac of NK cells were standardized to H3-K9-Ac of Hick Control cells. Values for Cancer Pre and Control are presented in box plots where the black line represents the median and the tinted area box represents 50% (interquartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Cancer Pre n= 27, Control n= 20, Cancer Post n=16.
Figure 28 Exploratory Analysis of HDAC2-p in NK cells of High Stress v. Low Stress Women.

Analysis of HDAC2-p total levels in NK cells of High stress and Low stress women was determined by flow cytometry. HDAC2-p mean fluorescent intensity (MFI) is standardized to total HDAC2 MFI in CD56+ NK cells. High Stress n=3, Low Stress n=2.
Supplemental Figure 2 Representative Intracellular IFN gamma levels in NK cells Cancer Pre Group.

Intracellular IFN gamma levels were determined by flow cytometry. Representative histogram of intracellular IFN gamma levels of Cancer Pre Group. Black line represents IFN gamma levels in NK cells and light grey shaded histogram represents negative staining cells. Approximately 90% of NK cells stain positively for intracellular IFN gamma in total Cancer Pre group.
Table 6

Demographics characteristics.

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<table>
<thead>
<tr>
<th>Income&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>10,000-29,000</td>
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<td>30,000-59,000</td>
<td>7</td>
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<td>60,000 and higher</td>
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<td>Stage of Cancer&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<td>-----------------------------------</td>
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<td>Stage 0</td>
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<tr>
<td>Stage I</td>
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<td>Stage IIA</td>
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<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Surgery only</td>
<td>6</td>
</tr>
<tr>
<td>Surgery + radiation therapy</td>
<td>3</td>
</tr>
<tr>
<td>Surgery + hormonal therapy</td>
<td>2</td>
</tr>
<tr>
<td>Surgery + radiation therapy +</td>
<td>13</td>
</tr>
<tr>
<td>hormonal therapy</td>
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</tr>
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</table>

<table>
<thead>
<tr>
<th>Surgery Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td>Breast conserving</td>
<td>19</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>8</td>
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</table>

SD= standard deviation. <sup>a</sup>=missing data for some women in cohort; answers were omitted and no imputation technique was used for missing data. *p=0.05, p value for Cancer v. Control. BMI= Body Mass Index
Table 7

Correlations for Cancer Pre and Control Groups Combined (a).

<table>
<thead>
<tr>
<th></th>
<th>Salivary Cortisol</th>
<th>NKCA-CD56+ NK cells</th>
<th>Intracellular IFN gamma of CD56+ NK cells</th>
<th>Intracellular IFN gamma of CD4+ T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary Cortisol Waking Rise</td>
<td>1</td>
<td>-0.311*</td>
<td>0.426**</td>
<td>0.431**</td>
</tr>
<tr>
<td>NKCA-CD56+ NK cells</td>
<td>-0.311*</td>
<td>1</td>
<td>-0.370*</td>
<td></td>
</tr>
<tr>
<td>Intracellular IFN gamma of CD56+ NK cells</td>
<td>0.426**</td>
<td>-0.370*</td>
<td>1</td>
<td>0.849**</td>
</tr>
<tr>
<td>Intracellular IFN gamma of CD4+ T lymphocytes</td>
<td>0.431**</td>
<td>0.849**</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Values are Pearson r value, n=44 for Cancer Pre and Control groups combined. Cancer Pre (Cancer women with increased PSS and decreased NKCA prior to radiation treatment); Control (decreased PSS and increased NKCA) See Materials and Methods for full description. *p<0.05, ** p<0.01. Cancer Pre n= 24, Control n= 20.
Table 8
Correlations for Cancer Pre and Control Groups Combined (b).

<table>
<thead>
<tr>
<th>NKCA</th>
<th>Perforin MFI of NK cells</th>
<th>Global H4-K8-Ac of NK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCA</td>
<td>1</td>
<td>0.358*</td>
</tr>
<tr>
<td>Perforin MFI of NK cells</td>
<td>0.358*</td>
<td>1</td>
</tr>
<tr>
<td>Global H4-K8-Ac of NK cells</td>
<td>0.298*</td>
<td>0.441**</td>
</tr>
</tbody>
</table>

Values are Pearson r value, n=44 for Cancer Pre and Control groups combined. Cancer Pre (Cancer women with increased PSS and decreased NKCA prior to radiation treatment); Control (decreased PSS and increased NKCA) *See Materials and Methods for full description.* *p<0.05, ** p<0.01. Cancer Pre n= 24, Control n= 20.
Supplemental Table 4.
Surgery Effects on Cancer Women at T2.

<table>
<thead>
<tr>
<th></th>
<th>Breast Conserving Lumpectomy</th>
<th>Mastectomy</th>
<th>p value</th>
<th>n</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>17.3 ± 2.5</td>
<td>20.7 ± 3.5</td>
<td>0.467</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>CES-D</td>
<td>10.8 ± 3.0</td>
<td>12.5 ± 3.3</td>
<td>0.745</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>NKCA</td>
<td>87.0 ± 18.4</td>
<td>94.4 ± 37.1</td>
<td>0.841</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>IFN gamma-NK cells</td>
<td>0.31 ± 0.04</td>
<td>0.28 ± 0.06</td>
<td>0.724</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. p value for Breast Conserving lumpectomy v. Mastectomy.
**Supplemental Table 5.**
Radiation Treatment Effects on Cancer Women at T3.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Radiation Treatment</th>
<th>No Radiation Treatment</th>
<th>p value</th>
<th>n</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>15.7 ± 2.7</td>
<td>14.7 ± 3.0</td>
<td>0.788</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>CES-D</td>
<td>12.9 ± 4.8</td>
<td>9.7 ± 4.7</td>
<td>0.655</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>NKCA</td>
<td>140.7 ± 32.7</td>
<td>81.0 ± 38.3</td>
<td>0.261</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>IFN gamma-NK cells</td>
<td>0.20 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.529</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. p value for Radiation Treatment v. No Radiation Treatment.
**Supplemental Table 6.**

IL-6 levels in Cancer Pre and Control groups.

<table>
<thead>
<tr>
<th></th>
<th>Cancer Pre</th>
<th>Control</th>
<th>p value</th>
<th>n</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Produced IL-6 Fold Increase</strong></td>
<td>989.7 ± 479.2</td>
<td>172.18 ± 114.0</td>
<td>0.115</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td><strong>Percent IL-6+ of CD14+ monocytes</strong></td>
<td>11.0 ± 1.3</td>
<td>6.6 ± 1.4</td>
<td>0.039</td>
<td>17</td>
<td>16</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. p value for Cancer Pre v. Control. Cancer Pre (Cancer women with increased PSS and decreased NKCA prior to radiation treatment); Control (decreased PSS and increased NKCA) *See Materials and Methods for full description.*
### Supplemental Table 7.

Selection Criteria.

<table>
<thead>
<tr>
<th></th>
<th>PSS-T1</th>
<th>NKCA-T1</th>
<th>PSS-T2</th>
<th>NKCA-T2</th>
<th>Time point selected</th>
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<tbody>
<tr>
<td>004</td>
<td>13</td>
<td>4</td>
<td>13</td>
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<td>006</td>
<td>24</td>
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<td>1</td>
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<tr>
<td>009</td>
<td>27</td>
<td>79</td>
<td>34</td>
<td>59</td>
<td>2</td>
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<tr>
<td>10</td>
<td>19</td>
<td>146</td>
<td>17</td>
<td>192</td>
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</tr>
<tr>
<td>11</td>
<td>18</td>
<td>45</td>
<td>31</td>
<td>78</td>
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<td>13</td>
<td>103</td>
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</table>

For other data selected missing psychological or immune data determined selection.
### Supplemental Table 8.
Other Cortisol Parameters

<table>
<thead>
<tr>
<th>Time</th>
<th>Cancer Pre</th>
<th>Control</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Awakening</td>
<td>0.44 ± 0.16</td>
<td>0.42±0.11</td>
<td>0.942</td>
</tr>
<tr>
<td>~30 minutes after awakening</td>
<td>0.45±0.05</td>
<td>0.36±0.03</td>
<td>0.237</td>
</tr>
<tr>
<td>Noon</td>
<td>0.16±0.04</td>
<td>0.09±0.01</td>
<td>0.203</td>
</tr>
<tr>
<td>5 pm</td>
<td>0.10±0.03</td>
<td>0.07±0.01</td>
<td>0.447</td>
</tr>
<tr>
<td>Bedtime</td>
<td>0.09±0.05</td>
<td>0.06±0.01</td>
<td>0.622</td>
</tr>
<tr>
<td>Area Under the Curve</td>
<td>1.45±0.23</td>
<td>1.33±0.20</td>
<td>0.732</td>
</tr>
</tbody>
</table>

Data are mean values ± S.E.M. p value for Cancer Pre v. Control. Cancer Pre (Cancer women with increased PSS and decreased NKCA prior to radiation treatment); Control (decreased PSS and increased NKCA) See Materials and Methods for full description.
CHAPTER 7

DISCUSSION

The data presented herein identifies global histone 4 lysine 8 acetylation (H4-K8-Ac) as an epigenetic modification associated with NK cell lytic function. Additionally, HDAC2 was identified as a potential regulator of global H4-K8-Ac in NK cells. The discussion section is divided by treatment systems (short-term, high dose dexamethasone (Dex) treatment of NK92 cells, chronic Dex treatment of NK92 cells and ex vivo analysis of peripheral blood mononuclear cells (PBMCs) derived from women with breast cancer). In vitro Dex treatment systems results will be discussed independently, before a brief comparison of the two systems (Specific Aim 1 and Specific Aim 2). Ex vivo analysis of PBMCs cells derived from women with breast cancer focuses on psychological, immune and epigenetic analysis of Cancer Pre, Control and Cancer Post, as this selected grouping systems identifies a population of women with increased psychosocial distress (Specific Aim 3). Finally, the discussion section ends with a comparison of the in vitro and ex vivo systems.

Short-Term, High Dose, Dex Treatment of NK92 Cells:

Therapeutic doses of glucocorticoids (GCs) depress the level of NK cell activity (NKCA) in the peripheral blood of treated individuals [178] and physiological concentrations of GCs suppress NKCA of PBMCs [4-6]. This inhibition of NKCA is time
and dose dependent [4]. In the short-term, high dose, Dex treatment system similar concentrations and length of exposure of Dex were used as was assessed in Holbrook *et al.* It is well-established that acute stress is associated with a reduction in NK cell functional activity [64, 179, 180], which may be mediated by increased adrenergic activity [181], as well as by the GC hormone, cortisol [182]. This investigation focused on the effects of short-term, high dose treatments with the synthetic GC, Dex, on the cellular function of the human NK cell line, NK92. The findings demonstrate reductions in; NKCA, granule constituents, adhesion to tumor targets and interferon gamma (IFN gamma) production. Each of these reductions was associated with reduced global acetylation of histone 4 lysine 8 (H4-K8-Ac). Global reductions in H4-K8-Ac associated with Dex treatment suggests that global acetylation patterns may reflects H4-K8-Ac patterns at promoter regions of NK cell effector genes (i.e. perforin and granzyme B). This was shown to be the case as reductions in global H4-K8-Ac were associated with decreased H4-K8-Ac at the proximal promoter regions of perforin and granzyme B genes, with concomitant reduction in gene transcription [6].

It is well known that GCs can induce gene transcription, yet their major effect on the immune system is to suppress transcription. The number of immune response genes regulated by GCs is conservatively estimated at more than 1000 [45, 183-185]. GCs can decrease cytokine, chemokine, and receptor expression by PBMCs [186]. These effects are primarily due to the GC action at the transcriptional level. Although activated
glucocorticoid receptors (GR) can directly interact with pro-inflammatory transcription factors like AP-1 (Fos-Jun heterodimers) and NFκB (p65-p50 heterodimers) [187-189], the primary effect of GCs are downstream of transcription factor binding to DNA with gene repression a consequence of the reversal of histone acetylation [48, 190]. Activated GR repress gene expression by direct inhibition of histone acetyl transferase (HAT) activity and by active recruitment of histone deacetylase (HDAC) complexes [48]. Most importantly, physiologically elevated GC levels induce HDAC expression in a time and dose dependent manner [48] resulting in deacetylation of histones, increased tightening of DNA, and reduced expression of immune response genes [191]. Moreover, the effect of GCs on chromatin structure and on immune response genes is reversible with HDAC inhibitors (HDACi) [131-133]. By repressing a downstream target of transcription factor activation (chromatin accessibility), GCs reduce gene expression irrespective of the exact transcription factor requirement for an individual gene. Hence, the data presented herein suggest that one likely explanation for the GC induced reduction in NKCA and in IFN gamma production is decreased transcription of immune effector genes due to reduced proximal promoter accessibility.

The results presented herein demonstrate the effect of short-term, high dose Dex in this human NK cell line. Further, Dex at the examined concentration and period of treatment profoundly reduced the degree of H4-K8 acetylation as judged by western blot analysis. Such a global effect of this GC suggests a profound reduction in overall
functional NK cell activity. In actuality, reductions in functional activity, although significant, approximated 50%. This was true for NKCA, for cytokine production and for the accessibility of the promoters IFN gamma, perforin and granzyme B [6]. This observation suggests that other effects in addition to transrepression may be ongoing as well and may influence the outcome of short-term, high dose Dex treatment and its impact on immune function. Additionally, treatment of NK92 cells with an HDACi (TSA) increased both global H4-K8-Ac, NKCA and IFN gamma levels in the presence of short-term, high dose Dex concentrations. These data suggest that global acetylation changes mediated by alterations in HDACs are implicated in altered NK cell lytic function. No differences in global H4-K8-Ac MFI were measured when NK92 cells were treated with TSA alone when compared to untreated cells (Table 3). TSA alone did significantly increase NKCA and IFN gamma levels when compared to untreated cells (Table 4 and 5). Since TSA inhibits all classes of HDACs it’s possible that treatment of NK92 cells with TSA alone causes increases in acetylation of other histone residues resulting in increased NKCA and IFN gamma production. Investigation into other histone residue acetylation status is assessed below in ex vivo analysis of NK cells derived from women with breast cancer. TSA inhibits all classes of HDACs, therefore although treatment with Dex and TSA suggests that global H4-K8-Ac and NK cell functional changes are a result of HDACs it did not answer the question as to which HDAC is inhibited. Currently, there are not any HDAC specific inhibitors available, thus no further HDACis were investigated.
In contrast to the results described herein, previous work has shown that HDACi (valproic acid and suberoylanilide hydroxamic acid) reduce the cytotoxic activity of NK cells [192]. NK cell inhibitory effects of HDACi were associated with reduced surface expression and function of specific activating receptors (NKp46 and NKp30) responsible for the induction of NK cell-mediated cytotoxicity. This inhibitory effect was at the transcriptional level as judged by real time PCR. These effects were only seen with IL-2 activated NK cells, with no effect on fresh NK. No effect of these HDACi was observed for LFA-1 or for IL-2 receptors. Surface expression levels of inhibitory receptors including KIR and NKG2A were not influenced by HDACi treatment, indicating a specific effect of HDACi on NK cell activating receptors. These differences, with regard to the results of this study, may be explained in multiple ways. The likely difference is that TSA reversed the effect of dexamethasone within a one to six hour period, while the effects of valproic acid and suberoylanilide hydroxamic acid were observed in NK cells cultured for 4 days with the HDACi.

The effect of short-term, high dose Dex treatment on NK92 cells was not limited to NKCA and IFN gamma. Quantitative reductions were also demonstrated for the lymphocyte adhesion molecule, LFA-1, which is known to mediate binding of NK92 cells to K562 [174], as well as the essential granule constituents, perforin and granzyme B [175]. These quantitative reductions were not universal in that the adhesion molecule, CD2 (also know to mediate binding of NK92 cells to K562) [174, 175] was not affected.
In sum, these results associate short-term, high dose Dex treatment induced epigenetic histone modification with NK cell function. The acetylation status of H4-K8 was found to correspond with NKCA, an adhesion molecule known to mediate the interaction of NK with tumor cells and granule constituents. The function of NK cells against tumor cells is carried out first by engagement of the tumor target, followed by exocytosis of NK cytolytic granule content and/or production of immune effector cytokines. Short-term, high dose Dex treatment of NK92 cells also resulted in decreased pro-inflammatory cytokine intracellular and produced levels (IFN gamma). The observation reported herein, demonstrate the effect of short-term, high dose Dex treatment to be at the epigenetic level with reduced transcription of immune effector gene products required for optimal functional activity of NK cells, leading to a reduction in NK cell functional activity. Further it may be that dose and time of treatment as described herein approximate the effect of short term or acute physiological effects of this GC and that greater dose and/or more prolonged treatment may reveal a more profound outcome and was explored next.

**Chronic Dex Treatment of NK92 Cells:**

This investigation focused on the effects of increased time, but decreased concentrations of Dex on functional activity of NK92 cells. Treatment of NK92 cells
with high dose Dex ($10^{-7}$) for longer than 24 hours resulted in reduced viability. Thus, decreased concentrations of Dex ($10^{-10}$) were used to assess increased exposure of NK92 cells to Dex treatment. The findings demonstrate reductions in; NKCA and granule constituents (perforin and granzyme B). Interestingly, these decreases in NK cell lytic activity were associated with increased IFN gamma production, resulting in a dichotomous NK cell phenotype. Reductions in NKCA, perforin and granzyme B were associated with reduced global acetylation of H4-K8-Ac.

GCs impact global acetylation patterns through recruitment of chromatin remodeling proteins. GCs enter cells by passive diffusion and bind to the ubiquitously expressed intracellular GRs. After a GC binds to GR, ligand activated GR is translocated to the nucleus. In the nucleus activated GR regulates the transactivation or transrepression of GC-responsive genes [193]. Ligand activated GR has also been shown to impact gene expression through interaction with other chromatin remodeling complexes such as HATs and HDACs. In the data presented herein GR induced alterations of class I HDACs (HDAC1, HDAC2, HDAC3) were investigated. Class I HDACs were chosen because previous work has found class I HDACs can interact with GR [49-51] and also alterations in class I HDAC function can impact NK cell lytic activity [141]. In the chronic Dex treatment of NK92 cells, increased nuclear localization of HDAC2 corresponded with decreased global H4-K8-Ac and decreased NKCA (Figures 7, 9, 10 and 12). Increased HDAC2 nuclear localization was measured by
immunofluorescence and flow cytometric analysis of increased phosphorylation of HDAC2 (HDAC2-p), which serves as a nuclear localization signal [176]. Increased HDAC2-p was measured at Day 2 (Figure 12), with nuclear localization of HDAC2 persistent at Day 5 (Figure 10). These data suggest that HDAC2 nuclear localization associated with decreased global acetylation of H4-K8. Additionally, time course analysis suggests that this occurs rapidly, beginning as early as Day 2 of the treatment. Analysis of HDAC1 measured no changes in total protein levels, whereas measurement of HDAC3 revealed significant decreases to total protein level, suggesting that it is HDAC2 responsible for these global decreases in H4-K8-Ac. Analysis of other classes of HDACs will need to be done to confirm that HDAC2 alone is responsible for global changes in H4-K8-Ac.

Cellular localization patterns of GR were investigated in chronic Dex treated NK92 cells. Analysis of GR revealed no differences in total protein or cellular localization of GR when comparing chronic Dex treated NK92 cells and untreated NK92 cells [194]. GR cellular localization patterns may not have been observed due to the natural cyclic activity of GR, continuous cycling in and out of the nucleus [195-197]. Studies have shown that GR rapidly translocates in and out of the nucleus during normal cellular processes, making measurement of GR co-localization and nuclear localization difficult.
Alterations in global histone acetylation patterns can be mediated by GCs interaction with HATs [136, 137]. As a result cellular levels of p300, a HAT known to interact with GR, were investigated in chronic Dex treatment of NK92 cells. No changes in total p300 levels were measured throughout the chronic Dex treatment (Figures 8 and 11). Additionally, p300 localized primarily to the nucleus in chronic Dex treatments. No relationships between p300 total levels or cellular localization and global H4-K8-Ac were identified. Analysis of other HATs remains to be determined.

In sum, these results presented herein found chronic Dex treatment caused a dichotomous NK cell phenotype, characterized by decreased NKCA and increased intracellular IFN gamma. Additionally the global acetylation status of H4-K8 was found to correspond with decreased NKCA. The observation reported herein, demonstrate the effect of chronic Dex treatment to be at the epigenetic level with reduced transcription of NK cell lytic gene products that are required for optimal NCKA.

Comparison of Short-Term, High Dose and Chronic Dex Treatment Systems:

The effect of psychosocial distress on NK cell function has been, in large part, attributed to elevations in GCs produced by stress-induced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis [1-3]. GCs have been shown to impact gene expression through interactions with chromatin remodeling proteins, such as
HDACs and HATs. Due to limited human sample numbers, *in vitro* systems to study the effects of GCs on NK cell function were established. The effects Dex on the cellular and molecular function of the human NK cell line, NK92 were used. Two *in vitro* systems were developed; a short term, high dose Dex treatment system and a chronic Dex treatment system. Both systems measured dramatic NK cell functional changes following Dex treatment. Additionally, both short-term, high dose Dex treatment and chronic Dex treatment of NK92 cells revealed the novel contribution that GCs can alter the epigenome which mediate alterations in NK cell function.

In both the short term, high dose Dex treatment system and the chronic Dex treatment systems significant decreases in NK cell lytic activity as determined by NKCA and intracellular levels of granule constituents (perforin and granzyme B) were found. This is consistent with previously published results that NKCA is especially responsive to alterations in GCs [15, 63, 105]. Additionally, global decreases in H4-K8-Ac were associated with decreased NKCA for both treatment systems suggesting that GCs alter NKCA through alterations in this epigenetic modification. Interestingly, with short-term, high dose Dex treatment, IFN gamma levels were decreased; whereas with chronic Dex treatment, IFN gamma levels were increased. These opposite results suggest that pro-inflammatory cytokine regulation by Dex is highly dependent on dose and duration of GC exposure. Interestingly, these varied results could explain discrepancies in the literature in regard to psychosocial distress and pro-inflammatory cytokines, where some
studies have measured increased pro-inflammatory cytokine levels and others measured decreased pro-inflammatory cytokine levels [5, 7-9, 74, 75]. In those studies where decreased pro-inflammatory cytokines were found, extreme increases in GCs may have resulted in decreased pro-inflammatory phenotype. In studies where increased pro-inflammatory cytokines were found (as is in Specific Aim 3), moderate levels of GC levels may be the cause. This theory is further supported by the fact that GCs have been shown to be both immunosuppressive and immune-enhancing [9]. Development of these two in vitro systems allowed for analysis of global epigenetic modifications associated with altered NK cell function. Additionally, comparison of the two systems allowed for a comparison of how dose and duration of GCs can impact NK cells.

**Ex Vivo Analysis of Peripheral Blood Mononuclear Cells (PBMCs) Derived from Women with Breast Cancer:**

It is well-established that the diagnosis of breast cancer has emotional consequences characterized by increased perceived stress, increased depressive mood and increased anxiety. Several studies have shown that these emotional consequences [22], termed in this dissertation as psychosocial distress, can lead to alterations in immune cell function [5, 13-15]. NK cells are highly sensitive to increases in psychosocial distress [15, 63, 105], with NKCA inversely correlating with perceived stress [107]. NK cell
dysregulation is significant in that NK cells contribute to host protection from cancer [102, 103, 198, 199] and NK cell activity has been correlated to increased survival in women with metastatic breast cancer [109-114]. Hence, the psychological response to a diagnosis of breast cancer diagnosis has clear biological consequence for women with breast cancer, as it may negatively impact their prognosis.

This investigation focused on the effects psychosocial distress on functional activity of NK cells with the hypothesis that global epigenetic patterns reflect a molecular epigenetic mechanism by which immune function was altered. This study identified a dichotomous phenotype in the NK cells of women with breast cancer, demonstrating decreased lytic activity and increased intracellular IFN gamma. Additionally, global acetylation of H4-K8-Ac was identified as an epigenetic modification which correlated with NK cell lytic activity and intracellular perforin. Exploratory analysis identified HDAC2 as a potential factor contributing to decreased global H4-K8-Ac and NKCA in women experiencing psychosocial distress. Women with breast cancer (Cancer Pre=prior to radiation treatment, See Materials and Methods) had increased PSS and decreased NKCA. These decreases in NKCA positively correlated with both reduced cytoplasmic perforin and global nuclear H4-K8-Ac. Additionally, in women with breast cancer who reported extremely high PSS, there was a significant reduction in global H4-K8-Ac when compared to Control women who reported low PSS (Figure 25). It is interesting to note that although H4-K8-Ac changes were observed globally, acetylation changes of other
histone residues were not observed, specifically no changes were observed at histone 3 lysine 9 (H3-K9) acetylation (Figure 27). This is of note as other studies have found relationships between H3-K9-Ac and granule components [126]. However in these studies, H3-K9-Ac was investigated in promoter regions of granule component genes (e.g. perforin and granzyme B), therefore H4-K8-Ac may serve as a better global marker of altered NK cell function. Investigation into H3-K9-Ac in the promoter regions of granule component genes has not yet been determined. These findings suggest that global acetylation alterations at H4-K8 were residue specific, suggesting that H4-K8-Ac serves as an epigenetic marker for NK cell lytic function. Analysis of global H4-K8-Ac in other immune cell populations did not measure relationships between acetylation status and function. No relationships between global H4-K8-Ac and intracellular perforin and granzyme B in CD8+ T lymphocytes were measured. These data suggest that global H4-K8-Ac patterns relate to alterations in NK lytic function, but not lytic function of CD8+ T lymphocytes.

The results presented herein demonstrate that the women with breast cancer prior to radiation treatment (Cancer Pre) have increased perceived stress as measured by a self-report questionnaire (perceived stress scale, PSS). During periods of increased perceived stress, activation of the HPA results in elevated circulating adrenal derived GCs, primarily cortisol. Salivary cortisol levels, which correlates with plasma cortisol levels [200, 201], was collected to determine diurnal cortisol rhythms in the Cancer Pre, Control
Cortisol rhythms are characterized by an initial spike approximately 30 minutes after waking, this initial spike is termed the cortisol awakening rise (CAR). GC levels then gradually subside throughout the course of the day, reaching a minimal level in the evening, just prior to bedtime. Increases in PSS in the Cancer Pre group corresponded with decreased NK cell lytic function as measured by NKCA when compared with both the Control and Cancer Post groups. These decreases in NKCA negatively correlate with salivary CAR, thus women with lower NK cell lytic activity have higher salivary CAR. This increase in CAR, may indicate that immune cells were exposed to higher levels of cortisol for prolonged periods during the day. Repeated exposure of NK cells to increased CAR may have allowed immune cells to be epigenetically primed for immune dysregulation. Other cortisol parameters still need to be assessed. However, CAR has been shown to be related to increased HPA activation [55, 56]. Additionally, HPA activation has been shown to be associated with increased psychosocial distress [57], suggesting that CAR was the appropriate cortisol parameter to assess. These correlations between NKCA and CAR suggest that women with increased PSS (Cancer Pre) have decreased NKCA as a result of increased NK cell exposure to cortisol. In contrast, NK cells of women with decreased cortisol waking rise (Control) may have been exposed to lower cortisol levels resulting in increased NKCA. Further, since NKCA positively correlates with global H4-K8-Ac, cortisol may impact gene transcription through recruitment of HDACs, leading to decreased histone acetylation.

Exploratory analysis of NK cells derived from women with breast cancer investigated
relationships between HDAC2-p and global H4-K8-AC. HDAC2-p is a marker of both nuclear localization and enzymatic activity [176]. Breast cancer women with increased PSS and decreased global H4-K8-Ac also had increased nuclear HDAC2-p. These finding are the first to identify HDAC2 as a potential mediator of decreased global H4-K8-Ac and decreased NK cell lytic activity measured during periods of psychosocial distress. Additional analysis to confirm these results is necessary.

Salivary cortisol levels, however, were not related to PSS scores and there are several possible reasons for this. As enrollment numbers were low, the study at this time may be under powered to measure these relationships. Additionally, in the majority of the women, a positive waking rise was measured, however, a small percentage of women had a negative waking rise. This could be due to improper salivary collection or could be an individual specific phenotype characterized by a negative waking rise or a blunted cortisol response which has been reported previously in women with breast cancer [59, 60]. At this time not enough women with negative waking rises to evaluate this group independently, but would be interesting to investigate this as total sample size increases. Finally, several different polymorphisms have been identified in the human GR gene, the cognate receptor for GCs. GR is comprised of three domains; the amino-terminal transactivating domain, the DNA binding domain and the carboxy-terminal ligand binding domain [202]. The three most characterized polymorphisms are ER22/23K, N363S and BcII. ER22/23K is an amino acid alteration from an arginine to a lysine and
N363S is an amino acid switch from an asparagine to a serine, both of these polymorphisms are located in the amino-terminal transactivating domain [203, 204].

BeII identifies a DNA switch from a cytosine to a guanine located in the linker region between the transactivating domain and the DNA binding domain. All three of these GR polymorphisms are associated with altered GC sensitivity. Characterization the GR sequence and the number(s) of polymorphisms observed in the women with breast cancer may identify a population of women more or less susceptible to fluctuates in cortisol. This would allow for further investigations into relationships between PSS and salivary cortisol within the population of breast cancer women most susceptible to alterations in cortisol rhythm.

The effect of surgery or radiation treatment on psychological analysis and immune function measured herein was investigated. Studies have shown that an interval of 10-12 days after surgery provided ample time for surgical or anesthesia-induced effects to dissipate [110, 153-162]. Nevertheless, type of surgery was investigated for impact on both psychological states and immune function in the total Cancer group. The majority of the women enrolled in the study were treated with breast conserving lumpectomy with a small number of women treated with mastectomy (Table 6 and Supplemental Table 4). No differences in perceived stress and depressive mood were measured when comparing the women that underwent breast conserving lumpectomy versus women that underwent mastectomy at T2 (Supplemental Table 4). Differences
were investigated at T2 as this was the time point immediately following surgery (Figure 13). Additionally, when immune functions were analyzed no differences were measured in NKCA and intracellular IFN gamma levels in the NK cells when comparing the women that underwent breast conserving lumpectomy versus women that underwent mastectomy (Supplemental Table 4). Radiation treatment effects were also investigated in the Cancer group at T3 (Supplemental Table 5). Psychological and immunological analysis did not occur during radiation treatment, however the closest time point, T3, occurred 6-8 weeks following the completion of radiation treatment. The majority of the women enrolled in the study were treated with radiation (Table 6), with a small percentage undergoing surgery alone or surgery plus hormonal treatment. No differences in perceived stress, depressive mood, NKCA or intracellular IFN gamma levels of the NK cells were measured when comparing women that received radiation treatment and those that did not (Supplemental Table 5). These data suggest that neither surgery nor radiation treatment impacted psychosocial distress and immune dysregulation.

Perceived stress was not the only determinant of psychosocial distress associated with the diagnosis of breast cancer; increases in depressive mood (CES-D) were also measured. However, analysis of relationships between depressive mood, immune function and epigenetic modifications revealed no relationships among these parameters. PSS is a measure of global stress appraisal over the previous month, where as CES-D assesses depressive mood for the previous week. Additionally, when analyzing PSS and
CES-D within all the total groups, PSS was significantly elevated in the Cancer group at T1 and T2 (Figure 14), however CES-D was only significantly elevated at T1 (Figure 15). Therefore PSS may better capture alterations in psychosocial distress experienced by the women with breast cancer over a period of a month. Additionally, these brief alterations in depressive mood may more accurately depict a temporary change in mood state not indicative of chronic depression. Interestingly, previous studies have reported dichotomous immune phenotypes in individuals with chronic depression [205]. This reported immune phenotype was characterized by decreased NKCA and increased pro-inflammatory cytokines, specifically IL-6, IL-1 and TNF alpha. Unlike data presented herein, this phenotype in chronic depression was not measured within a single immune cell population (e.g. NK cells). Additionally, in chronic depression this dichotomous phenotype was not predicted to be controlled by a single biological hormone (e.g. cortisol). Thus, unlike previous reports the data presented herein suggest the novel contribution that cortisol awakening response is associated with a dichotomous phenotype in NK cells in women with breast cancer. Analysis of immune cell function and epigenetic modifications within the total Cancer and total Control groups found no relationships. This is likely due to the varied perceived stress trajectories of the Cancer group. Additionally, several of the Control women recruited reported high perceived stress making differences between global H4-K8-Ac in the total Cancer versus Control groups difficult to discern. Due to these reasons, immune, cortisol, epigenetic and chromatin remodeling protein analysis was performed on the selected groups (described
in *Materials and Methods* section). Final conclusions await completion of the full study as these resulted are highly limited due to inadequate power.

NK cells derived from women with breast cancer (Cancer Pre group) had demonstrably increased intracellular IFN gamma levels when compared to IFN gamma levels in NK cells of the Control group (*Figure 22*). In women with breast cancer IFN gamma levels negatively correlated with NKCA, which strongly implies a dichotomous phenotype in the NK cells of women experiencing psychosocial distress. Additionally, ~90% of the CD56+ cells exhibited both intracellular IFN gamma (*Supplemental Figure 2*) and intracellular perforin (*Figure 24*), further implying that the same population of NK cells exhibited reduced tumor cell lysis and increased production of IFN gamma.

Investigation into relationships between cortisol awakening rise (CAR) and intracellular IFN gamma in NK cells did measure significant positive correlations (*Table 7*). Additionally, CAR inversely correlated with NKCA (*Table 7*). These data suggest that cortisol induced the increases in intracellular IFN gamma and decreases in lytic activity in NK cells. Increases in intracellular IFN gamma levels were not limited to NK cells, these increases were also measured in CD4+ T lymphocytes (T helper lymphocytes). Additionally, significant positive correlations were measured between CAR and intracellular IFN gamma in T helper lymphocytes (*Table 7*).

Relationships between intracellular IFN gamma levels and epigenetic modifications were investigated; however significant correlations were not measured. No
relationships were measured between global H4-K8-Ac and IFN gamma, further suggesting that acetylation at this histone residue is responsible for altered NK cell lytic activity. In NK cells, intracellular IFN gamma levels related to histone 3 serine 10 phosphorylation (H3-S10-P) (Pearson r =0.248, p=0.15, df=35), whereas in T helper lymphocytes IFN gamma levels related to H3-K9-Ac (Pearson r=0.33, p=0.125, df=23). These data suggest that cortisol may produce these effects on IFN gamma through alteration in modifications of the tail of H3. This preliminary analysis corresponds with previous work in the field. Other studies have shown relationships between acetylation of H3 relating to IFN gamma in CD4+ T lymphocytes [125]. Those studies measured acetylation of H3 at the IFN gamma promoter regions. Therefore continued analysis of IFN gamma in CD4+ T lymphocytes and global H3-K9-Ac could find the novel contribution of relating a global modification to pro-inflammatory cytokine production. Further analysis to confirm these results is necessary.

IFN gamma was not the only pro-inflammatory cytokine measured in the ex vivo analysis of NK cells derived from women with breast cancer. Tumor necrosis factor alpha (TNF alpha) and interleukin-6 (IL-6) were also measured. No differences were measured in NK intracellular or produced TNF alpha levels. Additionally, TNF alpha levels were also measured in the plasma and other immune cell populations (CD4+ T helper lymphocytes and CD8+ cytotoxic T lymphocytes). In all cases no differences in TNF alpha levels were measured when comparing Cancer Pre, Control and Cancer Post
groups. These data suggest that pro-inflammatory cytokine, TNF alpha is not altered during periods of psychosocial distress.

IL-6 levels were also measured in *ex vivo* analysis of women with breast cancer. Produced IL-6 was measured in both in the plasma and also by *ex vivo* stimulation of peripheral PBMCs in the Cancer Pre and Control groups. No differences were measured in the IL-6 levels in the plasma when comparing the Cancer Pre and Control groups. A trend approaching significance of increased produced IL-6 levels was measured in the Cancer Pre group when compared with the Control group (*Supplemental Table 6*). For this assays total PBMCs were stimulated for 48 hours before supernatants were collected and ELISA analysis determined produced IL-6 levels. Once trends in produced IL-6 were measured, intracellular flow cytometry was then used to determine which PBMC sub population(s) was producing IL-6. Initial PBMC sub populations investigated were NK cells, CD4+ T helper lymphocytes and CD8+ cytotoxic T lymphocytes, however minimal intracellular IL-6 levels were detected in these PBMC sub populations. As a result, intracellular IL-6 levels in CD14+ monocytes were also investigated. A significant increase in the percentage of IL-6 producing CD14+ monocytes was measured in the Cancer Pre group when compared to the Control group (*Supplemental Table 6*). As intracellular IL-6 staining was not started until differences in produced levels were observed, sample size limitations prevent comparison analysis with the Cancer Post group. Analysis of intracellular IL-6 levels remains to be determined. It is also worth
noting that although CD14+ monocyte cells produce IL-6, intracellular flow cytometric analysis identified another population of PBMCs which also stained positively for intracellular IL-6. This population has not been identified and remains a future direction; however, NK cells, CD4+ T helper lymphocytes and CD8+ cytotoxic T lymphocytes have been eliminated as possibilities. When the CD14+ monocytes were identified as an IL-6 producing population of PBMCs, epigenetic analysis on this population also began. Global H3-K9-Ac of CD14+ monocytes were measured by intracellular flow cytometry. Global H3-K9-Ac patterns were investigated as relationships between H3 modifications and IFN gamma were observed. No differences in global H3-K9-Ac levels in CD14+ monocytes were measured when comparing the Cancer Pre with the Control group. Relationships were also investigated between percentage of IL-6 producing CD14+ monocytes and global H3-K9-Ac of CD14+ monocytes. Body mass index (BMI) independently relates to IL-6 levels [206], therefore BMI was controlled for in correlation analysis. Exploratory analysis between percentage of IL-6 producing CD14+ monocytes and global H3-K9-Ac of CD14+ monocytes measured a positive relationship approaching significance (Pearson r= 0.789, p=0.062, df=4) between these two variables. These data suggests that global H3-K9-Ac patterns may relate to intracellular IL-6 levels in CD14+ monocytes. Additional analysis on CD14+ monocyte cells will confirm this result. The data presented herein suggests that while H4-K8-Ac is associated with lytic activity, modifications of H3 (H3-S10-p and H3-K9-Ac) may be associated with pro-inflammatory cytokines (IFN gamma and IL-6).
Comparison of In Vitro and Ex Vivo Analysis:

_In vitro_ analysis provided a screening tool for potential epigenetic modification and chromatin remodeling proteins associated with altered GCs. As human samples were limited, this served as a critical tool for determining which modifications and enzymes to investigate as well as allowed for assay development. The chronic Dex treatment system best reflected NK cell phenotypes observed in the Cancer Pre group and thus was most utilized. Although the chronic Dex treatment system best represented the human cohorts, it was not identical. For example, chronic Dex treatment reduced granzyme B levels in NK92 cells, however no differences were measured in granzyme B levels in the NK cells derived from the Cancer Pre group compared to the Control group. Additionally, NK92 cells produced IL-6, while NK cell derived from women with breast cancer produced minimal IL-6.

The two _in vitro_ systems allowed for the dose and time of GC exposure to NK cells to be assessed, suggesting the NK cell lytic activity was impacted early in treatments and is less dependent on GC levels. Both short-term, high dose and chronic Dex treatment decreased NKCA of NK92 cells. Interestingly, the impact of GCs on pro-inflammatory cytokines was dose and time dependent, with short-term, high dose Dex treatment decreasing IFN gamma levels and chronic Dex treatment increasing IFN gamma levels. This differential effect would be more difficult to identify in the salivary cortisol collections from the selected groups. Further analysis of the PBMCs derived
from women with breast cancer did allow for assessment of multiple immune cell populations, which was done \textit{in vitro}. Finally, comparison of the \textit{in vitro} and \textit{ex vivo} systems highlights the importance of multiple systems to represent human data, but also suggests that while model systems provide an important research tool they cannot completely mimic systems in the human body.
CHAPTER 8

FUTURE DIRECTIONS

Identification of HDAC2 as a mediator of decreased global H4-K8-Ac and decreased lytic activity in NK cells offers numerous therapeutic possibilities. Inhibition of HDAC2 could be used in treatment of individual experiencing psychosocial distress, exhibiting decreased NK cell function and/or women with breast cancer. The breast cancer women enrolled in this study have early stage breast cancer, resulting in minimal problems with tumor growth and progression. Therefore, a therapeutic inhibitor of HDAC2 would most likely not be recommended for this population of women. However, psychosocial distress and immune dysregulation measured in these women are also measured in women with advanced metastatic breast cancer. It would be interesting to investigate HDAC2 inhibitors as a means of increasing NK cell lytic activity to prevent tumor cell growth and metastasis in women with advanced breast cancer. Interestingly, a class of drugs termed HDAC inhibitors (HDACi) are already being investigated in a number of different disease states, one of which is breast cancer [207, 208]. Nearly all HDACis in clinical trials at this time interfere with activity of class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8). Inhibition of HDACs in tumor cells has been associated with decreased tumor cell proliferation and survival. Therefore, these HDACis offer
therapeutic potential for women with metastatic breast cancer to decrease tumor cell
growth while simultaneously enhancing NK cell lytic activity.

Investigation into the upstream protein kinase(s) responsible for phosphorylation of HDAC2 could also offer potential therapeutic opportunities. HDAC2 can be phosphorylated at four different serine residues; 394, 411, 422 and 424 [209]. Phosphorylation of serines 394, 422 and 424 is associated with enzymatic activity, with the link between phosphorylation of serine 394 (S394) being the strongest [176]. Casein kinase 2 alpha 1 (CK2alpha1) has been identified as a protein kinase responsible for phosphorylation of HDAC2 at S394 [176, 210]. Unlike other HDACs which can be phosphorylated by a number of different kinases, CK2 is unique in its ability to phosphorylate HDAC2. Investigation into CK2 activity could provide a novel pathway to directly inhibit only HDAC2. The data present herein suggests that inhibiting CK2 and in turn HDAC2-p could also lead to increased NK cell lytic activity. Links between NK cell and CK2 inhibitors are limited with one study measuring increased NK cell lytic activity when target tumor cells were pre-treated with CK2 inhibitors [211]. Interestingly, this study measured increases in NK cell lytic activity to be associated with death mediated ligands and not granule components. One potential future direction could be to investigate if inhibition of CK2 impacts NK cell lytic activity, intracellular perforin and global acetylation of H4-K8 in the presence of Dex. Additionally, CK2 activity has also been found to be elevated in cancer tissues [212]. Inhibition of CK2
activity is associated with decreased tumor proliferation and envasion in both esophageal and prostate cancer [213, 214]. CK2 promoted tumor cell growth by phosphorylation of cellular proteins other than HDAC2. Relationships between CK2 and HDAC2-p in tumor cell progression have not yet been identified. Although inhibition of CK2s in breast cancer has not been investigated these results suggest that interfering with CK2 could also lead to decreased breast cancer cell growth. Together these data suggest that CK2 inhibitors could also provide a novel cancer therapeutic by decreasing tumor cell growth and increasing NK cell activity to individuals with cancer.

Relationships between PSS and GCs may not have been measured because GCs may not have mediated the immunologic effects. Psychosocial distress upregulates other neurotransmitters other than cortisol such as catecholamines. Stimulation of the sympathetic nervous system results in the production of catecholamines such as epinephrine and norepinepherine. Epinephrine and norepinepherine can impact immune cells either through direct innervation of lymphoid organs or by secretion into the blood stream [215]. Epinephrine and norepinepherine exert their effects through engagement with beta-receptors [216]. There are five different beta-receptors that have been identified; beta 1, beta 2, beta 3, alpha 1 and alpha 2. These beta-receptors are G coupled protein receptors that upon activation induce a signaling cascade leading to increases in cAMP accumulation, activating protein kinase A within immune cells [217]. T lymphocytes, NK cells and B lymphocytes have all been shown to express beta 2
adrenergic receptor [218, 219]. The impact of these catecholamines on NK cells has not been well investigated and results are varied. Some studies found catecholamines decreased NK cell lytic function as measured by NKCA and mRNA levels of granzyme B and perforin [217, 220]. Other studies found catecholamines increased NK cell activity [221]. Analysis of epinephrine and norepinepherine on NK cell epigenetic modifications remains to be investigated. As neurotransmitters, such as epinephrine and norepinepherine, are upregulated during psychosocial distress understanding how neurotransmitters impact NK cell function and epigenetic modifications, alone or in combination with cortisol is a future direction for this laboratory.
CHAPTER 9
CONCLUSIONS

In sum, these results associate GC induced epigenetic histone modifications with NK cell function. The acetylation status of H4-K8 was found to correlate with not only NKCA but intracellular perforin levels in both ex vivo analysis of NK cells from women with breast cancer and Dex treatment of NK92 cells. Increased nuclear HDAC2 was associated with decreased global H4-K8-Ac in NK cells, identifying HDAC2 as a potential mediator of decreased global H4-K8-Ac. Additionally, a dichotomous phenotype of decreased lytic activity and increased IFN gamma was also identified in NK cells from women with breast cancer. This dichotomous phenotype correlated with cortisol awaking rise, suggesting that activation of the HPA and cortisol production mediates this effect. Exploratory analysis found that epigenetic modifications on H3 may relate to increased IFN gamma. Identification of epigenetic modifications associated with altered NK cell function highlights the importance of investigation into relationships between the epigenome and psychosocial distress. Such analysis has not been previously accomplished and this mechanistic insight will advance the science of psychoneuroimmunology. In addition, the findings and technology generated from this project can spur future translational studies that can develop epigenetic-based approaches as potential indicators of immune cell function.
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VITA

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