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Characterization of Chronic Glucocorticoid Exposure on Natural Killer Cell Function: A Model for Stress Induced Alterations on the Epigenetic Landscape

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CHARACTERIZATION OF CHRONIC GLUCOCORTICOID EXPOSURE ON NATURAL KILLER CELL FUNCTION: A MODEL FOR STRESS INDUCED ALTERATIONS ON THE EPIGENETIC LANDSCAPE

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LIST OF ABBREVIATIONS

GCs	glucocorticoids
HPA	hypothalamic-pituitary-adrenocortical
NK	Natural Killer
GR	glucocorticoid receptor
IFN-g	interferon gamma
TNF-alpha	tumor necrosis factor alpha
IL	interleukin
NKCA	Natural Killer cell activity
H4K8Ac	histone 4 lysine position 8 acetylation
HDAC	histone deacetylase
TSA	Trichostatin A
HAT	histone acetylases
NcoR	nuclear receptor co-repressor
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
PBMC	peripheral blood mononuclear cells
LU	lytic units
PMA	phorbol 12-myristate 13-acetate
PHA	phytoheamagglutin
BSA	bovine serum albumin
PBS	phosphate buffer solution

H3K9Ac	histone 3 lysine 9 acetylation
H3K4me3	histone 3 lysine 3 tri-methylation
H3S10P	histone 3 serine 10 phosphorylation
IgG	immunoglobulin gamma
LAC	leukocyte activation cocktail
LPS	lipopolysaccharide
MFI	mean fluorescent intensity
NKp30	Natural Killer cell activation receptor; anti-CD337
NKp46	Natural Killer cell activation receptor; anti-CD335
LFA-1	lymphocyte adhesion molecule; CD11a
FBS	fetal bovine serum
Dex	dexamethasone
CFSE	carboxyfluorescein diacetate, succinimidyl ester
RPM	revolutions per minute
PFA	paraformaldehyde
HRP	horseradish peroxidase
PSS	Perceived Stress Scale
CES-D	Center for Epidemiologic Studies–Depression
HDAC2-p	phosphorylated histone deacetylase 2
HDACi	histone deacetylase inhibitor
GRE	glucocorticoid response element
CD4+ T	T helper lymphocytes
BMI	Body mass index

CK2	Casein kinase 2
FACS	Fluorescent activated cell sorting
HiCK	Human intracellular cytokine
CAR	Cortisol awakening response
AUC	Area under the curve
H3	histone 3
H4	histone 4

ABSTRACT

The diagnosis of breast cancer is attributed with increased stress, depressive mood, anxiety, mood and sleep disturbance. Bi-directional communication networks between the brain and the immune system allow for one's emotions to impact immune function. Psychological stress can impact immune function through the release of glucocorticoids that affect the immune system in both a time and dose dependent manner. One cell type particularly susceptible to both psychological stress and glucocorticoids are NK cells. The purpose of this study was to assess the impact of chronic glucocorticoid (GC) treatment on NK cells and use this *in vitro* system as a tool to investigate the regulation of the immune system during periods of psychological stress.

Chronic *in vitro* treatment of NK92 cells produced a dichotomous NK phenotype in which enhanced proinflammatory cytokine production was accompanied by reduced NK cell lytic activity (NKCA). The transcriptional profile of chronic GC treated NK cells reflected this dichotomous phenotype with mRNA and protein levels for interferon gamma (IFN-g) and for IL-6 increased while mRNA and protein levels for perforin and granzyme B decreased. This dichotomy was at the individual cell level and was the first demonstration of such. For perforin and granzyme B, time course analysis revealed reduced transcription related to recruitment of histone deacetylase 2 (HDAC2) with concomitant reduction in histone 3 lysine 9 acetylation (H3K9Ac). In contrast, for IL-6

and IFN-g, increased transcription upon cellular activation related to increased H4K8Ac with concomitant loss of HDAC1 and HDAC3 at genomic loci. Hyper-acetylation of cytokine loci increased the sensitivity of these regions to nuclease digestion demonstrating increased accessibility of the chromatin structure, which was primed for enhanced transcription. These results demonstrate chronic exposure to GC to impact NK cell effector function through the modification of the epigenetic status of NK cell effector genes through HDAC redistribution and subsequent epigenetic changes in a promoter specific manner.

These observations were used to investigate the alterations in immune function during periods of psychological distress, at or within 10 days of cancer diagnosis. The perceived stress of cancer diagnosis was associated with increased daily cortisol production and thus enhanced activity of the hypothalamic-pituitary-adrenal gland (HPA) axis. A similar dichotomous immune phenotype characterized by decreased NKCA and enhanced proinflammatory cytokine production was observed in the peripheral blood mononuclear cells (PBMC) of these individuals and was related to the increased activation of the HPA axis. Epigenetic analysis of PBMC demonstrated that the enhanced production of the proinflammatory cytokines IFN-g and IL-6 was related to H3K9Ac within critical regulatory regions. The mediation pathway of enhanced cytokine production by perceived stress through H3K9Ac was tested using the Sobel test. These results demonstrate that increased proinflammatory cytokine production by stress is partially mediated by promoter specific acetylation patterns on H3K9 within critical regulatory regions. In conjunction with pathways elucidated *in vitro*, epigenetic mechanisms regulate immune function during periods of psychological stress.

CHAPTER ONE

LITERATURE REVIEW

THE IMMUNE SYSTEM

The immune system is the network of biological structures and cells that protects an organism from external threats such as pathogens and internal threats such as tumorigenesis. Any condition that disrupts the normal homeostasis of the immune system can lead to pathogenesis by microorganisms, auto-immunity, and cancer. The cells of the immune system are divided into two traditional categories; those of the innate and adaptive components. If an invading pathogen is able to breach the physical barriers which protect an organism the cells of the innate immune system are the first responders and are poised to initiate a robust and non-specific response to aid in pathogen destruction and clearance. These cells include neutrophils, monocytes, macrophages, dendritic cells, and natural killer (NK) cells. Activation of the innate immune system provides the signals and mechanisms required for the initiation of the adaptive immune response. Cells of the adaptive immune system are recruited and activated via a number of mechanisms to provide a pathogen specific response to clear the invading organism. These adaptive immune cells, mainly T cells and B cells, possess an additional ability to remain dormant in the body poised to detect and eliminate the specific pathogen which they previously encountered, in a mechanism known as immunological memory. Each cell of the immune system utilizes is own

unique repertoire of cellular responses to aid in pathogen elimination. In a similar manner, cells of both the innate and adaptive compartments patrol the body and eliminate abnormal cells, either infected or cancerous, from healthy tissues. The role of these many cell types in cancer detection, control, and eradication, as well as the mechanisms which control these processes are an intense area of research.

Hematopoiesis

Decades of research have illustrated that the cells of the immune system, including NK cells, develop through a regulated cascade known as hematopoiesis, outlined in Figure 1. During this process, patterns of gene expression are reprogrammed in stages that ultimately restrict cell potential, leading to the fully differentiated specialized cells of the immune system. The entire mammalian blood system is generated from a single primary pool of hematopoeitic stem cells (HSCs). HSCs are the only cells with both self-renewing capability and the capacity to differentiate into the more than ten mature cell types in the blood. These processes must be regulated in a highly orchestrated fashion to maintain the HSC and blood cell pool throughout life. The differentiation of HSCs into blood cells occurs in a hierarchical manner and includes clear-cut intermediate levels of lineage potential which decreases throughout the differentiation process. The developmental network serves to provide the organism with stable intermediates and precursors to supply the appropriate downstream lineage cells when needed throughout life. HSCs differentiate into multipotent

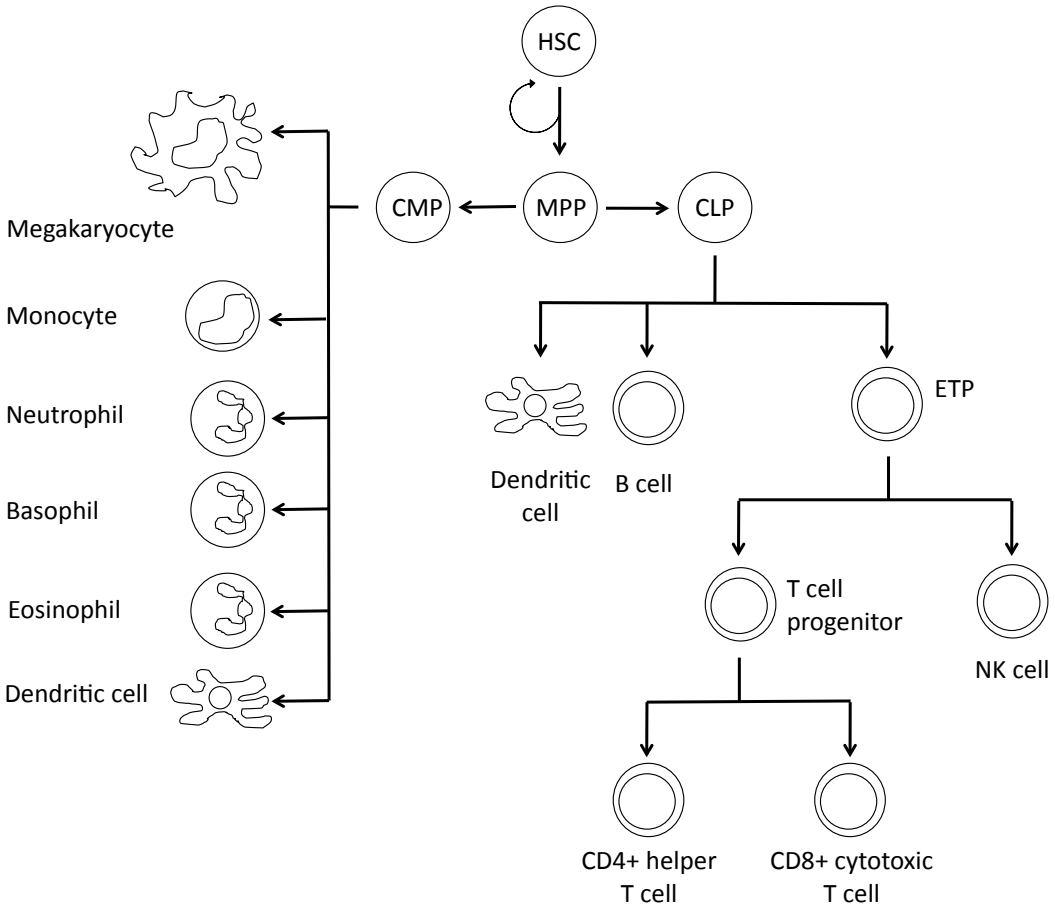


Figure 1. Schematic of hematopoiesis. Hematopoeitic hierarchy. Adapted from Kuby Immunology Sixth Edition. The hematopeoeitic stem cell (HSC) is the only cell in the hierarchy capable of self-renewal and pluripotency. HSC give rise to the multi-potent progenitor (MMP) which maintains multipotency but lacks self renewing potential. The MMP gives rise to both the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). The CMP can differentiate into all cells of the myeloid lineage while the CLP gives rise to all of the cells of the lymphoid lineage including the early T cell progenitor (ETP). The ETP differentiates into all T cell populations as well as NK cells. Not all intermediate and terminally differentiated cell populations are shown.

progenitor cells (MPPs) which lose their self-renewing properties but still maintain multipotency. MPPs are then directed to differentiate into a specific branch either the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP) via regulated networks and integration of signals from the host and microenvironment of the bone marrow niche. The CMP has the potential to differentiate into all the myeloid cells including erythrocytes, megakaryocytes, granulocytes, and macrophages. The CLP can differentiate into three specific lymphocyte lineages B cells, T cells, and NK cells [1]. CLP can differentiate into the bipotent T/NK progenitor (T/NKP) [2]. This T/NKP progenitor can give rise to the early T cell progenitor (ETC) and thus the naïve CD4+ and CD8+ T cell pool, as well as the other CD3+ T cell populations. Furthermore, this T/NKP gives rise to NK cells that share many functional properties with CD8+ cytotoxic T lymphocytes. Although they share many functional attributes of T cells, the differentiation of NK cells is a unique developmental pathway demonstrated by the presence of NK cells in RAG1 [3] or RAG2 [4] knockout mice, scid mice [5], as well as athymic nude mice [6]. NK cells are an innate lymphocyte not only important in the surveillance and elimination of abnormal cells but a critical link between the innate and adaptive immune components.

Natural Killer (NK) cells

NK cells are classified as innate lymphocytes that comprise approximately 15% of peripheral blood mononuclear cells (PBMCs) and are characterized phenotypically by the presence of CD56 and lack of CD3 surface expression. Two main subsets of NK

cells are found in human characterized by the levels of CD56 expression. There are $CD56^{bright}$ and $CD56^{dim}$ NK cells which differ in function, phenotype, and tissue localization. $CD56^{dim}$ NK cells constitute >90% of circulating NK cells in the blood. These $CD56^{dim}$ cells express perforin, killer Ig like receptors (KIRs), and the CD16 FcγRIII and are thought to be more cytolytic and less immuno-regulatory [7]. $CD56^{bright}$ NK cells exhibit more immuno-regulatory functions through the secretion of cytokines, mainly IFN-g [8]. Bone marrow transplantation studies have suggested that $CD56^{bright}$ cells are precursors to the $CD56^{dim}$ populations [9,10]. Independent of the role $CD56^{dim}$ vs. $CD56^{bright}$ NK cells play in the immune response, NK cells were originally discovered through their unique ability to directly lyse cancer cells and virally infected cells without prior exposure [6,11].

NK cells exhibit direct cytolytic activity through a complex process involving four main steps outlined in Figure 2. First NK cells must bind and form a stable conjugate with the target cell (Figure 2A). Conjugate formation by the NK cell with a target cell is a process mediated by integrins and immunoglobulin superfamily of molecules including CD2, CD11a (LFA-1), CD11b, CD11c, and CD28 [12-14]. Experiments have demonstrated that blocking these interactions with antibodies both suppressed adhesion and decreased target cell lysis by NK cells [15]. In the second step, stable conjugate formation is supported by and occurs concomitantly while the NK cell integrates a number of activating and inhibitory signals from ligands expressed on the target cell surface (Figure 2B). Third, NK cells release the contents of their cytolytic granules into the immunological synapse formed during stable conjugate formation

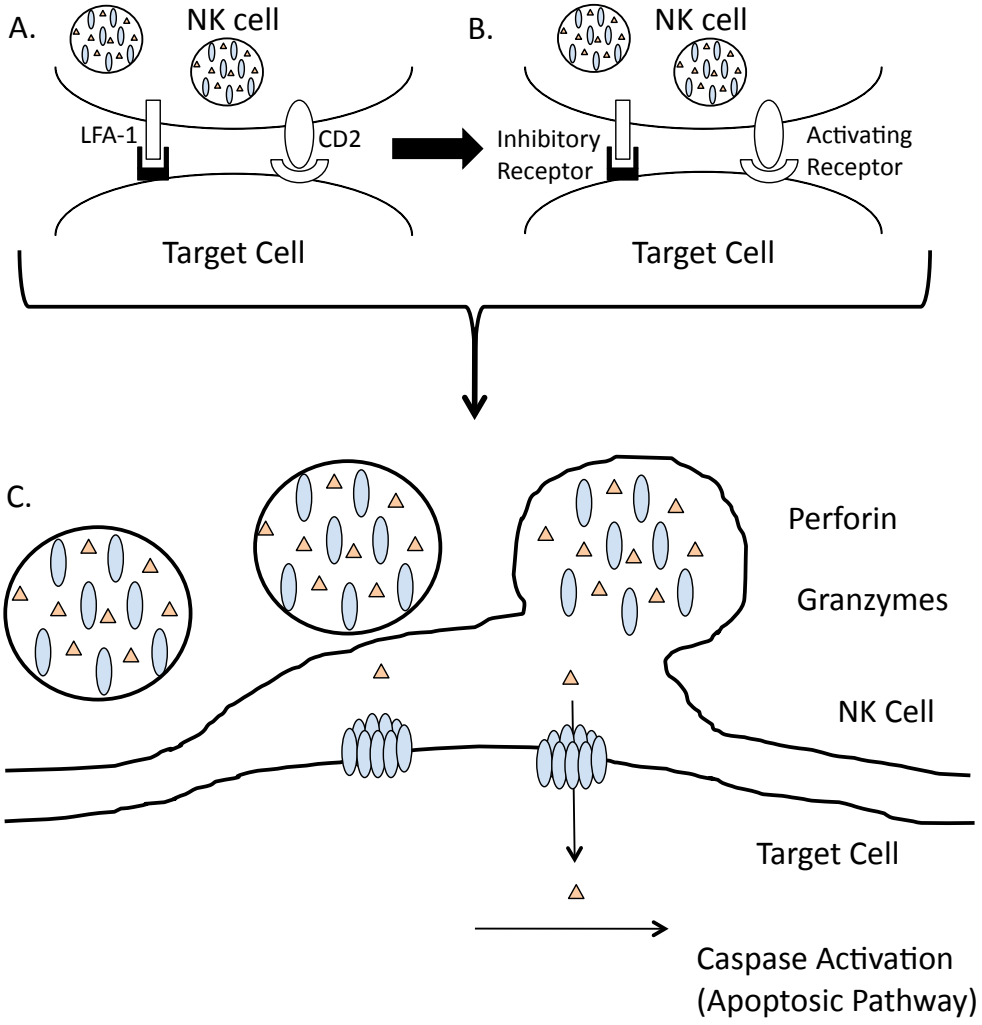


Figure 2. Schematic of natural killer cell lytic activity. (A) Natural Killer cells bind to target cells via LFA-1 and CD2. (B) NK cells integrate the expression of activating and inhibitory ligands presented on the target cell via germ-line encoded activating and inhibitory receptors. (C) Activation of NK cells leads to the release of cytolytic granules containing perforin and granzymes into the immunological synapse formed at the junction between the NK cell and the target cell. Molecules of perforin multimerizes on the plasma membrane of the target cell forming a pore-like structure. Granzymes then enter the target cell cytoplasm and active the caspase system inducing apoptosis.

(Figure 2C). In the final step, perforin and granzymes coordinate to activate the caspase system and thus apoptosis of the target cell (Figure 2C).

The entire process of target cell lysis is contingent on the ability of an NK cell to determine if the target cell is healthy tissue or abnormal in some way, be it transformation or infection. In order to determine whether a cell is infected, cancerous, or healthy tissue, NK cells possess a unique array of cell surface proteins that either stimulate (activating receptors) or dampen (inhibitory receptors) their activity when bound to their respective ligands.

Killer immunoglobulin like receptors (KIRs)

The effector functions of NK cells are controlled by germline encoded receptors that do not undergo somatic recombination, referred to as killer immunoglobulin like receptors (KIRs) [16-21]. KIRs fall into two main categories based on their structure: immunoglobulin-like and lectin-like receptors. Neither structure is specific to the type (activating or inhibitory) of receptor and thus activating and inhibitory receptors fall into both categories. NK cells integrate the signals from both these activating and inhibitory receptors to elicit the appropriate response either target cell killing or no cell killing (Figure 3). The activating receptors possess cytoplasmic tails that contain ITAMs (immunoreceptor tyrosine based activating motif). In contrast, inhibitory receptors possess cytoplasmic tails containing ITIMs (immunoreceptor tyrosine based inhibitory motif). Inhibitory receptors are mainly composed of molecules that recognize MHC class I expression on target cells. Lack of MHC class I

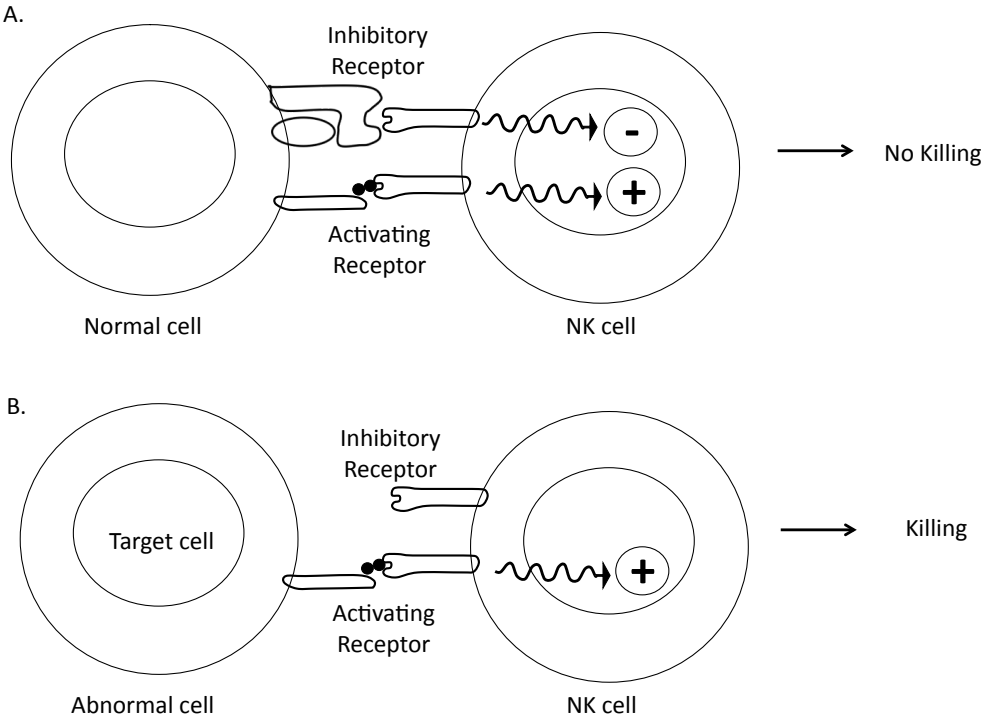


Figure 3. Integration of activating and inhibitory receptor by natural killer cells. Natural killer cells integrate the expression levels of both activating and inhibitory ligand expression on the surface of target cells through engagement to germ-line encoded activating and inhibitory receptors. Binding of an activating receptor to its ligand induces an activation signal, indicating by a plus sign, that results in killing. However, engagement of an inhibitory receptor with its ligand results in an inhibitory signal, indicated by a minus sign, that opposes the activation signal thus preventing NK cell killing. Loss of inhibitory ligand expression, a state normally associated with infection or transformation, allows for the activation signal to predominate resulting in target cell destruction.

on a potential target cell, which many times occurs during infection or cellular transformation, results in a lack of inhibitory signaling and thus results in NK cell activation and killing (Figure 3B). This lack of MHC class I expression and NK cell activation has been termed "the missing self hypothesis" [18]. This posits that when a cell lacks or exhibits reduced surface expression of self MHC molecules (missing self), NK cells are activated and kill the target cell due to lack of inhibitory signaling via ITIMs within the NK cell.

In addition to the inhibitory receptors there are also a wide variety of activating receptors present on NK cells. Recently, a number of activating ligands have been identified including molecules that are upregulated on the cell surface in response to DNA damage [22], cellular stress [23-25], and viral or bacterial invasion [26,27]. It is believed that these activating ligands are upregulated and presented on the surface when a cell senses stress or abnormal processes. The expression levels of these ligands are then sensed and integrated into the complex signaling cascade within the NK cell, ultimately determining whether the cell is killed or spared. More recently, the contribution of synergistic activation from multiple receptors is uncovering the dynamic and diverse array of signals that activate NK cells [28-30]. In summation, these NK cell receptor ligands are used to judge whether the normal processes of a cell are being altered due to cellular transformation or infection and regulate the activation of NK cells and their cytolytic activity.

Cytolytic Granules: Perforin and Granzyme

The activation of NK cells culminates in the controlled release of cytoplasmic granules, which contain perforin and granzymes. Perforin and granzymes are constitutively expressed in natural killer cells and are contained within electron dense cytoplasmic granules even in resting NK cells. Release of cytotoxin containing granules is directed toward the immunological synapse formed at the junction of the stable conjugate between the NK and target cells [31]. The granule-exocytosis pathway results in the activation of the caspase system and, ultimately, apoptosis of the target cell [32-35]. Two prevailing mechanisms have been suggested for the perforin/granzyme mediated cell lysis pathway. In the first model perforin is released into the immunological synapse and undergoes a conformational change when it encounters the target cell plasma membrane. This conformational change exposes an amphipathic domain that inserts into the target cell plasma membrane. Monomers of perforin then polymerize to form cylindrical pores between 5-20nm in diameter at neutral pH in the presence of calcium [36]. Further research revealed that perforin shares sequence and structural homology to the complement component C9 [37-39]. This pore is thought to allow granzymes, also released into the immunological synapse, access to the target cell cytoplasm. The granzymes, a class of serine proteases, cleave and activate members of the caspase system inducing apoptosis of the target cell [40].

In a second pathway, granzymes are thought to bind the mannose-6-phosphate receptor. Granzyme binding to the mannose-6-phosphate receptor results in internalization of the complex [41]. It is believed that this endocytic pathway would

result in vesicles that also contain perforin monomers; thus the formation of perforin mediated pores within this vesicle allows granzymes entry to the cytoplasm [41]. Ultimately, both pathways require perforin and result in the activation of the caspase pathway and subsequent apoptosis of the target cell via granzymes.

The important role of perforin in NK cell immunomodulation has been elucidated by experiments in mice and natural human mutations in the perforin gene. In a seminal study, perforin deficient mice showed a marked immunodeficiency [42]. Other mouse studies have confirmed the importance of the perforin pathway in defense against viral infection and cancer [43-45]. Additionally, human diseases in which patients lack or have a mutated forms of perforin exhibit a decrease in immunosurvelliance mechanisms [46]. This work in total emphasizes the critical role of perforin in the control of both tumorogenesis and viral infection.

The perforin gene, *PRF1*, is encoded on the human chromosome 10 band q22 and is expressed by NK, CD8+ T cells, γδ T cells, and NKT cells. The upstream 5' regulatory region of the *PRF1* contains two distinct enhancer regions located at -15kb and -1kb from the transcriptional start site. Perforin expression is regulated by STAT3 [47], STAT4 [48,49], STAT5 [50], and NF-κB [51]. STAT5 binding has been demonstrated at both enhancer regions of the perforin gene [52]. NF-κB, STAT1a, and STAT4 binding has been demonstrated at the -1kb enhancer [49,50]. STAT3, a key transcription factor of NK cells, seems to be involved in the constitutive expression of perforin and can bind the -1kb enhancer region [53]. IL-2 mediated signaling can activate both NF-κB and STAT5 [50,52]and enhance perforin gene expression. Additionally, IL-12 mediated signaling via STAT1a and STAT4 upregulate perforin

gene expression [49,50]. Although perforin is required for NK cell lytic activity, it alone is not sufficient for cytolytic function, but is critical for granzyme entry into the target cell.

Granzymes are a family of serine proteases and comprise a large portion of the proteins contained within NK cell cytotoxic granules. There are 5 known human granzymes (A, B, H, K, and M) each with distinct substrate specificity and regulation. NK, NKT, and γδ T cells are the only immune cells that constitutively express granzymes; expression is thought to be restricted to the lymphoid lineage. Granzyme A and B have been the most extensively studied of the granzyme family. Granzyme A and B have been shown be cytolytic *in vitro* at micromolar and nanomolar concentrations, respectively [32,54]. Granzyme B can induce apoptosis by activating both caspase-3 and -7 [55]. The cytolytic activity of Granzyme A is controversial as *in vitro* cytotoxicity only occurs at micromolar amounts, requires specific conditions, and is independent of the caspase system [56,57]. Emerging evidence links Granzyme A release during infection or disease with an inflammatory response via an extracellular mechanism. Granzyme B is regulated by type I interferons [58], IL-2 [59], IL-12 [59,60], IL-15 [61], and IL-18 [62,63]. The constitutive expression of granzymeB in CD8+ cytotoxic lymphocytes is regulated by the transcription factors AP-1, IKAROS, RUNX1, CREB1, and Eomes via binding to a hypersensitivity site in the granzymeB promoter approximately -250bp from the transcriptional start site [64,65]. Although not directly tested, it is assumed that granzymeB is regulated in a similar manner in NK cells as that demonstrated for CD8+ CTL. Furthermore, STAT3 has been hypothesized to regulate granzyme B via binding at the -250kb hypersensitivity site due to the presence

of a STAT binding element, but this has not been directly tested. The constituitive and cytokine induced regulation of both perforin and granzymes are thus vital to the cytolytic function of NK cells.

NK cells and Cytokines

NK cells not only have direct cytolytic activity but also produce a number of immunoregulatory cytokines [66,67]. NK cells have now been found to produce and secrete a variety of immunomodulatory cytokines such as IFN-γ, TNF-α, IL-10, IL-6, IL-5, IL-8, IL-1β, and TNF-β [47]. Although there is crosstalk between the signaling pathways that regulate lytic molecule and cytokine gene expression the pathway for cytokine secretion is distinct from that of the granule constituents [68]. The direct killing of infected or transformed cells is only one way NK cells are able to contain an infection and promote the appropriate immune response. The role of IFN-g secreted from NK cells and its role in the immune response is the most studied. IFN-g production by NK cells stimulates macrophage activation. Macrophage activation upregulates MHC expression and enhances phagocytosis, these mechanisms are thought to aid in clearance of apoptotic cell debris, tissue regeneration, and promote the adaptive immune response through the presentation of pathogen and/or tumor specific antigens. Furthermore, IFN-g activates CD8+ CTLs. As CTLs are the adaptive counterpart to NK cells, it is thought that IFN-g production from NK cells promotes an antigen specific immune response to the pathogen or tumor. IFN-g production from NK cells is also thought to direct the differentiation of naïve CD4+ T cells. IFN-g inhibits Th2 and

promotes Th1 differentiation of naïve CD4+ T helper cells. Th1 cells also secrete IFN-g and aid CD8+ CTL activation and thus an antigen specific response. The initial recognition of infection or tumorogenesis by NK cells and their subsequent release of IFN-g provides a critical link between the innate and adaptive immune systems.

Like all cytokines IFN-g transcription is tightly regulated to ensure that its production is maintained for the appropriate length of time and to the desired concentration. The regulation of IFN-g transcription has mostly come from studies investigating the differentiation of CD4+ T helper cells into the Th1 lineage in mouse models and cell lines and is extensively reviewed [69]. These studies have revealed that the IFNG locus is composed of approximately 150,00 bp of DNA. Within the *IFNG* locus analysis of DNase hypersensitivity sites have revealed a number of cis-regulatory elements critical for differentiation and transcriptional activation [69]. These cis-regulatory elements are conserved non-coding sequences (CNSs) which encode binding sites for transcription factors involved in the development of permenant epigenetic modifications important for the differentiation and structure of this locus throughout the differentiation process. In naïve CD4+ T cells the *IFNG* locus contains DNase hypersensitivity sites at -22kb, -34kb, and -70kb downstream of the transcriptional start site (TSS). Additional DNase hypersensitivity sites are located upstream of the TSS at +17-19kb, +30kb, +40kb, +46kb, and +66kb. As the *IFNG* locus becomes fully differentiated DNase hypersensitivity sites open up at the proximal promoter, about -5-6kb, and -34kb downstream of the TSS [69]. As NK cells are able to rapidly secrete IFN-g upon stimulation and do not require cellular proliferation for this function it is believed the *IFNG* locus contains an *IFNG* locus similar to that of fully differentiated

CD4+ Th1 cells. One peticular enhancer 22 kilobases downstream in mice and 17 kilobases downstream in human has been demonstrated to be required for IFN-g transcription in both T cells and NK cells [70,71,72]. IL-2 [73], IL-12, IL-15 and IL-18 have been shown to upregulate IFN-g in NK cells [74,75,76]. These changes in gene expression have been attributed to NF-κB [77], STAT4 [78], STAT1 [79], STAT3 [80], and T-bet [81]. The bindings sites for NF-κB, STAT1, STAT3, and STAT4 are contained within the proximal promoter, -5kb enhancer, and -22kb enhancer regions of the *IFNG* locus and are critical for the regulation of IFN-g production.

More recently NK cells have also been shown to produce IL-6 [47], although NK cells have been known to be responsive to IL-6 for years [82]. The *IL6* gene is controlled via elements in the proximal promoter including an IL-1-response element as well as a CRE and NF-IL6 binding sites. The *IL6* gene is rapidly upregulated by LPS, IL-1, TNF, IL-2 [83], and IL-6 itself [84]. Most studies in the regulation of IL-6 have been elucidated in monocytes, which are the main producers of IL-6 within the immune cell pool. The contribution of IL-6 production by NK cells and its role in the immune response remains unclear.

NK cells can also produce TNF-alpha in response to external stimuli such as target cell recognition and is implicated in the clearance of both intracellular pathogens and tumors. The *TNF* gene is clustered with lymphotoxin (LT)-α and LT-β, also members of the TNF superfamily, on human chromosome 6 within the MHC class III region. The transcription of the *TNF* gene is regulated via the recruitment of transcription factors and other co-activators to the 5' proximal promoter upstream of the TSS [53]. Two additional hypersensitivity sites -1.7kb and +1.4kb have also been

identified and might contribute to the regulation of *TNF* transcription [53]. The proximal promoter contains a binding site for AP-1, AP-2, CREB, and NF-κB and these factors have all been shown to upregulate TNF-alpha [85]. Unlike the other cytokines it has been shown in monocytes that TNF-alpha is constituitively expressed yielding productive transcripts; however, the mRNA is destabilized by its interaction with the RNA binding protein, tristetraprolin [86]. This minimizes TNF-alpha production in the absence of stimuli but also contributes a negative feedback loop to resolve the TNF-alpha production after cellular activation with LPS, TNF-alpha, and IL-2 [86,87].

Conclusions

Natural killer cells constitute a critical member of the immune system. Their ability to patrol the body using germline encoded receptors to detect abnormal cells is critical for host survival. Constituitive expression and storage of lytic molecules within cytoplasmic granules allows for immediate and effective irradication of abnormal cells without prior exposure. Additionally, NK cells are able to secrete a wide variety of immunoregulatory cytokines to modulate the activity of other immune cells and the subsequent immune response. Like all cells of the body NK cells are regulated by the neuroendocrine systems. Understanding how hormonal systems regulate NK cell effector function remains an active area of research. This dissertation examines a particular hormone, glucocorticoids, and how they regulate both NK cell cytolytic function as well as cytokine production.

GLUCOCORTICOIDS AND THE GLUCOCORTICOID RECEPTOR

Introduction

Hormonal systems regulate human physiology and are a mechanism by which the brain comminicates with multiple body systems to maintain host homeostasis. Glucocorticoids (GC) are a class of steroid hormones whose name derives from their role in glucose metabolism (gluco), synthesis from the adrenal cortex (cortic), and their steroid structure (oid). Glucocorticoids regulate many aspects of human physiology including metabolism, sleep, the cardiovascular system, as well as the immune system.

The HPA axis and Diurnal Cortisol Rhythms

Cortisol is the main glucocorticoid in human and its production and secretion is regulated by the hypothalamic pituitary adrenal gland (HPA) axis. Activation of the hypothalamus results in the secretion of corticotropin-releasing hormone (CRH). CRH then triggers the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH then travels through the vascular system to the adrenal glands situated above the kidneys. ACTH then stimulates the production and secretion of cortisol by the zona fasciculate within the adrenal cortex. The activity of the HPA axis is regulated in a circadian manner via circadian oscillators, which yields a distinct 24 hour cycle of cortisol secretion from the adrenal cortex known as the cortisol diurnal rhythm [88,89,90]. Figure 4 is a graphical representation of a typical diurnal cortisol rhythm in a

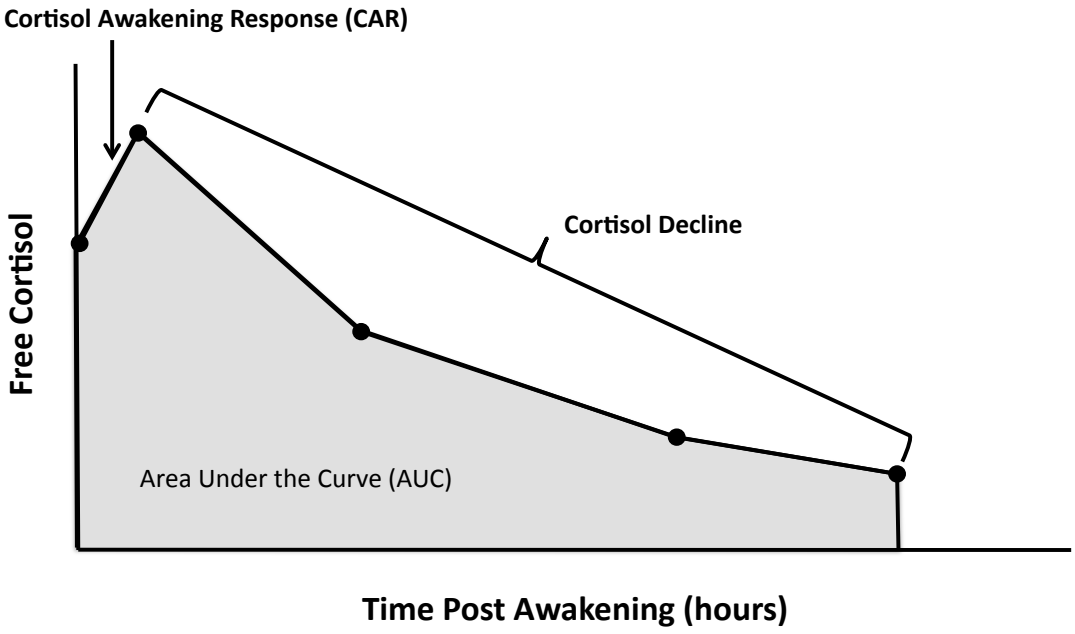


Figure 4. Schematic of Typical Diurnal Cortisol Rhythms. A typical diurnal cortisol rhythm is characterized by an initial spike approximately 15-45 minutes post-awakening, termed the cortisol awakening response (CAR). Cortisol levels then gradually subside throughout the day into the night, reaching a minimal level in the hours preceeding awakening. The area under the curve (AUC) represents the total cortisol levels throughout the day (shaded in grey).

healthy individual. Cortisol levels rapidly increase in serum and body fluids after awakening, termed the waking rise also known as the cortisol awakening response (CAR). This 50-75% increase in cortisol occurs within 30 to 45 min post awakening when cortisol levels are at their peak. Cortisol levels then decline throughout the day and into the night until cortisol levels are lowest in the hour preceding awakening. The typical diurnal rhythm is detected in about 75% of healthy individuals [91]. Cortisol is a lipophilic hormone ligand that can freely diffuse through the plasma membrane and thus exert its biological effects through its binding to the cytoplasmic glucocorticoid receptor.

The Glucocorticoid Receptor (GR)

Glucocorticoids mediate their effects through the binding and activation of the glucocorticoid receptor (GR). GR mRNA can be detected in almost all tissues in both mice and humans, and therefore, it is assumed that functional GR protein is also present and thus has the potential to regulate gene expression in almost all known cell types[92,93,94]. GR is a member of the nuclear receptor superfamily and is a ligand activated transcription factor which is maintained in the cytoplasm via a complex of inhibitory cytoplasmic heat shock proteins including both HSP70 and HSP90[95]. GR contains a zinc-finger motif DNA-binding domain, a dimerazation domain, and a ligand-binding domain. Upon ligand binding GR undergoes a conformational change, dissociates from the inhibitory complex, and translocates to the nucleus via its nuclear localization signal. Once in the nucleus, GR interacts with DNA in a sequence specific manner and directly regulates gene transcription through the recruitment of co-activators

and co-repressors. GR can both positively and negatively regulate transcription through its zinc-finger motif and binding to glucocorticoid response elements (GREs) and negative glucocorticoid response elements (nGREs), respectively [96]. The role of GRE and nGRE in the transcriptional regulation by GR has been further complicated with research demonstrating that sequence variation in the GREs can alter GR structure and activity in a promoter specific manner [97]. The number of genes negatively regulated by glucocorticoids is conservatively estimated at more than 100 while those that are positively regulated are conservatively estimated at over 400 [98-101]and more liberal estimates place the number of GC regulated genes at greater than 1,000 [102-104]. Additionally, GR can interact with other transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) and repress transcription indirectly via trans-repression [105-107].

GR activity is regulated at multiple levels. GR is encoded by the NCR1 gene and through alternative splicing results in two main mature mRNA forms which ultimately result in the translation of two distinct proteins, GR-alpha and GR-beta. GR-beta lacks the carboxy-terminus ligand binding domain and is thus insensitive to ligand and is hypothesized to act as a dominant negative isoform. The GR-beta isoform is not ubiquitously expressed, is hypothesized to be involved in glucocorticoid resistance, and still is under intense investigation to understand its precise biological role(s). Once ligand activated and translocated to the nucleus, GR-alpha is thought to be degraded by the proteasome and thus GR is thought to have a rapid turnover to facilitate integration of rapidly changing GC levels [108]. Furthermore, GR is both phosphorylated and acetylated which provides further levels of regulation via these posttranslational

modifications. Although the regulation of GR is complex the effects of GC on the immune system have been used pharmacologically for decades.

Glucocorticoids and Inflammation

Many GR target genes are involved in modulation of the immune system and the inflammatory response. For this reason the potent immunosuppressive effects of glucocorticoids have been used clinically to treat a wide variety or immunologic based diseases such as asthma, allergies, auto-immunity, and arthritis. GR has been shown to downregulate a number of pro-inflammatory cytokines, including IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, TNF-alpha, and GM-CSF [109]. Conversely, GC upregulate the expression of a number of anti-inflammatory genes including; Lipocortin-1, CC10, IL-1 receptor antagonist, and IkappaB, through a number of mechanisms dependent on the gene of interest [109]. Of the pro-inflammatory genes down-regulated by high glucocorticoid concentrations, the IL-6 promoter is the only promoter demonstrated to contain a GRE and recruit GR when glucocorticoids are present [110]. With the absence of cononical GREs within pro-inflammatory genes other mechanisms to explain the decrease in proinflammatory production by GR were investigated.

Decades of research have uncovered a wide variety of mechanisms by which GR can modulate the transcription of genes independent of direct DNA binding. One such mechanism involves the modulation of signaling cascades such as the JAK-STAT and NF-κB pathways. GR has been shown to interact with IκBα maintaining NF-κB in an inactive state in the cytoplasm [111,112]. GR has now been shown to interact with a

number of members of the Jak-STAT pathway these interaction have mainly been shown to inhibit the stimulated production of inflammatory mediators [113,114]. The described mechanisms mainly involve the inhibition of nuclear translocation of the above signaling pathways resulting in decreased proinflammatory cytokine production.

Another mechanism has been described involving the direct interaction between GR and transcription factors such as NF-κB and AP-1 known as tethering. In this mechanism, GR can be recruited to genomic regulatory regions via this tethering mechanism and can thus increase or descrease the transcription of genes independent of GREs via transactivation or transrepression, respectively. It is believed that this tethering involves GR monomers rather than the GR dimers recruited to GREs and the direct regulation of GR responsive genes such as GILZ [115].

For decades GC were deemed potently anti-inflammatory; however, recent research suggests that GC can in fact be pro-inflammatory in nature dependent on both the dose and duration of GC exposure [116]. Furthermore, although GC alone can be anti-inflammatory, GC can synergize with cytokines to potentiate the inflammatory response [109]. Additionally, the finding that some individuals become insensitive to the anti-inflammatory effects of glucocorticoids also have demonstrated that the universal anti-inflammatory effects of GC are oversimplified. The development of insensitivity to GC has been termed glucocorticoid resistance. Mechanistically the resistance of individuals to GC has been attributed to alterations in the levels of GR within a given cell type. GR is known to auto-regulate its own expression and GC administration results in the down-regulation of GR mRNA and thus GR protein, resulting in cells becoming insensitive to further GC administration. Furthermore, GR is

regulated by post-translational modifications and has been demonstrated to be both phosphorylated and acetylated. Phosphorylation of GR at has been shown to alter the activity of

Glucocorticoids and Natural Killer cells

A number of studies have demonstrated the effects of GC on NK cells. We have previously shown that incubation of the NK cell line, NK92, with dexamethasone for 24 hours decreased NK cell lytic ability in a dose dependent manner [117]. We confirmed these results in a second NK cell line YT-Indy [118]. These results have also been demonstrated with cortisol on NK3.3 cells which exhibited reduction in NKCA via decreases in the mRNA for both perforin and granzymes [119]. NK cells isolated from PBMC of humans and treated with dexamethasone also exhibited decreased NKCA [120]. NK cells isolated from PBMC and incubated with cortisol had decreased expression of NKp46 and NKp30 [121]. Direct treatment of human with cortisol resulted in a loss of circulating NK cells from the blood, implying that GC also can mediate redistribution of NK cells *in vivo* [122]. In addition to the effects of GC on NK cell lytic activity we have published that 24 hour dexamethasone treatment also inhibited the unstimulated and stimulated production of IFN-g by NK92 cells in a dose dependent manner [117]. All these studies used high concentrations of GC with incubations lasting no more than 24 hours. As the effects of GC are known to be both dose and time dependent, the effects of extended GC treatment on NK cells remains unclear. A relationship between circulating cortisol and NK cells has also been observed

demonstrating that NK cell lytic activity is regulated by the diurnal cortisol rhythms [123].

Conclusion

Glucocorticoids represent a critical regulator of immune function and thus have been used for decades pharmacologically. Specifically for Natural Killer cell the effects of glucocorticoids have been demonstrated to regulate both lytic activity as well as cytokine production. Furthermore, correlations between circulating cortisol levels and NK cell effector function *in vivo* indicate the physiological role glucocorticoids play in the immunoregulation of NK cells. Many mechanisms have been proposed for the immune regulation by GC but come mainly from suprapysiological concentrations over short periods of time. The effects of GC are both dose and time dependent and thus exhibit significantly different phenotypes when these parameters are altered. Furthermore, these mechanisms cannot fully explain the effects on immune function seen during alterations in the activity of the HPA axis. This dissertation examines how glucocorticoids regulate the epigenome of NK cells and how these epigenetic mechanisms might be relevant during periods of altered immune function when HPA activity is dysregulated.

EPIGENETICS

The DNA of eukaryotic cells is composed of repeating units of nucleosomes referred to as chromatin. One hundred and fourty seven base pairs (bp) of DNA wraps around a dimer of four core histone proteins, H2A, H2B, H3, and H4. Each of these histone proteins as well as the DNA sequence itself are subject to multiple posttranslational modifications which alter chromatin structure and function. These covalent posttranslational modifications work by altering the overall charge of the nucleosome particle and/or create docking sites to recruit regulatory protein complexes. Histone modifications constitute a "Histone Code" which is read by the cell machinery to bring about a specific transcriptional profile. The term epigenetics describes the process by which changes in this histone code impart heritable, but also reversible, changes in gene expression without changes to the DNA sequence itself. These changes in chromatin structure yield two distinct types of genomic regions in the nucleus of a eukaryotic cell; condensed transcriptionally silent, heterochromatin, and less condensed transcriptionally active, euchromatin.

Each of these regions is characterized by a specific epigenetic pattern which maintains this structure and gene expression pattern, even through cellular division. The DNA itself can be methylated on CpG islands and has been demonstrated to strongly correlate with regions of heterochromatin and transcriptional repression [124]. DNA methylation is mediated by a class of enzymes known as DNA methyltransferases (DNMTs). Further research has demonstrated that methylated DNA provides docking sites for proteins which not only maintain DNA methylation but recruit histone

modifying enzymes which inhibit histone acetylation and promote methylation of specific histone residues, maintaining heterochromatin condensed structure and gene repression.

Histone proteins can be phosphorylated, acetylated, methylated, ubiquitynated, and sumoylated. Phosphorylation and acetylation of histone proteins generally accompany gene transcription. Sumoylation of histone protein accompanies repression while ubiquitynation and methylation are implicated in both activation and repression of transcription. Methylation of histone proteins occurs on lysine residues and comes in three forms; mono (me1), di (me2), and tri-methylation (me3). Methylation of lysine residues occurs via histone methyl transferases (HMTs). Methylation of histone tails creates docking site for additional co-activator and co-repressor complexes. Methylated lysines are recognized by proteins which contain chromo-domains. These chromo-domain containing proteins recruit additional proteins and protein complexes which further modify the genomic locus for either gene silencing or gene activation.

Acetylation of histones has been the most widely investigated epigenetic modification. Two mechanisms for how histone acetylation regulates gene expression have been postulated. First the acetylation of histones occurs on lysine residues, which possess a positive charge. It is hypothesized that the positive charge associated with the lysine residue allows for condensation of the chromatin structure via interaction with the negatively charged DNA phosphate backbone or the neighboring nucleosome particles. Addition of an acetyl group by histone acetyltransferases (HATs) neutralizes this positive charge and results in decreased condensation and opening of the chromatin structure. The role of acetylation and chromatin accessibility has been demonstrated in

many models. Secondly, addition of the acetyl group on the lysine residue creates a docking site for other regulatory proteins. Research has revealed a common domain among some regulatory proteins known as a bromo-domain. The structure of these domains allows for recognition and binding to acetylated lysines but not unacetylated lysines. From these observations histone acetylation is linked with transcriptional activation and recruitment of co-activator complexes.

The Histone Code

The transcription of a given gene is dependent not on a single epigenetic mark but the summation of the marks present in a specific loci termed the "Histone Code". Figure 5 outlines the relevant histone modifications for this body of work. Transcriptionally inactive heterochromatin is marked by the inhibitory marks H3K9me3 and H3K27me3 (black circles) [125-128]. The structure of this region is compact and inaccessible to transcription factors and transcriptionally machinery, indicated by the tight grouping of the nucleosome particles (Figure 5). This structure is maintained within intergenic non-coding regions of DNA as well as in genes that are permanently repressed in a specific cell lineage. This is in contrast to poised euchromatic genes which maintain H3K27me3 but possess H3K4me3 (grey circles) as well. H3K4me3 is typical representative of transcription start sites and is strongly correlated with transcription potential [129,130]. These regions are termed bivalent as they have the potential to be expressed in a particular cell lineage, are more accessible than heterochromatin (indicated by a decrease in chromatin condensation), but are not

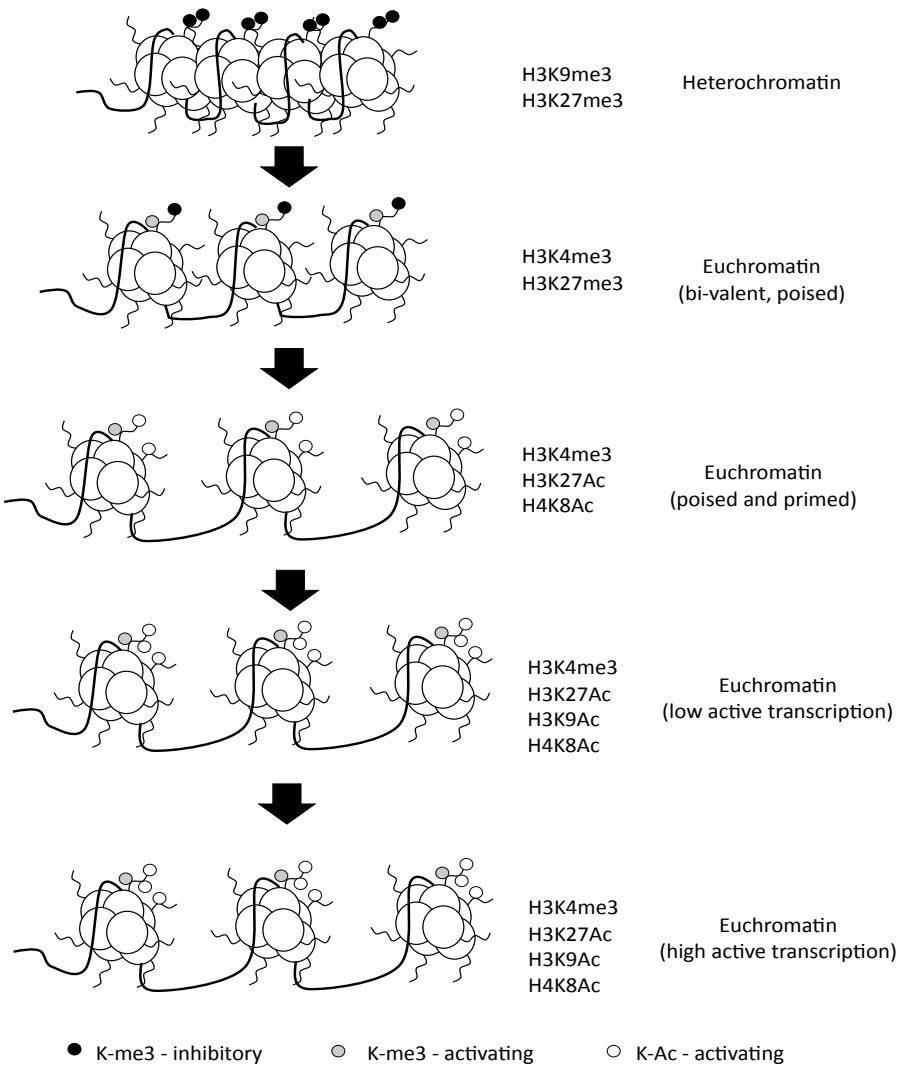


Figure 5. Diagram of histone tail modifications, changes in chromatin structure, and transcription. Heterochromatic regions of DNA are associated with the repressive histone tail modifications tri-methylated H3K9 and H3K27 (black circles) and exhibit a condensed chromatin structure. Bi-valent euchromatin is characterized by the loss of H3K9me3 and addition of H3K4me3 (grey circles) with slightly increased chromatin accessibility. These regions are not transcribed but are accessible and poised for potential transcription. Poised and primed euchromatin is characterized by the loss of H3K27me3 and addition of H3K27Ac and H4K8Ac (white circles). These regions exhibit greater accessibility than bi-valent euchromatin but remain to the most part untranscribed. The addition of H3K9Ac distinguishes poised and primed euchromatin from actively transcribed euchromatin and results in a completely accessible chromatin structure. The levels of H3K9Ac are highly predictive of the rate of transcription and can be further divided via this histone modification.

actively transcribed requiring further differentiation and activation signals [131]. Poised euchromatin can be further primed for future transcription through the acquisition of H3K27Ac and/or H4K8Ac (white circles). These modifications act via the mechanisms described previously opening up the chromatin structure and create docking sites for co-activator complexes. Lastly actively transcribed euchromatin possesses H3K9Ac (white circles) while maintaining all of the marks associated with poised and primed euchromatin. The degree of transcription highly correlates with the level of H3K9Ac and thus can be divided further by the levels of this specific transcriptional mark. Most work understanding this histone code and epigenetic patterns has come from studies in cell differentiation pathways. Specifically in NK cells the epigenetic marks associated with the KIR locus through the differentiation process have been the most widely studied [132]. Thus by characterizing the epigenetic modifications at specific genomic loci not only provides insight into the differentiation state of the cell but also the activation, transcriptional status, and future transcriptional potential of the gene.

Histone Modifying Enzymes

HDACs deacetylate proteins through removal of an acetyl modification from lysine epsilon-amino group. To date 18 HDACs have been described in humans. These HDACs are divided into 4 categories, Class I, II, III, and IV, based on their structural and enzymatic homologs in yeast and bacteria. HDAC1, HDAC2, HDAC3, and HDAC8 comprise the class I HDAC family based on their homology to the yeast Rpd3 family. HDAC1, 2, and 3 seem to be ubiquitously expressed while HDAC8 activity is

primarily seen in smooth muscle [133,134,135]. Classically it was thought that class I HDACs were localized to the nucleus; however, recent evidence demonstrates that HDAC1, HDAC2, and HDAC3 all have cytoplasmic activity and can shuttle in and out of the nuclear compartment [133]. Class II HDACs are subdivided into two groups; Class IIa (HDAC4, 5, 7, and 9) and Class IIb (HDAC6 and 10). Class II HDACs unlike class I HDACs seem to have tissue and developmental specific expression and are classified based on their homology to the yeast HdaI family [136]. Class II HDACs also dynamically shuttle between the cytoplasm and nuclear compartment [136]. Class IIb HDACs contain two enzymatic domains as compared to the one of class IIa proteins [136]. Furthermore, class IIa HDACs are expressed in the brain, HDAC6 is expressed primarily in the testis and HDAC10 is expressed in the liver, spleen, and kidneys [136]. Class III HDACs are also referred to as Sirtuins, based on structural and enzymatic homology to yeast SIR2 HDACs. These sirtuins are not homologous to class I, II, or IV HDACs. Furthermore the catalytic domains of this family require the cofactor NAD+ for activity. HDAC11 is the only member of class IV HDACs and its separate classification is based on a conservation of HDAC domain stucture but significant sequence divergence from the other HDAC families [137].

The role of histone deacetylases in the regulation of gene expression is highlighted by the findings using HDAC inhibitors (HDACi). Treatment of ESC with vaproic acid an HDACi resulted in increased pluripotency and was correlated with an increase in both global and gene specific H3K9 acetylation [131,138]. Although many reports highlight increases in gene transcription and histone acetylation upon HDACi treatment, there are an increasing number of reports indicating that HDACi can also

inhibit gene transcription [139]. Further research has elucidated that HDACs are not only involved in gene repression but are enzymatically inactive structural components of both co-activator and co-repressor complexes. Furthermore, HDACs are required to reset chromatin after each round to transcription by RNApol II and thus inhibition of HDACs can also decrease transcription [140]. These results highlight that HDACs are important regulators of gene transcription through their removal of acetyl group from lysines residues on histone proteins.

As HDACs are not solely confined to the nucleus it was postulated and confirmed that they have non-histone targets. Furthermore, their nuclear translocation is regulated via post-translational modifications in a manner similar to canonical transcription factors. Thus HDAC nuclear localization and gene regulation is dependent upon the integration of a complex array of signaling cascades. These findings are highlight by recent experiments with HDACi or siRNA knockdown experiments of specific HDACs. HDACi treatment promotes Treg conversion *in vivo* [141]. Furthermore, knock-down of HDAC6, HDAC9, and SIRT1 increased the suppressive activity of Tregs both *in vivo* and *in vitro* [142,143,144]. These along with other experiments highlight that global changes in HDAC activity and protein levels regulate cellular function. Thus HDACs integrate signals and augment cellular function and processes accordingly. Through modulation of HDAC localization and activity the glucocorticoid receptor and thus GC can regulated gene transcription through epigenetic modifications.

The Glucocorticoid Receptor and Epigenetics

Many studies have demonstrated that exposure to glucocorticoids results in altered transcriptional rates as well as changes in histone modifications both globally and in a gene specific manner [117,118]. These changes in histone modifications prompted investigations in the mechanisms by which GC mediate these changes. Research has demonstrated that GR can interact with HDAC1[145], HDAC2[146], and HDAC3[147]. The observations that GR can interact with histone modifying enzymes prompted investigation into whether GR can recruit or dissociate histone modifying enzymes to and from specific genomic loci. Experiments using the mouse mammory tumor virus (MMTV) promoter have clearly delineated that activation of GR results in recruitment of GR to the MMTV promoter region which is associated with an increase in HDAC1 recruitment. Furthermore, GR dependent HDAC1 recruitment decreased histone acetylation at the MMTV promoter [145]. Using this same system, but with a different concentration of ligand, other studies have demonstrated that activation of GR recruits HDAC3 to the MMTV promoter reducing the acetylation of both H3 and H4 [147]. Studies on the regulation of IL-1β demonstrate that activated GR recruits HDAC2 to the IL-1β promoter decreasing both H4 acetylation and transcription[146]. GR also recruits HDAC2 to the GM-CSF promoter to antagonize its transcription[146]. As GR can both upregulate and downregulate gene expression, it is not surprising that GR has also been found to interact with HATs and co-activator complexes, such as SRC-2 [148], pCAF [148], and CBP/p300 [149,150]. In summation of these studies GR can also recruit HATs in a gene specific manner, increase histone acetylation, and

transcription. In addition to histone acetylation, GR can also regulate both histone and DNA methylation. The role for GR dependent DNA and histone methylation has primarily been investigated in neuronal cell populations; however, recent research has also found that GC can alter DNA methylation at the Fkbp5 gene in non-neuronal cells potentially through the regulation of DNMT1[151]. Thus GR alters gene expression through changes in histone modifications via its association with histone modifying enzymes and subsequent recruitment or dissociation of these enzymes in a gene specific manner.

The experiments described above demonstrate that GR itself modifies the epigenetic landscape. Recently it has been demonstrated that genomic binding of the GR is targeted to pre-existing foci of accessible chromatin, and thus the epigenetic landscape impacts GR recruitment [152]. These results were confirmed using chromatin immunoprecipitation and nuclease digestion experiments that implicate cell-selective organization of the pre-existing chromatin structure as a critical factor in determining tissue specific receptor function [153]. Thus GR can further modify the pre-existing chromatin structure either enhancing or decreasing the relative accessibility of a specific genomic region. Through the modulation of GC levels in the body each tissue with its specific preexisting chromatin structure results in a tissue specific response from GR. GR can respond to these changes in GC and immediately impart epigenetic changes that effect gene transcription and cellular function in a tissue specific manner.

As previously described the subcellular localization and overall protein levels of HDACs also regulate cellular function. Furthermore, GR can interact with a number of HDACs. Thus GR can modulate cellular function through direct interaction with

histone modifying enzymes in a gene specific manner but also through changes in their subcellular localization or protein levels. These results highlight the multitude of ways GC signaling can impact cellular function through the modulation of the epigenetic landscape.

Conclusion

Epigenetics provides a critical regulator of cellular function via cell type specific regulation of gene transcription. This regulation occurs at multiple levels via changes in nucleosome charge, chromatin accessibility, and co-regulator binding sites. Many mechanisms have been proposed for the immune regulation by GC but these mechanisms cannot fully explain the effects on immune function seen during alterations in the activity of the HPA axis. Furthermore, epigenetics provides a means by which the evironment, such as the perception can impart changes in gene expression and thus cellular function. This dissertation examines how glucocorticoids regulate the epigenome of NK cells and how these epigenetic mechanisms might be relevant to the field of psychoneuroimmunology.

PSYCHONEUROIMMUNOLOGY

Decades of research have illustrated that the brain, and thus perception of the environment, can control the activity of the immune system. All primary and secondary lymphoid organs are directly innervated and provide a direct link between the nervous and immune systems. Receptors for neurotransmitters have been found on almost all immune cells including; macrophages[154], B cells[154,155], T cells[154,155], as well as NK cells[154,156]. Thus direct innervation of lymphoid organs and release of neurotransmitters provides direct regulation of the immune response by the nervous system. Furthermore, macrophages have been shown to produce catecholamines upon stimulation[157]. These findings highlight that the activities of the central nervous and immune systems are intimately linked. The brain also regulates the activation of the HPA axis and release of glucocorticoids, described previously. Furthermore, the GR is ubiquitously expressed by all immune cells investigated; therefore, glucocorticoids provide a second pathway by which the brain can regulate the activity of the immune system.

In the reverse, cytokines released from immune cells can regulate the nervous system. Cytokines can cross the blood-brain barrier (BBB) and cells of the nervous system possess the receptors to respond to these changes in cytokine levels. IL-1, IL-6, and TNF have all been found to induce fever by the preoptic hypothalamic region involved in thermoregulation, and is just one demonstration of such bi-directional communication[158]. Direct experimental evidence shows that in the absence of infection, LPS or cytokine administration into mice can impart behavioral changes

similar to those seen during infection. These behavioral changes associated with infection and cytokines have been termed sickness behavior and are characterized by anhedonia, lethargy, anorexia, fatigue, depression or irritability, increased pain sensitivity, sleep disturbance, and concentration difficulties[159]. This shared network of signaling molecules and receptors provide the basis of psychoneuroimmunology research and highlight the complex bi-directional communication between the central nervous and immune systems and are illustrated in Figure 6.

Breast Cancer Diagnosis and the Psychological Consequences

Cancer is the unregulated growth of abnormal cells within the body and is the second leading cause of death in the United States[160]. Among women breast cancer is the leading cancer diagnosis in the United States with 122 women per 100,000 diagnosed each year, with roughly 40,000 women dying of breast cancer annually[161]. After diagnosis breast cancer is staged between 0 and VI. These stages categorize the location, size, progression, and dissemination of the disease and are used to determine the appropriate treatment. Ductal carcinoma in situ (DCIS) or Stage 0 cancer is defined as a noninvasive carcinoma with abnormal cells located in the lining of the breast duct with no evidence of spreading to the adjacent stroma. A body of literature has demonstrated that the diagnosis of cancer is accompanied by psychological consequences, which are both short and long-lived[162,163]. A diagnosis of breast cancer is accompanied by an increase in stress perception, anxiety, fear, uncertainty, and mood disturbance[164-169]. These psychological consequences are independent of the

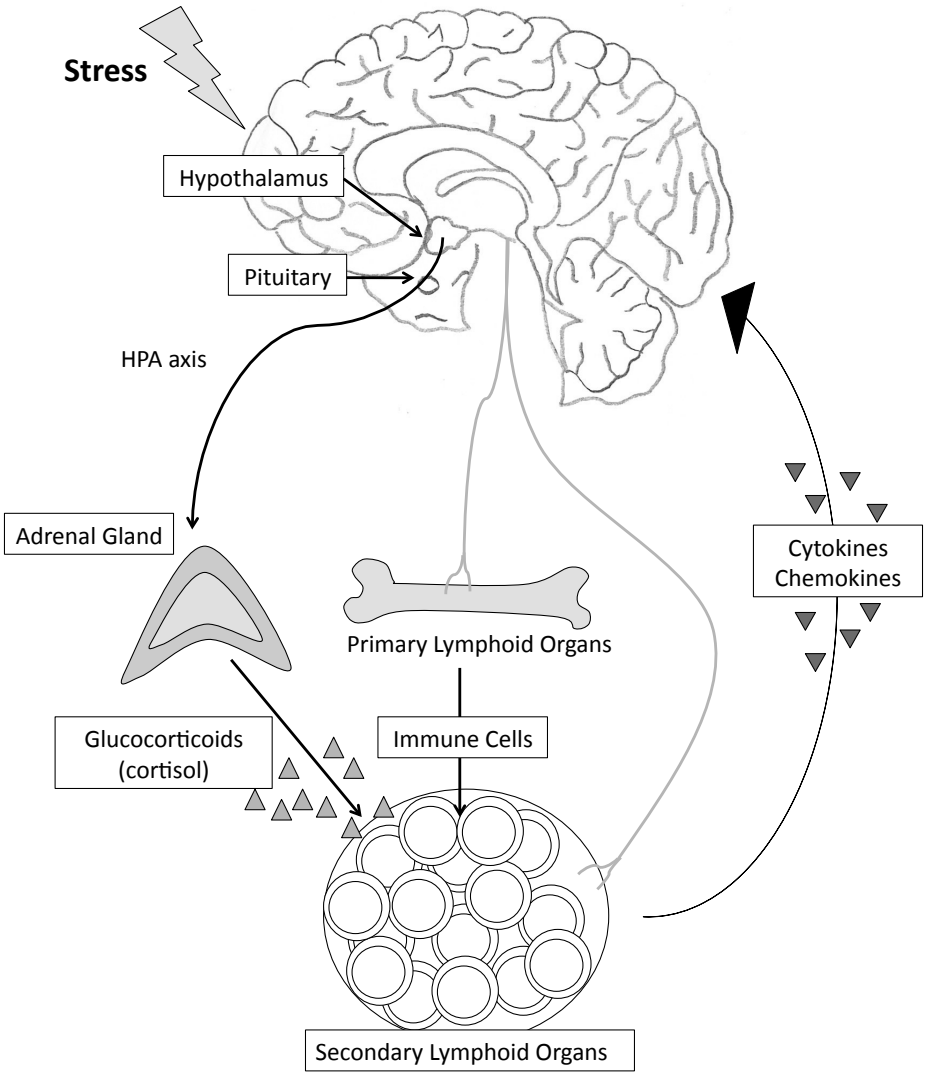


Figure 6. Bi-directional communication between the immune and central nervous systems. The brain perceives external stressors and activates the hypothalamic pituitary adrenal gland (HPA) axis resulting in the production and secretion of glucocorticoids by the adrenal cortex. The main glucocorticoid in human is cortisol. Cortisol diffuses through the circulatory system interacting with and modulating the activity of immune cells. Additionally, the perception of stress activates sympathetic nerves which directly innervate both primary and secondary lymphoid organs providing a direct link between the central nervous and immune systems. In the reverse, cytokines and chemokines secreted from immune cells are released into the circulator system and can pass through the blood brain barrier effecting both behavior and physiology.

disease state as women diagnosed from stage 0 to stage IV all report increases in psychological distress[170]. The individual response to the diagnosis of breast is diverse, in that there are both variations in the intensity and duration of the distress associated with diagnosis[171]. This variation makes understanding both the psychological and physiological outcomes of distress complex. For some individuals the diagnosis of breast cancer elicits an appropriate allostatic response defined as "the active process of responding to a challenge to the body by triggering chemical mediators of adaptation"[172]. This appropriate emotional and biological response allows the body to both adapt to the stressor and return to allostasis. However, if the distress is too great the resulting "wear and tear on the body and brain that results from chronic dysregulation (over activity or inactivity) of mediators of allostasis" leads to increased allostatic load [172]. Psychosocial distress is reported in approximately 80% of women diagnosed with breast cancer during the initial treatment phase[173]. Although some women continue to exhibit negative psychological distress after cancer treatment, ample evidence shows that many women have reductions in distress upon treatment completion[163,174].

Psychoneuroimmunology (PNI) has demonstrated that psychological distress impacts multiple organ and body systems and specifically impairs immune function. Psychological distress can have a number of biological outcomes, most importantly for this study the immune dysregulation observed during stress could adversely affect cancer control[165]. Reciprocal neuro-immune pathways and shared receptor and

signaling systems link the brain and immune system and provide a network whereby one's emotions to impact immune function.

Psychological Distress and the HPA axis

One hallmark feature of all known life forms is their ability to respond and adapt to their environment. Cortisol has effects both physiologically, as previously discussed, and affects aspects of cognitive as well as emotional networks[175,176,177,178]. During periods of stress, the brain initiates the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which produces a cascade of neuroendocrine products resulting in altered circulating adrenal derived glucocorticoids (GC). Modulation of the HPA axis and thus the production and secretion of the glucocorticoid, cortisol, is one mechanism that allows the brain to perceive the environment and respond (either to the benefit or detriment of the organism). Under normal conditions allostasis is maintained resulting in proper immune cell function and homeostasis. If the perception of stress is chronic, disruption of the diurnal cortisol rhythm occurs[162,179], resulting in increased circulating GC[180,181,182]. Many psychological disorders such as depression and post tramatic stress disorder (PTSD) are characterized by alterations in HPA regulation. Additionally, HPA axis activation and increased cortisol is observed in individuals experiencing psychological distress. Increases in psychological distress and depressive mood were associated with both an increase in the cortisol awakening response and activation of the HPA[183,184,185].

More specifically, for the stress attributed to the diagnosis of breast cancer a relationship between increased mood disturbance and increased evening cortisol was found[186]. Other studies have demonstrated that the analysis of total cortisol levels throughout the day, by calculating the area under the curve of the diurnal cortisol rhythm, is decreased in individuals with metastatic breast cancer who report increased perceived stress[179,187,188]. These experiments not only highlight the complex relationship between psychological stress and the HPA axis but implicate its dysregulation in the immune outcomes observed during periods of stress.

Although stress is traditionally associated with increased cortisol secretion and HPA axis activity, there is a growing body of literature finding differential interplay between cortisol rhythms and psychological disturbances. Low cortisol levels, or a hypoactive HPA axis, have been observed in a number of stress related disorders [189,190] such as; posttraumatic stress disorder (PTSD) [191,192], chronic fatigue syndrome[193,194,195], burnout [196,197], and irritable bowel syndrome[198]. Hypocortisolism can be the result of several mechanisms, but a recent meta-review concluded that hypocortisolism commonly develops out of long term chronic stress, where an initial stage of a hyperactive HPA axis eventually evolves into a hypoactive HPA axis [188]. Although mechanisms at different levels of the HPA axis are potentially capable of producing a hypocortisolemic net effect, one of the earliest and most common findings in this development might be an exaggerated negative feedback response of the HPA axis [189].

Psychological Distress, Natural Killer cells, and Inflammation

Psychological stress reduces NKCA, the response of NK cells to cytokines, IFN-g synthesis, antibody responses, and T-lymphocyte responses [199,200,201,202,203]. Our lab and others have shown that the diagnosis of breast cancer results in significant psychosocial distress in women that is accompanied by dysregulated Natural Killer cell activity (NKCA) and cytokine production[202,203,204,205]. Women with breast cancer who report greater distress exhibit lower basal and augmented NKCA and reduced T cell proliferative responses[204,205]. The implications of altered immune function from cancer diagnosis are noteworthy because both NK cells and IFN-g have been shown to contribute to tumor control and destruction[206,207,208,209]. Abnormal cells arise continually throughout life and increase with age. Under normal homeostatic conditions the body controls cancer through immune surveillance. Studies reveal that NK cells can effectively protect against primary solid tumors[210,211]. Autopsy has revealed that even in healthy women, breast cancer cells can be identified in the bone marrow suggesting that the tumor cells are kept in check while the woman is alive [212,213].

The immune dyregulation associated with stress is of particular relevance to breast cancer because epithelial tumors are responsive to the immune protective effects of NK cells and IFN-g[206,207,208,209]. The importance of NK cell activity is highlighted by findings involving the relationships between cancer and NKCA. First, high levels of NKCA in cancer patients correlate with a good prognosis[214,215,216,217]. Impaired NKCA also correlates with invasiveness of human malignancy[218]. NK cell infiltration into primary tumors is associated with

fewer metastases to the lymph nodes and less lymphatic invasion[214]. Lastly, overall survival for patients with high and low NKCA was 71 vs. 30 weeks, respectively and NKCA was an independent prognostic factor for overall survival [215]. These findings illustrate the relevance of NK cell dysregulation during stress as NK cell activity has profound implications for cancer free survival.

Conclusions

The mechanisms for the NK cell dysregulation associated with stress remain incompletely understood. While abundant research focuses on the treatment of the cancer it is clear that treating the stress of cancer diagnosis is of critical relevance. Therefore, determining the mechanism(s) by which distress precipitates negative health outcomes is critical in potential avenues for advancement of treatment cancer free survival. Epigenetic regulation through the glucocorticoid receptor provides one potential mechanism for the regulation of NK cell function in response to their environment in the context of psychological stress.

CHAPTER TWO

MATERIALS AND METHODS

In vitro Analysis: Cell Culture

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*) was 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 (FBS) (Gibco Laboratories, Grand Island, NY), penicillin, 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 IL-2 (100 units/ml). The human erythroleukemic like cell line, K562, was obtained from the American Type Culture Collection, Rockville, MD. K562 cells were maintained in suspension in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100ug/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.1 mM non-essential amino acids and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY)

Cellular Treatment.

NK92s, cultured at $2.5x10^5$ cells/mL, were treated with or without dexamethasone (Sigma Aldrich, St. Louis, MO) (10^{-10} M) for 4 days in the presence of IL-2 (100 units/mL), every 48 hours cultures were collected, washed, and resuspended in fresh media to a concentration of $2.5x10^5$ cells/mL supplemented with or without dexamethasone and IL-2 (100 units/mL). For the final 24 hours of treatment cells were collected, washed, and resuspended to a concentration of $2.5x10^5$ cells/mL with or without dexamethasone in the absence of IL-2. Following treatment NK92s were washed with media and resuspended to 1 x 10^6 cells/ml and used for analysis. In all cases, cell number and viability were determined by exclusion of 0.1% Trypan blue. Viability was maintained between 85 and 95% in all cases. In some experiments cultures described above were subjected to an additional 24hr treatment in the presence of dexamethasone (10^{-7}M) to determine the sensitivity of the cells to further GC stimulation (switch to -7). For RU-486 treatments cells were cultured as described above in the presence or absence of 10^{-10}M RU-486 (Sigma Aldrich, St. Louis, MO) dissolved in EtOH or EtOH alone as vehicle throughout treatments.

Natural killer cell activity (NKCA)

K562 tumor cells were radioactively labeled with 100 uCi of [^{51}Cr] (New England Nuclear, Boston, MA). Radiolabled K562 cells were washed and then incubated for 4 hr

with NK92 cells. Following incubation, the supernatants were removed using a Skatron harvesting press (Skatron Inc., Sterling, VA) and the associated radioactivity was determined. Effector to target ratios for NKCA was 2, 1, 0.5 and 0.25:1.

Results are 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.

Evaluation of cytokine production

Cytokines were measured in NK92 culture supernatants as described previously [117]. To assess stimulated cytokine production, NK92s (2.5X 10^5 cells/ml) were treated as described above and then stimulated with IL-2 (5,000 U) or IL-12 (40 pg/mL) for an additional 4 hours. Cell culture supernatants were harvested and aliquots of the supernatants were stored at -80 ^{0}C for subsequent cytokine determination by ELISA (R&D Systems, Minneapolis, MN).

Table 1. Primers used for gene expression studies

AMPLICON	PRIMERS (5'→ 3')
B-actin	Forward: ATGGGTCAGAAGGATTCCTATGTG Reverse: CTTCATGAGGTAGTCAGTCAGGTC
IFNG	Forward: TGGAAAGAGGAGAGTGACAG Reverse: ATTCATGTCTTCCTTGATGG
IL6	Forward: CAACCTGAACCTTCCAAAGATG Reverse: ACCTCAAACTCCAAAAGACCAG
PRF1	Forward: GGAGTGCCGCTTCTACAG Reverse: CGTAGTTGGAGATAAGCCTGAG
GZMB	Forward: AAGACGACTTCGTGCTGACA Reverse: CCCCAAGGTGACATTTATGG
HDAC1	Forward: GTCCAGATAACATGTCGGAGTACAGC Reverse: CGATGTCCGTCTGCTGCTTATTAAG
HDAC2	Forward: CCTCATAGAATCCGCATGACCCATAAC Reverse: AGACATGTTATCTGGTCTTATTGACCGTAG
HDAC3	Forward: CAAGCCATACCAGGCCTCCCAGC Reverse: GAGATGCGCCTGTGTAACGCGAG
HDAC4	Forward: CCTGCACAGACACGGGGAAGGTG Reverse: GAGCTGCTCTTCAGACAGCAAGC
SIRT1	Forward: TGCGGGAATCCAAAGGATAATTCAGTGTC Reverse: CCTCATCTTTGTCATACTTCATGGCTCTATG
CBP	Forward: TCAGTCAACATCTCCTTCGC Reverse: TGTTGAACATGAGCCAGACG
P/CAF	Forward: AGAACATTGCTTCGCTCGG Reverse: TGCCTCAAGTCCAGAAGAGG
N-COR	Forward: GCTGATGAGGATGTGGATGG Reverse: TTGGACTCTTGGATGTGCC
SMRT	Forward: TGTGGTTCATAAGCCATCTGC Reverse: CGGAATCTTCCCCTCCTCCC
p300	Forward: CATATTTCCTACGCCGGATCC Reverse: AGTATTCCGCTCGATTGTTTGC

Analysis of Gene Expression

Messenger RNA from NK92 cell line was obtained using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) was used to generate cDNA for quantitative real-time PCR from 500ng total RNA. Negative reverse transcriptase samples confirmed absence of contaminating DNA. All reactions were performed in triplicate using SYBRgreen supermix (Biorad). PCR cycles were denaturation at 94°C (30 s) and annealing at 55-61°C (30 s), no extension phase was necessary and performed using the Opticon 2 Real-Time Detector (Bio-Rad, Hercules, CA) and analyzed using the Opticon Monitor Software (Bio-Rad, Hercules, CA). The ΔΔC(t) method was used to determine changes in transcript levels between untreated and chronic glucocorticoid treated cells using beta-actin as a reference gene.

$$\text{Fold Change} = \frac{\text{Efficiency(target)} \wedge (\Delta C(t) \text{ target (control-treatment)})}{\text{Efficiency(reference)} \wedge (\Delta C(t) \text{ reference (control-treatment)})}$$

Immunofluorescent Flow Cytometric Analysis of Intracellular and Surface Proteins.

For surface stains NK92 cells (1.0×10^5/assessment) were incubated with antibodies for 30 minutes on ice (agitated after 15 minutes). Cells were then washed twice with 0.1%

BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) and resuspended in 1% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO). Surface staining antibodies included- anti-CD337 (NKp30) (AlexaFluor 647 conjugated) (BD Biosciences, San Jose, CA), anti-NKp46 (PE conjugated) (BD Biosciences), anti-IL12Rβ2 (PerCP conjugated) (R&D, anti-IL-2Rα/CD25 (Alexa488 conjugated), and anti-CD11a/LFA-1 (FITC conjugated) (BD Biosciences, San Jose, CA). For intracellular protein analysis, cells were washed twice with 0.1%BSA following surface staining, then 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 molecules of interest including anti-granzyme B (Alexa Fluor 647 conjugated) (BD Biosciences, San Jose, CA), anti-perforin (PE-conjugated) (BD Biosciences, San Jose, CA), and anti-glucocorticoid receptor (GR) (unconjugated) (Abcam, Cambridge, MA). 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 intracellular cytokine analysis of IFN-g (PE

conjugated) (BD Biosciences, San Jose, CA) and TNF alpha (PE-conjugated) (BD Biosciences, San Jose, CA), cells were incubated in leukocyte activation cocktail (BD Pharmigen, San Jose, CA) at 37°C for 4 hrs prior to permeabilization and antibody staining. Samples were analyzed with a FACSCanto (Fluorescence-Activated Cell Sorter (FACS) Core Laboratory (FCL) at the Cardinal Bernardin Loyola University Cancer Center) equipped with a 15mW argon-ion laser and a red diode laser using FACSDiva software for data acquisition. After staining, cells were analyzed by flow cytometry. 10,000-30,000 events were recorded and analyzed with FlowJo v8.4.1. Flow cytometric analysis was confirmed by microscopy.

Western Blot

For Western Blot analysis, nuclear and cytoplasmic compartments of NK92 cells were extracted from 1-3 X 10^6 cells via the Fermentas ProteoJET Cytoplasmic and Nuclear Protein Extraction protocol (Fermentas, Burlington, ON). Nuclei were lysed using Nuclear Lysis Buffer (Fermentas, Burlington, ON) both lysed nuclei and cytoplasm were both resuspended in Laemmlis SDS-Sample Buffer (4x) (Boston Bioproducts, Boston, MA). Samples were boiled for 10 minutes and proteins separated by electrophoresis with a 12% agarose gel and transferred to a nitrocellulose membrane for blotting. Proteins were visualized with anti-NFκB p65 (Abcam, Cambridge, MA), anti-cJun (Abcam, Cambridge, MA), anti-STAT4 (Santa Cruz, Santa Cruz, CA), anti-GR (Abcam, Cambridge, MA), anti-HDAC3 (Abcam, Cambridge, MA), and HRP conjugated goat anti-rabbit IgG secondary antibody (Millipore, Temecula, CA) and

chemiluminescence reagent (ThermoScientific, Rockford, IL). Quality of nuclear and cytoplasmic compartment extraction was determined by LamininB and GAPDH, respectively (Abcam, Cambridge, MA). Blot density was quantified using Image J software.

Chromatin Immunoprecipitation (ChIP) Assay.

NK92 cells (5 x 10^6) were cross-linked with 1% formaldehyde for 10 min and terminated by the addition of glycine to a final concentration of 125 mM for 5 min. The cells were washed twice with ice-cold PBS. Crosslinked nuclei were extracted using the Fermentas ProteoJET Cytoplasmic and Nuclear Protein Extraction protocol (Fermentas, Burlington, ON) and lysed with 500 µL of high salt lysis buffer (Santa Cruz, Santa Cruz, CA). The samples were sonicated using a Branson Sonifier 250 on ice for a total of four, 15 second cycles per sample. Sonicated samples were diluted and aliquoted into 100µl volumes. Pre-cleared supernatant with ChIP-Grade ProteinG Magnetic Beads (Cell Signaling, Danvers, MA) was collected and immunoprecipitated overnight at 4°C with the following antibodies: anti-acetyl-H4K8 (Millipore, Temecula, CA), anti-acetyl-H3K9 (Cell Signaling, Danvers, MA), anti-acetyl H3K27 (Abcam, Cambridge, MA), anti-trimethyl H3K4 (Cell Signaling, Danvers, MA), anti-trimethyl H3K9 (Cell Signaling, Danvers, MA), anti-trimethyl H3K27 (Cell Signaling, Danvers, MA), anti-HDAC1 (Abcam, Cambridge, MA), anti-HDAC2 (Abcam, Cambridge, MA), anti-HDAC3 (Abcam, Cambridge, MA), and control rabbit Ig (Cell Signaling, Danvers, MA).

ChIP-Grade ProteinG Magnetic Beads (Cell Signaling, Danvers, MA) were added and incubated an additional 4HR at 4°C. The immune complexes were collected, washed, and eluted. Cross-linking was reversed by heating at 65°C overnight and 10min at 95°C in elution buffer (Santa Cruz, Santa Cruz, CA). DNA was recovered using the MiniElute Reaction Cleanup Kit (Qiagen, Valencia, CA) and resuspended in 50 µl of dH_2O. Quantitative real-time PCR was performed using an Opticon 2 Real-Time Detector; PCR cycles were denaturation at 94°C (30 s) and annealing at 55-61°C (30 s), no extension phase was necessary. The DNA samples were amplified with primer pairs to the -22KB enhancer and proximal promoter of the *IFNG* locus, the proximal promoters of *PRF1*, *GZMB*, *TNF*, and *IL6*. PCR data was analyzed using Opticon Monitor 3 Software (BioRad, Hercules, CA). Modification levels were calculated as percent input using the equation.

Percent Input = 100 x 2^(C(t)untreated – C(t)treated cells).

Chromatin Accessibility

The EpiQ Chromatin Analysis Kit (BioRad, Hercules, CA) was used to determine the accessibility of the *IFNG* enhancer, *IL6* proximal promoter, *TNF* proximal promoter, *GZMB* proximal promoter, *GAPDH* gene, and *RHO* gene. The methodology supplied in the kit was as directed for cells in suspension. Briefly, 250,000 NK92 cells were collected from treatments and resuspended in the supplied EpiQ chromatin buffer and either left undigested or nuclease digested. DNA was then isolated and quantification of accessibility as a measure of intact genomic regions was performed by q-RT-PCR. The

EpiQ Chromatin Analysis Kit Data Analysis Tool was used to determine percent accessibility. The control primers for the genomic regions *RHO* and *GAPDH* were used as directed and used as 0% and >95% respectively.

AMPLICON	PRIMERS (5' → 3')
IFNG promoter	Forward: TCATCGTCAAAGGACCCAAGGAGT Reverse: ATGGTGACAGATAGGCAGGGATGA
IFNG enhancer	Forward: GAATTGGCTTGACACCTCTGTCCT Reverse: TTCCATCTCTCGGCAAAGAGCAGT
IL6 promoter	Forward: CAGAAGAACTCAGATGACTGG Reverse: GCTGGGCTCCTGGAGGGG
TNF promoter	Forward: CGCTTCCTCCAGATGAGCTC Reverse: TGCTCTCCTTGCTGAGGGA
PFR1 promoter	Forward: GGCACAGTTCCAAGCACTTCACAA Reverse: AGCCTCACTGTGCCTCAGTTTCTT
GZMB promoter	Forward: AGCCTGTTGCCTCTGTGAGAAAGT Reverse: TGGGATTTGCTGGCAACCTAGACA

Table 2. Primers used for ChIP and Chromatin Accessibility Studies

Immunofluorescent Microscopy

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*. Fluorescent images were visualized (500-3000 events per condition) on Amnis Imagestream. A mask on the nucleus was created; within this area, colocalization of HDACs and nuclear dye (DAPI) was measured by similarity (IDEAS software, Amnis).

Co-immunoprecipitation experiments

To determine protein-protein interactions the DynaBeads Co-Immunoprecipitation Kit (Invitrogen, Grand Island, NY) using anti-HDAC3 (unconjugated) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit IgG (Cell Signaling, Danvers, MA). Immunoprecipitates were collected via magnetic separation and analyzed by western blot analysis using the procedures described above in Western blot analysis section.

Ex vivo analysis of human PBMC

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 are treated with systemic chemotherapy, have recurrent breast cancer or other cancers (within the last 5 years), immune-based disease (e.g. multiple sclerosis, HIV), psychoses, cognitive dysfunction; are 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 respond to and recover from breast cancer diagnosis at four time points (See Figure 19). Data were collected at the 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[214,219,220,221,222]. 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 or approximately four months after diagnosis and the fourth time point (T4) was six months after radiation treatment completion or approximately 8 months after diagnosis. PBMCs were isolated from blood specimens obtained from these women by a ficol histopaque gradient density separation as described previously[223].

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[224]. Reliability (stability) was 0.85 and Cronbach alphas range from 0.75-0.86[224]. The PSS is widely used in studies of stress on immunity, including studies of the stress-immune response of women with breast cancer[203,205]. 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[205]. CES-D provided a measure of depressive symptoms. CES-D assesses frequency and duration of depressive symptoms[225]. Scores range from 0-60 and $\geq$16 suggests depression risk.

CES-D is widely used in studies of women with breast cancer[226,227]. It has good construct validity, good test-retest reliability [225]. In cancer patients and healthy controls CES-D showed internal consistency (alpha=0.87 and alpha=0.89), respectively[228].

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[229,230,231,232], 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[233]. Centrifuged samples were frozen (-20^0C) 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 is 3.75-6.41%. The minimal detectable cortisol concentration < 0.003 μg/dL. The diurnal cortisol rhythms generated a curve consisting of 4 trapezoids. The area under the curve was calculated by summing the area of these 4 trapezoids using the equation.

$$\text{Area}_n = (x_n - x_{n-1}) \times \frac{(y_n + y_{n+1})}{2}$$

$$\text{Area Under the Curve} = (\text{Area}_1 + \text{Area}_2 + \text{Area}_3 + \text{Area}_4)$$

Where x_1, x_2, x_3, x_4, and x_5 represent awakening, 30min post awakening, noon, 5pm, and bedtime, respectively. Additionally y_1, y_2, y_3, y_4, and y_5 representing cortisol levels at these timepoints.

Statistical methods

Data are expressed as means with the standard error of the mean (SEM) or standard deviation (SD) as noted. Main study variables were analyzed using Student's t test, a two-sided alpha of 0.05 was set for significance. The Statistical Package for Social Sciences (SPSS: version 13.0) was used for data analysis. Pearson correlations and regression analysis

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.

Wards Method of Classification

The Statistical Package for Social Sciences (SPSS: version 13.0) was used to generated standardized scores for variables used for classification. Clusters were generated by using the standardized scores to determine hierarchical clustering based on Euclidean mean squared distance. This is a statistical method that merges attributes into clusters based on the residual error within the differences of the instance attributes from those of another group. This method minimizes the variance of the differences in attributes within a cluster using the distance algorithm based on the sum of squares of the difference of the attributes. It joins cluster pairs whose merger minimizes the increase in the total sum of squares within-group error. The resulting groups were used for analysis and are statistically graphed using dendograms.

Cytokine Production (ELISA):

NK92 (2.5×10^5 cells/ml) cells were incubated in 24 well plates for 24 hours at 37 ^{0}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-g) <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. 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 4 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 aliqoted 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[223,234,235]. 10,000-30,000 events were recorded and analyzed with FlowJo v8.4.1.

CHAPTER THREE

EXPERIMENTAL RESULTS

Development of an *in vitro* system to investigate the effects of chronic glucocorticoid exposure on Natural Killer cell function

Previous studies have demonstrated that 24 hour incubation of human derived CD56+ cells as well as the NK cell lines; NK3.3, YT, and NK92, with dexamethasone or cortisol reduces NK cell lytic activity [117,118,119]. Furthermore, we have published that 24 hour incubation of the NK cell line, NK92, with dexamethasone resulted in a dose dependent inhibition of NK cell lytic ability [117,118]. Results from all studies concluded that this is in part mediated through a reduction in lytic molecule transcription, both perforin[117,119] and/or granzymes [119]. Furthermore, 24 hour treatment of both *ex vivo* cultured human derived CD56+ NK cells or NK cell lines treated with high concentrations of dexamethasone or cortisol resulted in decreased proinflammatory cytokine production[117,118,236,237]. Together these studies demonstrated that short term (24-hour) high concentration exposure to glucocorticoids is both immunosuppressive and anti-inflammatory in nature. However, these studies did not investigate the effects of longer duration glucocorticoid exposure on NK cells. Therefore, we developed a culture system to investigate the effects of chronic exposure to glucocorticoids, termed chronic GC treatment, for a total of 5 days and performed time course analysis, characterizing cytokine production and lytic activity of the NK92 cell

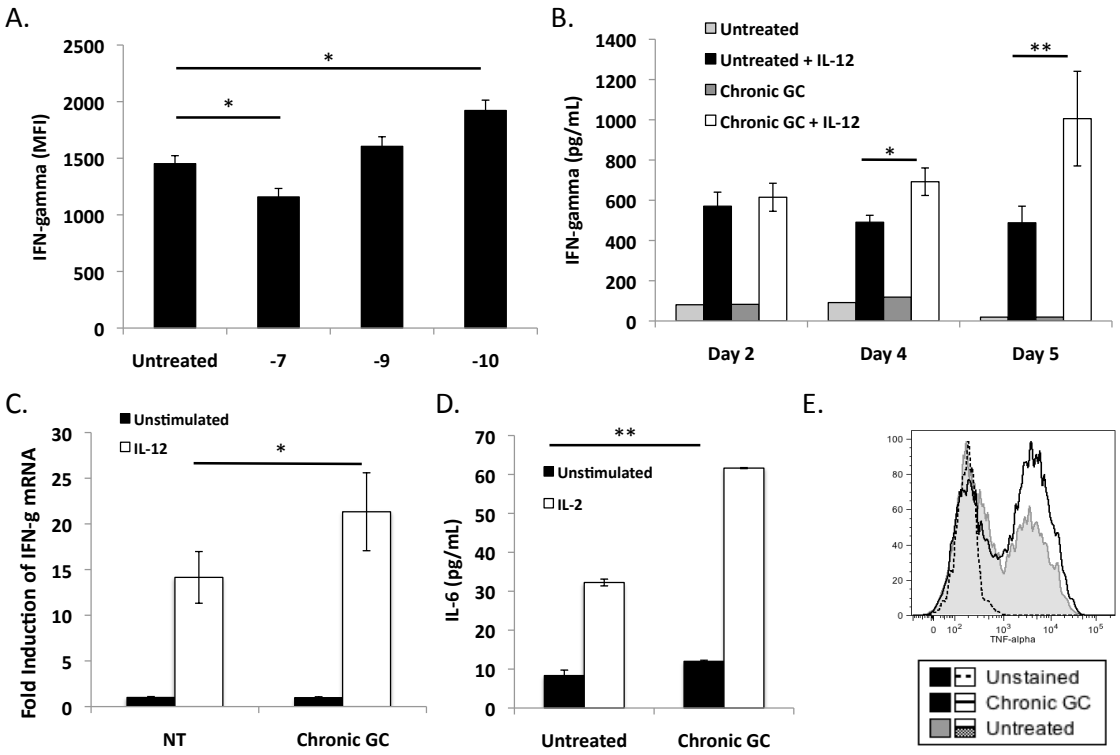


Figure 7. The effect of chronic glucocorticoid (GC) treatment on pro-inflammatory cytokine production by NK92. (**A**) MFI of IFN-g production as measured flow cytometry in untreated or chronic GC treated NK92 cells with a range of dexamethasone from 10^{-7} to 10^{-10} M .(**B**) IFN-g production as measured by ELISA from NK92 cells either untreated or treated with GC and harvested at various times and then either stimulated or unstimulated with IL-12 for 4 hours, n=3. (**C**) IFN-g transcript levels for IL-12 stimulated and unstimulated cultures with and without chronic (5 day) GC exposure. IFN-g transcript levels from unstimulated, untreated cells were set to 1. (**D**) IL-6 production as measured by ELISA from NK92 cells harvested from 5 day cultures either unstimulated or stimulated with IL-2 for 4 hours, n=3. *; $p<0.05$, **; $p<0.01$.

line. Treatment of NK92 cells for five days demonstrated that high concentrations of dexamethasone, both 10^{-7}M or 10^{-8}M, reduced IFN-g production as judged by a decrease in the mean fluorescence intensity (MFI) of IFN-g staining by flow cytometry (Figure 7A). In contrast, there was a trend for increased IFN-g production at dexamethasone (10^{-9}M) and a significant increase in IFN-g MFI was observed after 5 day treatment with 10^{-10}M dexamethasone (Figure 7A). Intracellular cytokine staining demonstrated that there was a 32±3.8% increase in intracellular IFN-g mean fluorescence intensity (MFI) in 5 day chronic GC (10^{-10}M) treated NK92 cells compared to untreated cells (Table 3). The shift in MFI was accompanied by a modest increase in IFNg positive cells (90% vs 85%) (Table 3) indicated that NK92s in culture shift as a population towards increased cytokine production. This apparent pro-inflammatory effect of GC on NK cells when exposed for extended time prompted further analysis. Therefore, for this body of work the effects of chronic 5 day GC treatment (10^{-10}M dexamethasone) were analyzed, termed chronic GC treatment.

NK cells were harvested at indicated days of treatment, stimulated with IL-12, and the production of interferon gamma (IFN-g) was analyzed in the culture supernatants by ELISA. Chronic GC treatment had no impact on basal IFN-g (Untreated vs. Chronic GC) production. However, when stimulated with IL-12, chronic GC treated cells exhibited increased IFN-g production (Untreated + IL-12 vs. Chronic GC + IL-12) reaching a 2-fold increase at day 5 (Figure 7B). This was not an IFNg specific phenomenon, as IL-2 stimulated IL-6 production increased from 32.2±1.8pg/mL to 61.6±2.4pg/mL after day 5 of chronic GC treatment (Figure 7D). Furthermore, flow cytometry demonstrated an increase in TNF-alpha MFI after chronic GC treatment

Table 3. Effect of chronic GC treatment on proinflammatory cytokine production and granule constituent levels in NK92 by flow cytometry

Values are mean fluorescent intensity (MFI) of intracellular proteins ± S.E.M., n= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI Untreated - MFI Chronic GC treatment)/(MFI Untreated) X 100. Statistically significant difference, Untreated vs. Chronic GC Treatment.

Mean Fluorescent Intensity (MFI) and/or Percentages of Cytokine and Intracellular Protein Positive Cells				
Cellular Protein	Untreated	Chronic GC Treatment (5 day)	P value	(%) Change
IFN-gamma (MFI)	1452 ± 71	1922 ± 91	0.0005	32.4 ± 3.8
IFN-gamma positive (%)	85.0 ± 1.0	90.0 ± 1.7	0.001	5.7 ± 0.37
Perforin (MFI)	7421 ± 192	6371 ± 138	0.017	-14.1 ± 1.86
Perforin Positive (%)	95.3 ± 0.29	94.3 ± 1.36	0.309	-0.98 ± 1.42
Granzyme B (MFI)	11009 ± 150	10115 ± 188	0.017	-8.13 ± 1.71
Granzyme B Positive (%)	98.2 ± 0.40	97.8 ± 0.31	0.318	-0.34 ± 0.31

(Figure 7E). IL-6 protein levels could not be analyzed by intracellular cytokine staining due to low intracellular accumulation of protein levels. Additionally, no mature active TNF-alpha was detected in culture supernatants, most likely due to a lack of TNF-alpha converting enzyme (TACE) expression. mRNA isolated and quantified from NK92 cells after 5 days of chronic GC treatment exhibited a 23- fold induction of IFN-g mRNA after IL-12 stimulation while untreated cultures exhibited only a 14-fold induction (Figure 7C). These results taken together demonstrated that chronic GC treatment primes NK cells to produce proinflammatory cytokines via enhanced transcription of cytokine genes after cellular stimulation.

Effect of chronic GC treatment on natural killer cell activity (NKCA)

NK cells not only produce proinflammatory cytokines but also have direct cytolytic activity. NK cells were harvested at indicated days during chronic GC treatment and NKCA assessed via chromium release. NK92 cells exhibited a time dependent decrease in NKCA with a decrease at day 4 (18%), and a significant decrease at day 5 (25%)(Figure 8A). Human NKCA is primarily mediated via granule dependent lysis of tumor cells; therefore, the mRNA levels of two key NK cell granule constituents, perforin and granzyme B, were assessed. mRNA expression and levels for both perforin (*PRF1*) and granzyme B (*GZMB*) were reduced throughout chronic GC treatment and observed as early as day 2 of treatment (Figure 8B). At day 5 of chronic GC treatment *PRF1* transcripts were reduced by 60% and *GZMB* transcripts were reduced 40% compared to untreated cells (Figure 8B). Flow cytometry confirmed reductions in both

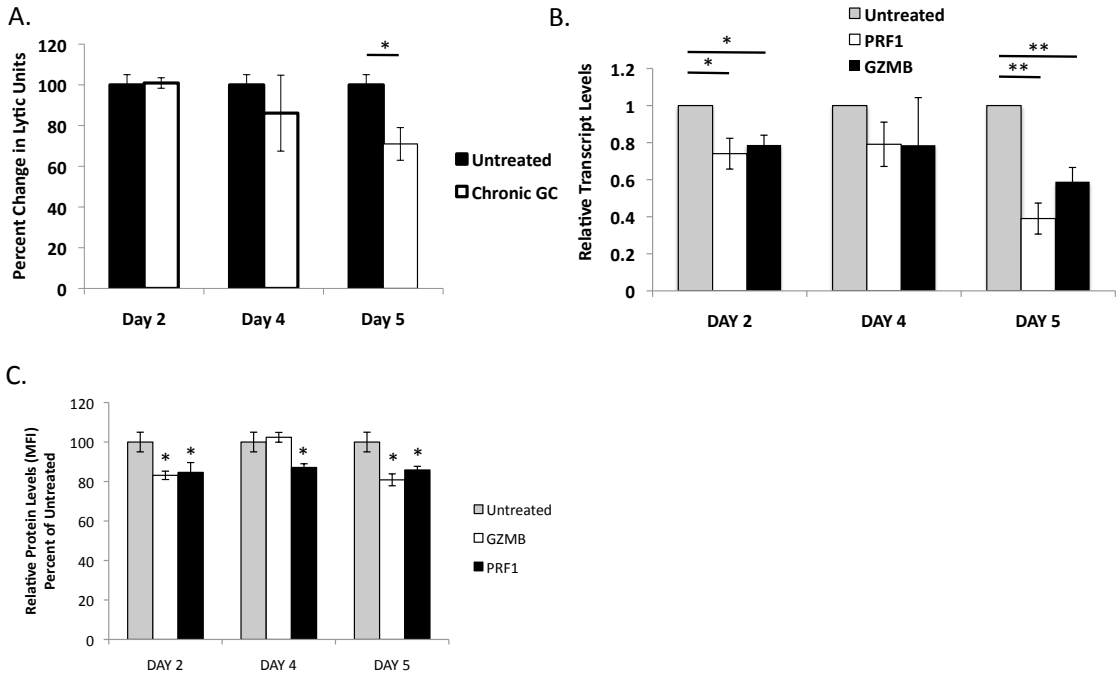


Figure 8. Effect of chronic glucocorticoid treatment on NKCA and lytic molecule production. (**A**) NKCA presented as percent change in lytic units ± SEM of NK92 harvested at indicated days of treatment for target K562 cells, n=3. (**B**) Relative transcript levels for two granule constituents, perforin (PRF1) and granzymeB (GZMB), in NK92 cells harvested at indicated days during treatment and standardized to transcript levels in untreated (grey bars) cells set to 1. (**C**) Flow cytometric analysis of granule constituent protein levels after 5 day chronic GC treatment. Graphs represent mean fluorescent intensity of PRF1 and GZMB levels compared to untreated cells set to 100%. *; $p<0.05$, **; $p<0.01$.

Table 4. Effects of chronic GC treatment on surface receptor expression by NK92 cells

Mean Fluorescent Intensity (MFI) and/or Percentages of cells expressing surface Proteins				
Cellular Protein	Untreated	Chronic GC Treatment (5 day)	P value	% Change
IL-2Ralpha	3854 ± 98	3806 ± 125	0.56	-1.26 ± 1.62
IL-12Rbeta2	722 ± 32	776 ± 16	0.17	7.5 ± 4.3
LFA-1	4861 ± 97	4745 ± 11	0.28	-2.4 ± 0.22
NKp30	784 ± 7.2	821 ± 6.1	0.008	4.7 ± 0.77
NKp46	176 ± 4.3	177 ± 3.0	0.75	1.0 ± 1.67
CD107a	10765 ± 443	10416 ± 257	0.28	-3.23 ± 2.39
CD107a (%)	11.7 ± 0.16	12.9 ± 0.59	0.23	4.0 ± 5.0

Values are mean fluorescent intensity (MFI) of surface proteins ± S.E.M., n= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI Untreated - MFI Chronic GC treatment)/(MFI Untreated) X 100. Statistically significant difference, Untreated vs. Chronic GC Treatment.

perforin and granzyme B protein levels after 5 days of chronic GC treatment (Figure 8C and Table 3). These data demonstrate chronic GC treatment to reduce perforin and granzyme B levels with concomitant reductions in NKCA. Furthermore, the percentages of perforin or granzyme B positive cells were unchanged after chronic GC treatment (Table 3). NKCA is also regulated by the expression of activating and inhibitory receptors on NK cells as well as the ability of NK cells to form stable conjugates with target cells and exocytose granules. I found no significant difference in the ability of NK92 cells after chronic GC treatment to form stable conjugates with target tumor cells using a flow cytometric assay presented as % conjugate formation (data not shown). Furthermore, chronic GC treatment had no impact on the ability of NK92 cells to exocytose granules as measured by CD107a surface expression after target cell contact (Table 4). The surface expression of NKp46 were unaffected by chronic GC treatment (Table 4). We did observe a small but significant increase in NKp30 expression; however, this increase in activating receptor expression could not explain the significant decrease in NKCA (Table 4). Hence, chronic GC treatment shifts NK cell effector function to a less cytolytic and more proinflammatory NK cell phenotype through the dichotomous transcriptional regulation of the respective effector genes.

Effect of chronic GC treatment on NK cell signaling pathways

The transcriptional changes described above prompted investigation into the effect of GC on signaling pathways that regulate NK cell effector function. IFN-g and

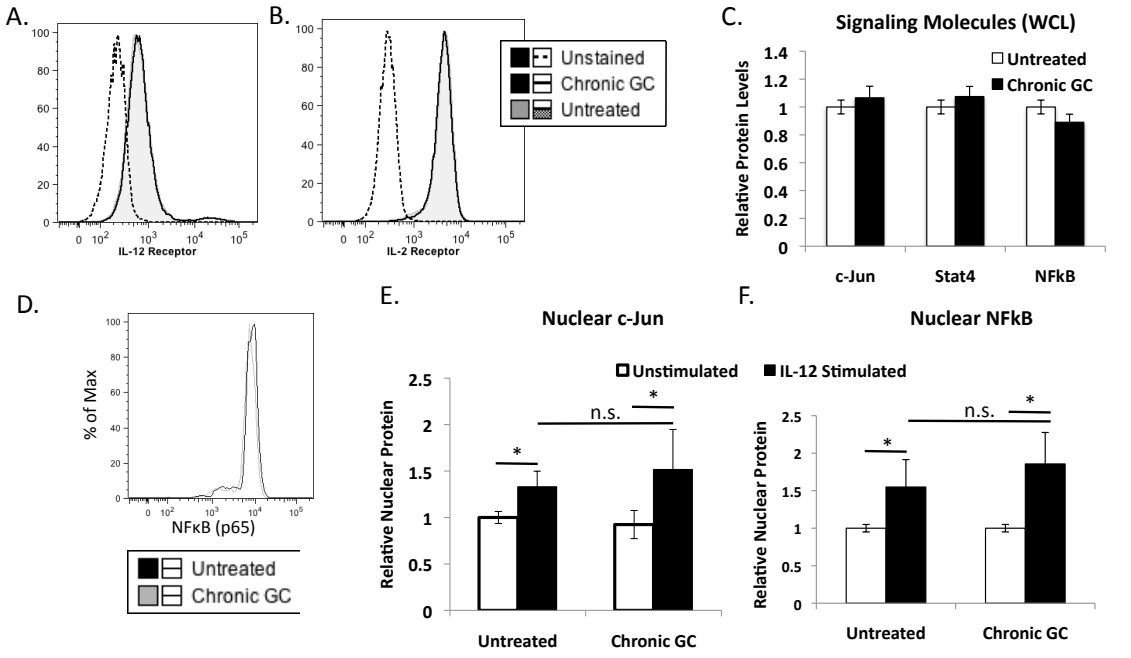


Figure 9. Effects of chronic GC treatment on proinflammatory cytokine signaling pathways. Flow cytometric analysis of (**A**) IL-12 and (**B**) IL-2 receptor surface expression for chronic GC treatment (5 days) or untreated cells. (**C**) ImageJ quantification of whole cell lysates probed by western blot analysis for c-Jun, Stat4, and NFkB levels in chronic GC treated and untreated cells. (**D**) Flow cytometric analysis of NFkB in 5 day chronic GC and untreated cells. ImageJ quantification of nuclear (**E**) c-Jun and (**F**) NFkBp65 in chronic GC and untreated cells prior to and after IL-12 stimulation.

IL-6 mRNA and protein were increased after cellular stimulation with IL-12 and IL-2, respectively (Figure 7A-D). At the cell surface, chronic GC treatment had no impact on the levels of the IL-12 receptor beta chain or the IL-2 receptor alpha chain, implying that chronic GC did not alter receptor density of NK92 cells for the cytokines used for stimulation. (Figure 9A-B). Downstream of receptor signaling is the activation and subsequent nuclear localization of the canonical proinflammatory signaling transcription factors NFκBp65 and AP-1 (c-Jun) as well as STAT4, the signaling molecule directly downstream of the IL-12 receptor. Western blot analysis and ImageJ quantification demonstrated 5 days of chronic GC treatment had no impact on the total cellular levels of these three transcription factors (Figure 9C). Flow cytometry confirmed that 5 day chronic GC treatment did not alter the cellular levels of NFκBp65 (Figure 9D). Western blot analysis and quantification of nuclear fractions isolated from 5 day chronic GC treated and untreated cells before and after IL-12 stimulation revealed that IL-12 stimulation significantly increased the nuclear localization of NFκBp65 and AP-1 (c-Jun) in both untreated and 5 day chronic GC treated cells (Figure 9E-F). However, no significant difference was observed in the nuclear translocation of either cJun or NFκBp65 between untreated and chronic GC treated cells stimulated with IL-12 (Figure 9E-F). These data demonstrate that NK cells exposed to chronic GC are able to sense and respond to cellular activation to the same degree as untreated cells.

Chronic GC induced histone modifications at proinflammatory regulatory regions

Post translational epigenetic modifications at genomic regulatory regions can affect both active transcriptional rates as well as priming genes for future transcription. GR have been shown to interact with histone modifying enzymes and to influence histone tail post-translational modifications at genomic regulatory regions [117,146,238]. To assess the effect of chronic GC treatment on histone tail modifications at the regulatory regions of NK cell effector genes, chromatin immunoprecipitation (ChIP) followed by qRT-PCR was performed. Enhanced production after 4 hours of IL-12 stimulation was observed after 5 but not 2 days of chronic GC treatment (Figure 7B); therefore, a comparison of the epigenetic patterns at cytokine regulatory regions (the promoter and -22KB enhancer region of the *IFNG* locus, and the *IL6* promoter) was performed. The levels of 6 histone modifications, referred to as the epigenetic pattern, in a particular region for untreated cells in comparison to chronic GC treated cells is presented in Figure 10. ChIP analysis demonstrated that in untreated cells all three regions exhibited moderate H4K8Ac, H3K27Ac, and H3K4me3; low but detectable H3K9Ac and H3K27me3; and undetectable/below background H3K9me3 levels. This epigenetic pattern is indicative of a poised promoter region defined as a gene able to be expressed in a specific cell lineage (undetectable H3K9me3 and H3K27me3) and poised for future transcription, moderate H3K9Ac and H3K27Ac [239]. The *IFNG* enhancer, which is critical for *IFNG* transcription[70,72], exhibited a significant increase in H3K4-trimethylation (me3) at day 2 of GC treatment (grey bars) (Figure 10A). However, at day 5 of chronic GC treatment (dark grey bars) the *IFNG* enhancer had similar levels of H3K4me3 as untreated but did exhibit increased H4K8Ac as well as a time dependent increase in H3K27Ac (Figure 10A). This pattern of acetylation for H4K8 and H3K27

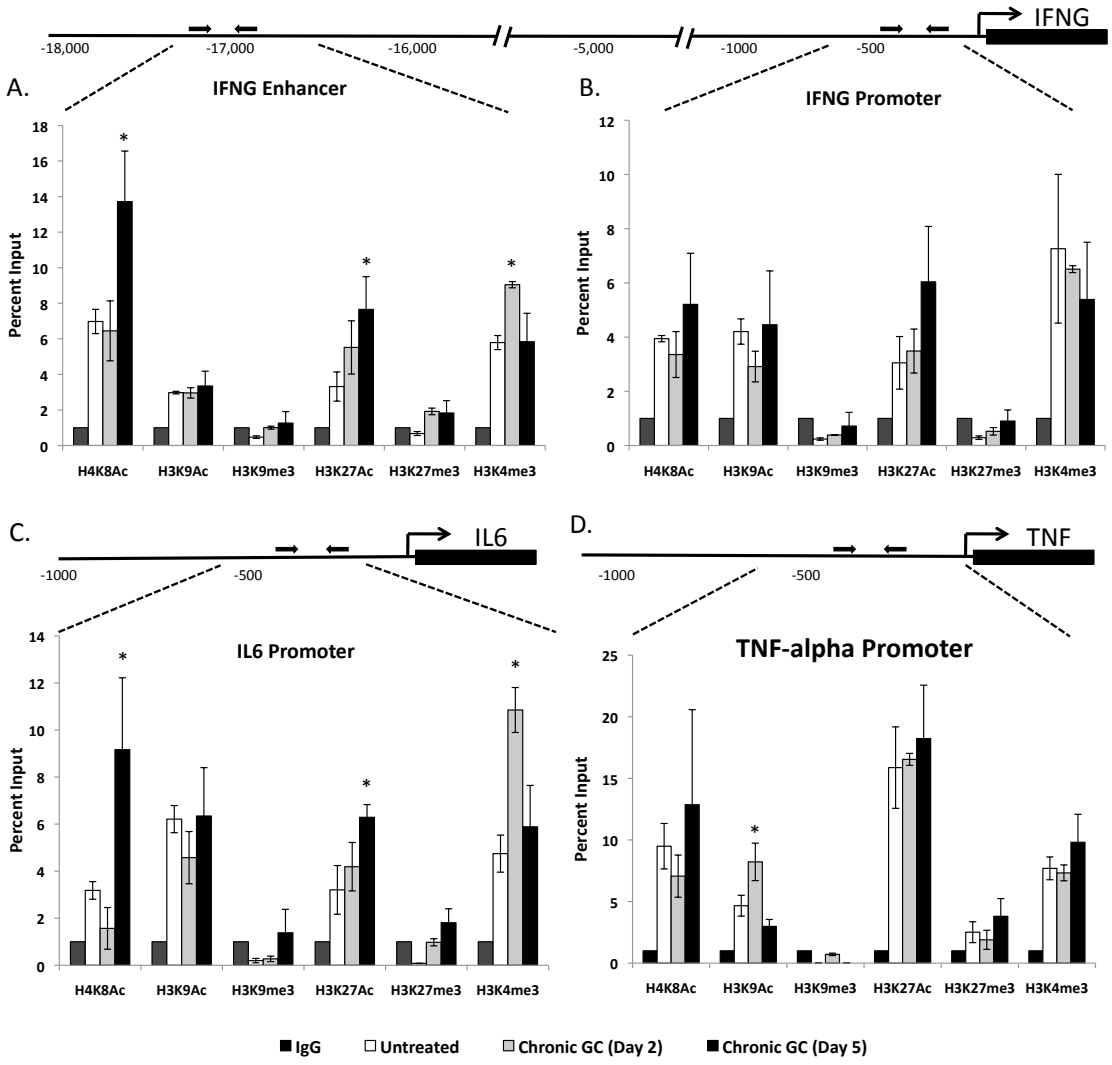


Figure 10. Histone modifications at NK proinflammatory regulatory regions. Schematic diagrams and percent input calculations of posttranslational histone modifications at the (**A**) *IFNG* enhancer, (**B**) *IFNG* promoter, and (**C**) the *IL6* promoter after day 2 (grey bars) and day 5 (dark grey bars) of chronic glucocorticoid treatment or in untreated cells (white bars) compared to the IgG (black bars) negative control immunoprecipitations. Untreated values are the average of the levels of each modification on both day 2 and day 5 from untreated cultures as no difference in the levels over time was observed. n=6 ± SEM *; $p<0.05$.

corresponded to the enhanced IFN-g production and secretion following chronic GC treatment and IL-12 stimulation (Figure 7A-C). No GC treatment effects were observed at the proximal promoter of *IFNG* (Figure 10B) or the TNF-alpha promoter (Figure 10D). The *IL6* promoter exhibited a similar chronic GC dependent change in histone modification as the IFNG enhancer; increased H3K4me3 at day 2 of GC treatment (gray bars) and after 5 days of treatment (dark gray bars) H4K8Ac was significantly increased as well as a time dependent increase in H3K27Ac (Figure 10C). These changes corresponded with the two fold increase in IL-6 production following chronic GC treatment and IL-2 stimulation (Figure 7D). These data indicate that proinflammatory gene regulatory regions become hyperacetylated following chronic GC treatment and that acetylation of histone proteins contributes to the enhanced proinflammatory cytokine production following cellular activation.

Chronic GC induced histone modifications at lytic molecule regulatory regions

The regulatory regions of the granule constituents exhibited a different epigenetic pattern compared to the cytokine loci in untreated NK92 cells, demonstrating high H4K8Ac, H3K9Ac, H3K27Ac, and H3K4me3 while H3K9me3 and H3K27me3 were at or below background (IgG) levels (Figure 11A-B). This pattern is indicative of genes undergoing active transcription (high H3K9Ac and H3K27Ac) with no heterochromatin marks (H3K9me3 and H3K27me3) [131,138,239]. The perforin (*PRF1*) promoter after 5 days of chronic GC treatment (dark grey bars) exhibited

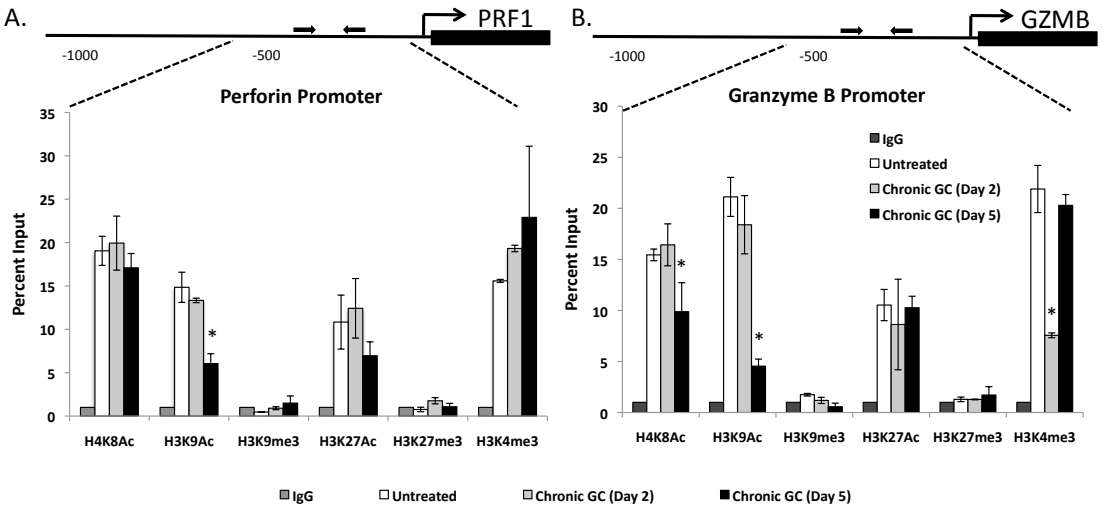


Figure 11. Histone modifications present at NK cell granule constituent regulatory regions. Schematic diagrams and percent input calculations of posttranslational histone modifications at the (**A**) proximal promoter of the PRF1 gene and the (**B**) proximal promoter of the GZMB gene after day 2 (grey bars) and day 5 (dark grey bars) chronic glucocorticoid treatment or untreated (white bars) cultures compared to the IgG (black bars) negative control immunoprecipitation. Untreated values are the average of the levels of each modification on both day 2 and day 5 from untreated cultures as no difference in the levels over time was observed. Data represents the average of three independent experiments performed in duplicate n=6 ± SEM *; $p<0.05$.

significantly reduced H3K9Ac, marking this region for reduced transcription corresponding with the reduction in perforin transcripts shown previously (Figure 11B and 8B). A trend for reduced H3K9Ac was observed at day 2 of GC treatment and was significant at day 5 of treatment, which is consistent with the gradual time dependent reduction in NKCA (Figure 11A and 8A). The proximal promoter of the granzymeB (*GZMB*) gene exhibited a similar pattern as the *PRF1* promoter for untreated cells, and including a significant reduction in H3K9Ac at day 5. Additionally, the *GZMB* promoter demonstrated a decrease in H4K8Ac (Figure 11B). Furthermore, H3K4me3 was reduced at day 2 but returned to untreated levels at day 5 of chronic GC treatment. The decrease in the active transcriptional mark H3K9Ac corresponded with the reduction in granzyme B mRNA after chronic GC treatment (Figure 8B). These data together suggest that chronic GC decreases NKCA at least in part through epigenetic modifications that result in the transcriptional repression of these two granule constituents.

Chronic GC induce changes in chromatin accessibility

Hyperacetylation has been shown to increase the accessibility of regulatory regions and to enhance the transcription of genes upon cellular activation. Chromatin from 5 day chronic GC treated and untreated cells was subjected to nuclease digestion or left undigested and qRT-PCR was used to quantify the degree to which each regulatory region was resistant to digestion compared to *GAPDH*, a constitutively open and

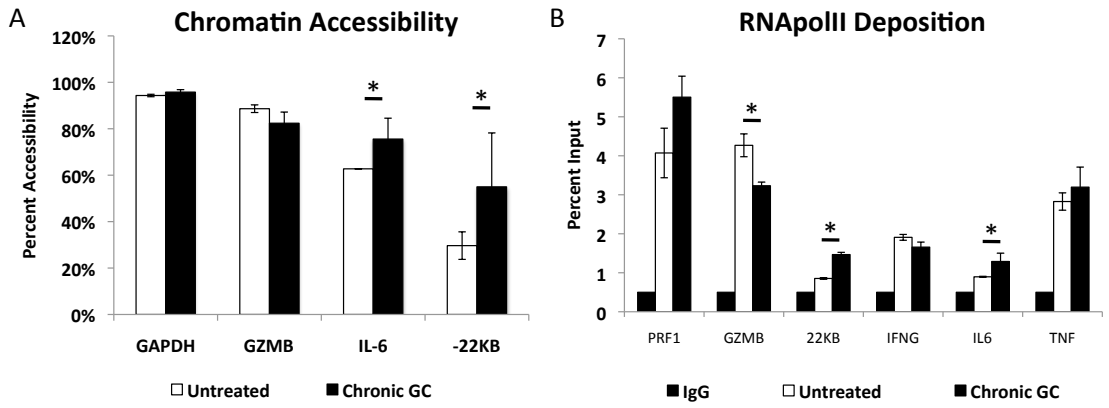


Figure 12. Effect of chronic GC treatment on chromatin accessibility and RNApol II deposition. (**A**) Percent change in sensitivity of indicated genomic regions to nuclease digestion as determined by the Bio-Rad EpiQ chromatin accessibility kit. Accessibility is calculated as the ability of nuclease to digest indicated regions setting GAPDH accessibility as 100% in untreated (white bars) and 5 day chronic GC treated cells (black bars). **(B)** Percent input calculation for RNApol II deposition at NK cell effector genes after day 5 (dark grey bars) chronic GC treatment or untreated (white bars) cultures compared to the IgG (black bars) negative control immunoprecipitation. Untreated values are the average of the levels of each modification on both day 2 and day 5 from untreated cultures as no difference in the levels over time was observed. Data represent the average of three independent experiments performed in duplicate n=3 ± SEM *; $p<0.05$.

transcribed gene. Chronic GC treatment increased the accessibility of the *IL6* promoter by 18% and increased the accessibility of the *IFNG* enhancer by 37% (Figure 12A). As no increase in transcription was observed prior to activation (Figure 7C) it is likely that this increased accessibility is the result of the hyperacetylation of proinflammatory cytokine regulatory regions (Figure 10A and C) which primes NK cells for enhanced cytokine production when stimulated. Analysis of the *GZMB* promoter yielded no significant difference in accessibility (Figure 12A). Although *GZMB* transcripts were diminished and the *GZMB* promoter exhibited decreased histone acetylation, chronic GC treatment did not appear to alter the chromatin structure, suggesting that granule constituent regulatory regions are affected by GC in a manner different from cytokine regulatory regions.

The changes in histone acetylation and chromatin accessibility, as well as the enhanced transcription of cytokine regulatory regions upon cellular stimulation prompted investigation into the depostition of RNApol II at these loci. ChIP demonstrated a quantitative increase in RNApol II deposition at the *IL6* promoter and *IFNG* enhancer after chronic GC treatment compared to untreated cells (Figure 12B). No changes were observed in cytokine loci (i.e. IFN-g and TNF-alpha promoters) that did not exhibit changes in histone acetylation upon chronic GC treatment. A significant decrease in RNApol II deposition was observed at the *GZMB* promoter which is in accordance with the significant decrease in transcripts following chronic GC treatment. Lastly, a significant increase in RNApol II deposition was observed at the *PRF1* promoter in chronic GC treated cells. The increased deposition of RNApol II at the cytokine regions without any increase in basal cytokine production implys that increase

represents stalled RNApol II at these loci. Again the granule constituents seem to be regulated in a different manner than the cytokine loci after chronic GC treatment.

Chronic GC induced effects on histone deacetylases (HDACs)

Histone acetylation is mediated by two classes of enzymes, histone acetyltransferases and histone deacetylases (HDACs). Modulation of these enzymes impacts gene transcription and cellular function [140,240,241]. GR can interact with members of the class I HDAC family[118,145], HDAC2[146] and HDAC3[147]. Therefore, the presence of HDACs 1, 2, and 3 at NK cell effector genes was assessed by ChIP. Both the *IL6* promoter and the *IFNG* locus had detectable levels of all 3 HDACs in untreated (white bars) cells (Figure 13A and B). At both day 2 and day 5 of chronic GC treatment the level of resident HDAC3 was significantly decreased at the IFNG enhancer and the *IL6* promoter (Figure 13A and B). Additionally, the level of HDAC1 at both the *IFNG* enhancer as well as the IL6 promoter was significantly decreased after 5 days (black bars) of chronic GC treatment compared to untreated cells (Figure 13A and B). Thus chronic GC treatment resulted in significant reductions in both HDAC1 and HDAC3 at the *IFNG* enhancer and *IL6* promoter corresponding to the increase in histone acetylation and accessibility previously observed (Figure 10A and C). These data imply that the hyperacetylation and increased chromatin accessibility observed at proinflammatory cytokine regulatory regions following chronic GC treatment is at least in part due to reductions in resident HDACs at these loci. The *PRF1* and *GZMB* promoter regions had extremely low but detectable levels of HDAC1 and moderate

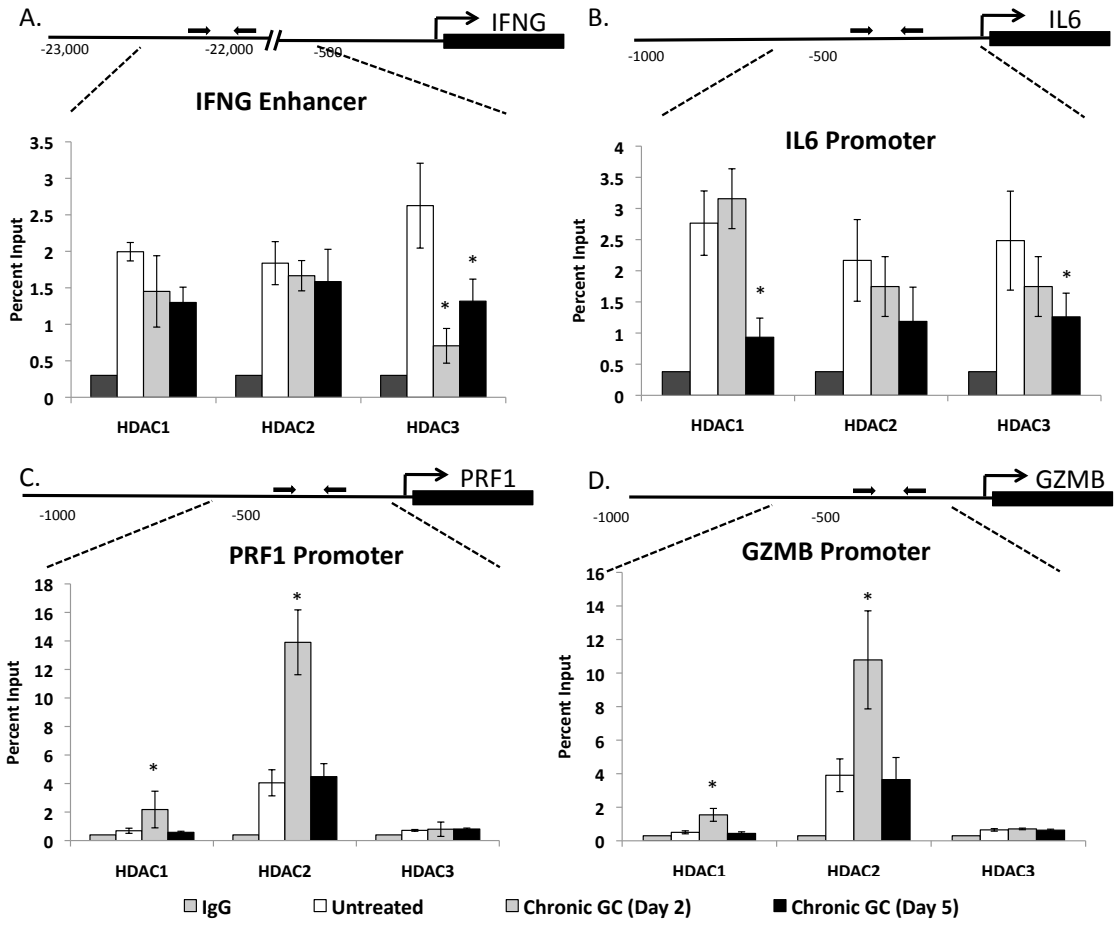


Figure 13. Class I histone deacetylase (HDAC) deposition at NK cell effector gene loci. Schematic diagrams and percent input calculations of HDAC1, 2, and 3 deposition at the (**A**) *IFNG* enhancer, (**B**) *IL6* promoter, (**C**) *PRF1* promoter, and (**D**) *GZMB* promoter after day 2 (grey bars) and day 5 (dark grey bars) chronic glucocorticoid treatment or untreated cultures (white bars) compared to the IgG (black bars) negative control immunoprecipitation. Untreated values are the average of the levels of each modification on both day 2 and day 5 from untreated cultures as no difference in the levels over time was observed. Data represents the average of three independent experiments performed in duplicate n=6 ± SEM *; $p<0.05$.

levels of HDAC2 as resident HDACs in untreated cells (white bars) (Figure 13C and D). After day 2 of chronic GC treatment there was a significant increase in the presence of both HDAC1 and HDAC2 at both the *PRF1* and *GZMB* promoters that returned to initial levels by day 5 (Fig 13C and D). Increased HDAC1 and HDAC2 deposition after GC treatment corresponded to the decreased histone acetylation observed during chronic GC treatment (Figure 11A and B). Together these data demonstrate that chronic GC exposure decreased the deposition of HDAC1 and HDAC3 at proinflammatory cytokine regulatory regions increasing acetylation and accessibility for enhanced cytokine production following stimulation. In contrast, chronic GC treatment increased HDAC1 and HDAC2 deposition at lytic molecule regulatory regions at day 2 of treatment decreasing histone acetylation at day 5 of GC treatment, which ultimately reduced NKCA.

Class I HDAC subcellular redistribution during chronic GC treatment

To investigate class I HDAC genomic deposition, the levels and subcellular distribution of these enzymes was assessed using cytometric and microscopic techniques. Representative histograms of flow cytometric analysis of cellular HDAC protein levelsafter 5 days of chronic GC treatment revealed that HDAC1 and HDAC2 protein levels remained unchanged (Figure 14A, *left and middle panels*). In contrast, HDAC3 staining was markedly reduced after 5 days of chronic GC treatment compared to untreated cells (Figure 14A, *right panel*). Quantitation of the relative MFIs for HDAC1, 2, and 3 at both day 2 and day 5 of chronic GC treatment revealed no change in either

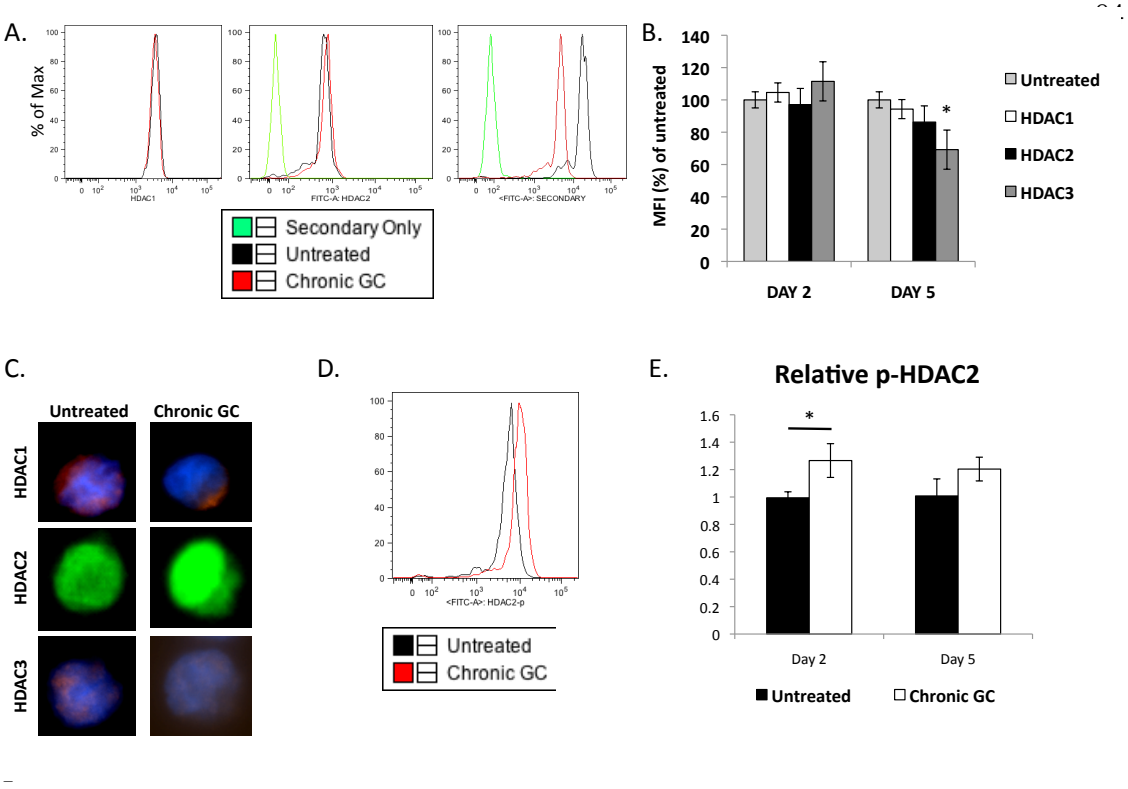


Figure 14. Class I HDAC levels and subcellular distribution following chronic glucocorticoid treatment. (**A**) Representative histograms of HDAC1, HDAC2, and HDAC3 staining in 5 day chronic GC treated and untreated cells. (**B**) Percent change in HDAC1, 2, and 3 MFI after day 2 and day 5 of chronic GC treatment. The MFI of each molecule in untreated cells is set to 100%. (**C**) Representative confocal pictures of HDAC1, 2, and 3 cellular staining in untreated and chronic (5 day) GC treated cells. (**D**) Representative histogram of phosphorylated HDAC2 in untreated and chronic GC treated cells harvested and analyzed by flow cytometry at day 2. (**E**) Quantification of phospho-HDAC2 in untreated and chronic GC treated cells at day 2 and day 5 where the MFI of phospho-HDAC2 in untreated cells is set to 1.

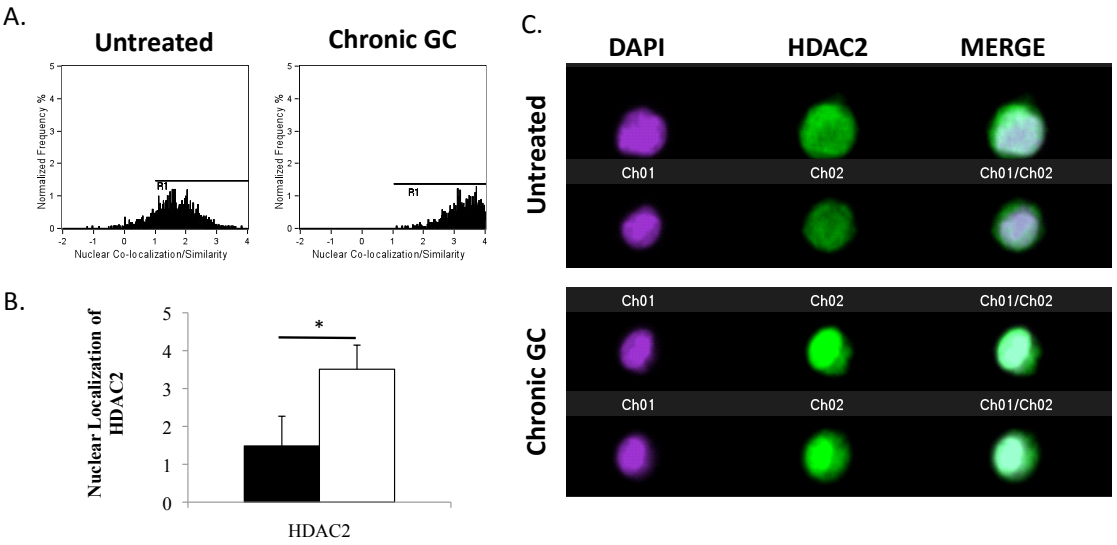


Figure 15. Chronic GC induced HDAC2 nuclear localization . (**A**) Graphs showing the similarity dilate (nuclear localization) of DAPI and HDAC2 from untreated and chronic GC treated cells harvested at day 2. (**B**) Quantification of similarity dilate (nuclear localization) medians from three independent experiments comparing the similarity of DAPI and HDAC2 signals in chronic GC treated or untreated cells harvested at day 2 of treatment. (**C**) Representative images of multispectral flow cytometry used for nuclear localization analysis.

HDAC1, 2, or 3 after 2 days of chronic treatment but a GC specific decrease in HDAC3 levels at day 5 (Figure 14B). Fluorescence microscopy confirmed that HDAC3 staining decreased after 5 day chronic GC treatment confirming the flow cytometry data (Figure 14C, *lower panel*). Chronic GC treatment induced a subcellular redistribution of both HDAC1 and HDAC2. After 5 days of chronic GC treatment HDAC1 exhibited a more intense cytoplasmic (Figure 14C, *upper panel*). Conversely, HDAC2 appeared to have an increase in nuclear staining after 5 days of chronic GC treatment (Figure 14C, *middle panel*). Phosphorylation of HDAC2 at serine 394 is associated with nuclear localization and enzymatic activity [242]. Using flow cytometry we measured the levels of phosphorylated HDAC2 at both day 2 and day 5 of chronic GC treatment as a measure of relative nuclear localization. A representative histogram of phospho-HDAC2 staining from day 2 of chronic treatment reveals the increase in phospho-HDAC2 MFI in chronic GC treated cells (Figure 14D). In accordance with the apparent increase in nuclear staining, quantification of the MFI of phospho-HDAC2 staining demonstrated increased levels of phospho-HDAC2 at both day 2 and day 5 of chronic GC treatment (Figure 14E). Using multispectral flow cytometry of individual cells, HDAC2 nuclear localization was assessed. Nuclear localization was defined by a positive similarityindex (co-localization), indicating the correlation coefficient between the two fluorescent signals: that of the nuclear dye DAPI and that of HDAC2 as a measure of nuclear localization. Untreated cells had a positive similarity index of 1.62 indicating that HDAC2 is both nuclear and cytoplasmic in untreated NK92 cells (Figure 15A, *left panel*). This is in comparison to the 3.5 similarity index of NK92 cells harvest at day 2 of GC treatment (Figure 15A, *right panel*). Figure 15B represents the quantification of

the similarity index of nuclear localization of HDAC2 from untreated and 2 day chronic GC treated cells. Representative images used for quantification of nuclear HDAC2 following chronic GC treatment from the AMNIS Image Stream and IDEAS software analysis are presented in Figure 15C. The first column shows DAPI, channel 01, staining in two representative cells from both untreated and 2 day GC treated cells. The second column, channel 02, illustrates HDAC2 staining and the third column, channel01/channel02, the merged images. The data demonstrate that chronic GC treatment increased nuclear HDAC2 at day 2 which continues throughout treatment. These data provide insight into the decreased acetylation and increased HDAC2 present at the *PRF1* and *GZMB* regulatory regions following chronic GC treatment. These results implicate the redistribution of HDAC 1 and 2 and the loss of resident HDAC3 to result in the changes observed in acetylation status of individual regulatory regions of NK effector genes after chronic GC treatment.

Regulation and role of GR after chronic GC treatment

GC have been shown to reduce the sensitivity of the glucocorticoid receptor (GR), making it unresponsive to subsequent high anti-inflammatory concentrations of GC [243]. Flow cytometry demonstrated no change in GR MFI at day 2 or day 5 of chronic GC treatment of NK92 cells when compared to untreated cells. A representative histogram is shown in Figure 16A and quantification of GR MFI in Figure 16B, with untreated cells set to 100%. To assess the functional activity of GR in NK cells following 5 days

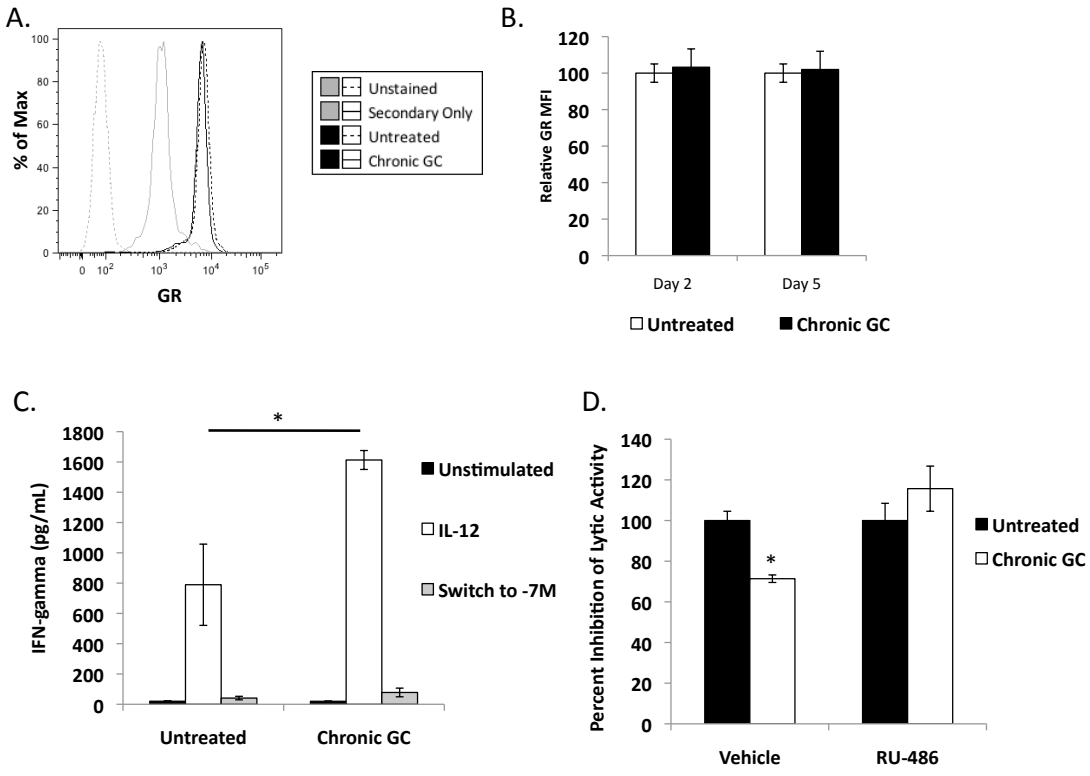


Figure 16. Effect of chronic GC treatment on GR signaling. (**A**) Representative histogram of GR protein levels in untreated and chronic GC treated NK92 cells assessed by flow cytometric analysis. (**B**) Quantification of MFI from flow cytometric analysis of GR levels in cells harvested at day 2 and day 5 from untreated and chronic GC treated cultures where the MFI of untreated cells is set to 100%. (**C**) IFN-g production from untreated (black bars) and chronic GC (white bars) treated cells with a subsequent addition of 4hr treatment with high concentration of dexamethasone (10^{-7}M) (switch to -7) during a 4 hour stimulation with IL-12. (**D**) Lytic activity of NK92 cells after 5 day chronic GC treatment or left untreated in the presence of RU-486 (10^{-10}M) throughout treatment.

of chronic GC treatment, cells were exposed to a high concentration of GC (10^{-7}M) during the 4 hours of IL-12 stimulation. Figure 16C demonstrates that when cells were exposed to 10^{-7}M GC the untreated and 5 day chronic GC treated NK92 cells wereequally reduced in their capacity to produce IFN-g when stimulated with IL-12. Addition of 10^{-10}M RU-486, a GR antagonist, throughout the 5 days of chronic GC treatment was able to reverse the GC dependent reductions in NKCA (Figure 16D). In contrast, RU-486 did not restore IFN-g production to untreated levels. NK92 cells cultured in the presence of RU-486 through the 5 day chronic GC treatment, exhibited a numerical increase in IFN-g MFI (Figure 17A, *white bars*). Flow cytometric analysis of HDAC3 MFI within chronic GC treated NK92 cells cultured in the presence of both RU-486 and chronic GC exhibited an even greater decrement in HDAC3 levels (Figure 17A, *black line*) and was inversely related to the production of IFN-g. When analyzed by co-immunoprecipititation, GR was demonstrated to directly interact with HDAC3 (Figure 17B). Taken together these data suggest that the effects on NKCA and on IFN-g production are via separate and distinct mechanisms.

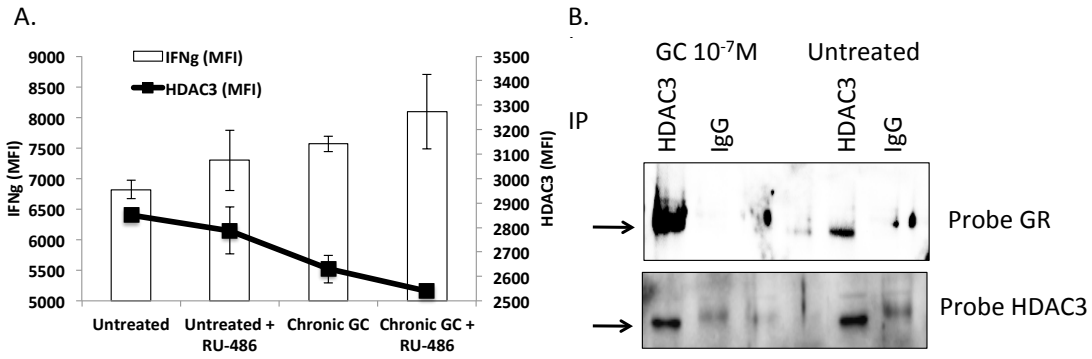


Figure 17. Interaction between HDAC3 and GR pathways. (**A**) MFI of IFN-g (white bars) and MFI of HDAC3 (black line) in NK92 cells with and without chronic GC treatment in the presence or absence of RU-486 (10^{-10}M), harvested and analyzed on 5 day of treatment. (**B**) Anti-IgG or anti-HDAC3 immunoprecipitations were performed on total cell extracts from 4 hour dexamethasone (10^{-7}M) treated or untreated NK92 cells followed by anti-GR (upper panel) or anti-HDAC3 (lower panel) Western blots.

Proposed Model for the effects of chronic GC treatment on NK92 cells

A model for the dichotomous effect of GC on NK cell effector function (Figure 18). After 2 days of GC treatment, increased levels of HDAC2 were observed in the nucleus (Figure 14E - 15) with HDAC 2 detected at the promoter regions of both *PRF1* and *GZMB* (Figure 13C-D). After 2, 4 and 5 days of GC treatment, transcription of perforin and granzyme B were reduced, with concomitant reductions NK cell cytolytic activity (Figure 8A-B). After 2 days of GC treatment no significant change in the acetylation status of either promoter regions was noted, however, a slight reduction in H3K9Ac was noted at day 2 and by day 5 of GC treatment, significant reductions in H3K9Ac were identified (Figure 11A and B), suggesting that acetylation status may relate to the maximal reductions in perforin and granzyme B levels as well as maximal reductions in NK cell cytolytic activity at day 5 of GC treatment. In contrast, the acetylation status of the enhancer region of *IFNG* and the promoter region of *IL6* was increased upon 5 days of chronic GC treatment (Figure 10A and C). This increase in acetylation was associated with a decrease in overall cellular levels of HDAC3 (Figure 14A-B), relocalization of HDAC1 to the cytoplasm (Figure 14C), and with reduced HDAC1 and HDAC 3 at the *IFNG* enhancer and the *IL6* promoter (Figure 13A and B). These epigenetic modifications coincided with increased transcription and protein levels of these proinflammatory cytokines when NK cells were stimulated (Figure 7A-C), suggesting that reduced occupancy by HDACs and increased acetylation status relate to priming of these promoters regions for increased transcription of these genes with cellular stimulation.

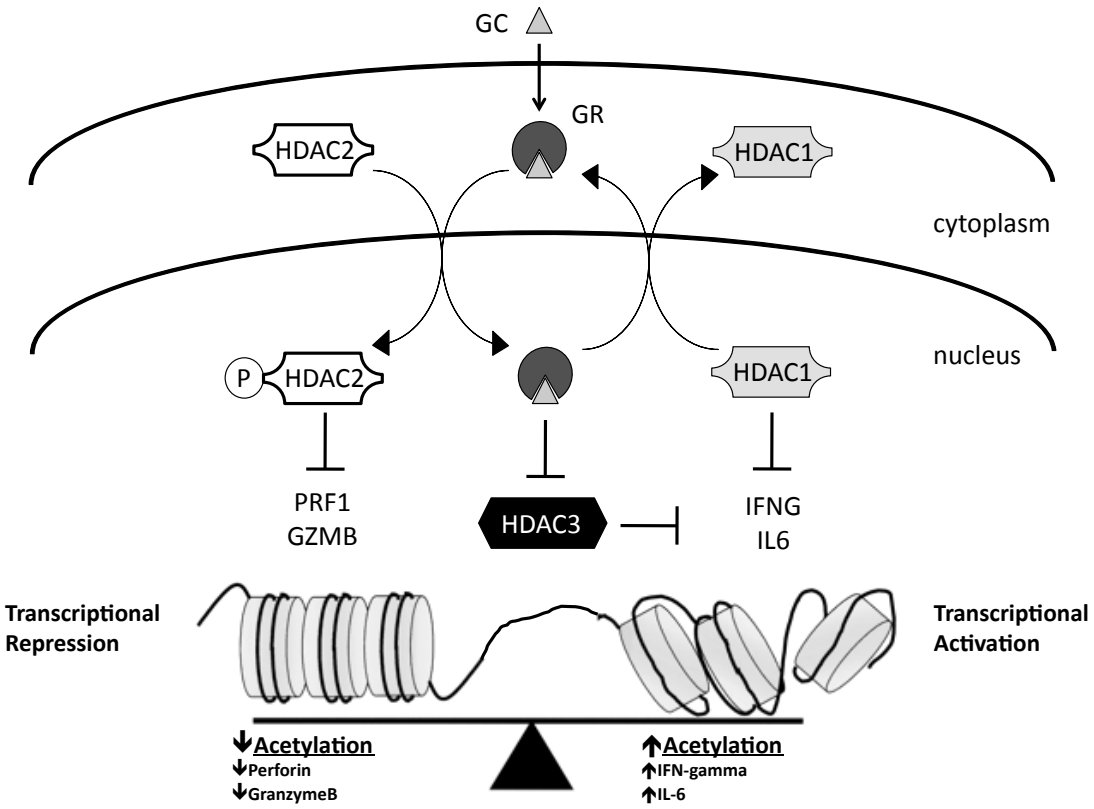


Figure 18. Proposed Model for the effects of chronic GC effects on NK92 cells

Analysis of ex vivo derived PBMC from women diagnosed with

Introduction

Breast cancer diagnosis results in increased stress, anxiety, fear, as well as mood and sleep disturbance[164,165,166,167,168,169,244]. Psychological distress reduces NKCA, the response of NK cells to cytokines, IFN-g synthesis, antibody responses, as well as T-lymphocyte responses[202,203,204,205]. We have shown that the diagnosis of breast cancer results in significant psychosocial distress in women which is accompanied by altered NKCA and cytokine production[202,203]. The effect of psychological distress on NK cells has been particularly attributed to stress-induced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis [245]. Individuals experiencing chronic psychological distress exhibit HPA dysregulation resulting in altered glucocorticoid (GC) levels[202,203] and disruption of the diurnal cortisol rhythm [162,179]. The effects of GC have been readily demonstrable in peripheral blood mononuclear cells (PBMC)[246] and are primarily due to GC action at the transcriptional level. Our *in vitro* data demonstrate that chronic exposure to GC dichotomously regulates the phenotypic profile of NK cells (See Figures 7-8). Chronic exposure to GC resulted in increased pro-inflammatory cytokine production upon stimulation which was associated with increased histone acetylation in critical regulatory loci. Furthermore chronic exposure to GC reduced NKCA and was associated with decreased histone acetylation at regulatory regions for lytic molecules. These *in vitro* findings were used to test hypotheses regarding the relationships between psychological stress, glucocorticoid production, and immune function using the diagnosis of breast

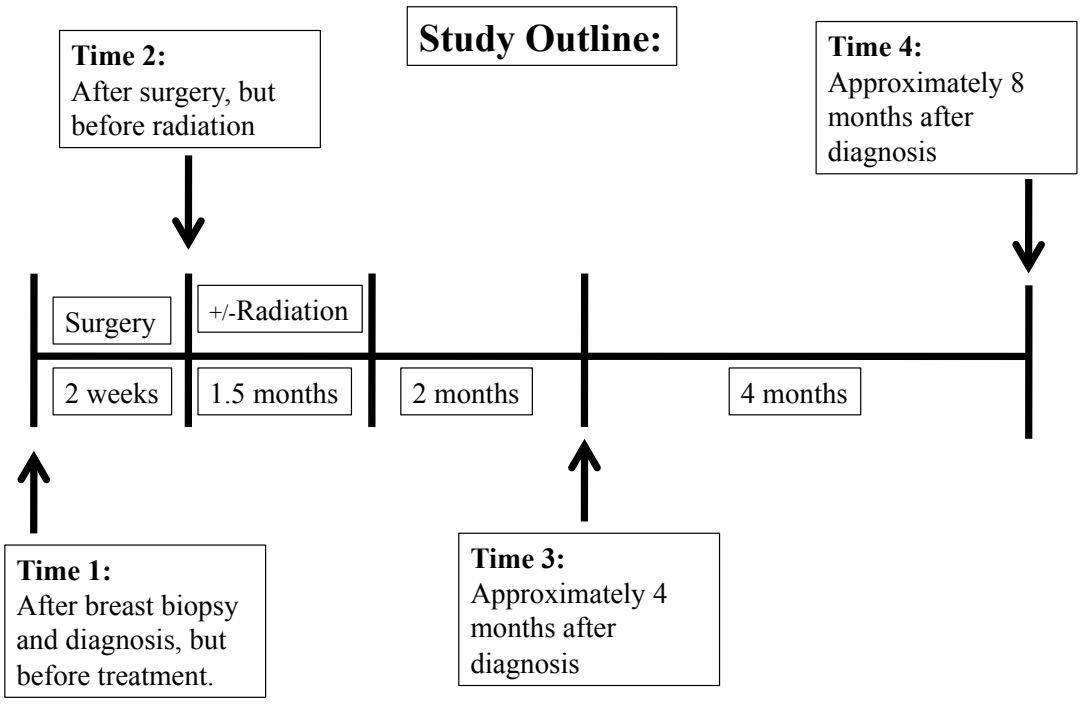


Figure 19. Subject Enrollment Time Line. Each arrow denotes a time period for psychological assessment, immunologica, and epigenetic analysis. Salivary cortisol assessment occurred 2 days prior to blood draw (not shown).

Table 5. Demographic characteristics of individuals enrolled in human study

	Cancer (N=29)	Cancer Free (N=27)
Age (years) mean ± SD	57.4 ± 9.0	56.2 ± 9.0
BMI	26.2 ± 8.6*	21.7 ± 8.0
Race	Frequency	Frequency
Caucasian	23	19
African American	3	3
Hispanic	2	1
PI/Asian	1	4
Marital Status[a]		
Married	23	14
Divorced/separated	1	6
Single	2	4
Widowed	2	1
Income[a]		
10,000-29,000	1	2
30,000-59,000	7	3
60,000 and higher	17	21
Stage of Cancer[a]		
Stage 0	19	
Stage I	5	
Stage IIA	0	
Treatment[a]		
Surgery only	6	
Surgery + radiation therapy	3	
Surgery + hormonal therapy	2	
Surgery + radiation therapy + hormonal therapy	13	
Surgery Type[a]		
Breast conserving	19	
Mastectomy	8	

SD= standard deviation. a=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. Cancer Free.

cancer as a model of psychological stress. I sough to determine whether the immune alterations during periods of stress could mechanistically be explained through epigenetic modifications. Therefore, the acetylation patterns at proinflammatory cytokine regulatory regions were assessed in women as they respond to the diagnosis of breast cancer in order to understand whether these epigenetic patterns related to stress, cortisol levels, and immune function.

Analysis of psychological assessments across timepoints

Women diagnosed with ductal carcinoma *in situ* (DCIS) and a matched control group were enrolled in the study. Enrollment criteria are outlined in Materials and Methods. Demographics, disease, and treatment characteristics are summarized in Table 5. Study participants were evaluated longitudinally at 4 time points (T1-T4, Figure 19). The Perceived Stress Scale (PSS), a measure of the general appraisal of stress, and the Centers for Epidemiologic Studies-Depression Scale (CES-D), a measure of depressive mood, were used for psychological assessments at each timepoint. The mean, standard deviation, range, and distribution of both perceived stress and depressive mood across all timepoints were analyzed between the cancer and cancer free groups and are presented in Table 6. Women in the Cancer group had increased PSS scores compared to the Cancer Free group at T1 (T1; $p<0.001$). These increases in PSS were evident at T2, T3, and T4 (Figure 20A). These results indicate that the perceived stress of breast cancer diagnosis is greatest at T1 and T2 at diagnosis and prior to radiation therapy, respectively. However, the perceived stress of breast cancer diagnosis is still elevated 4

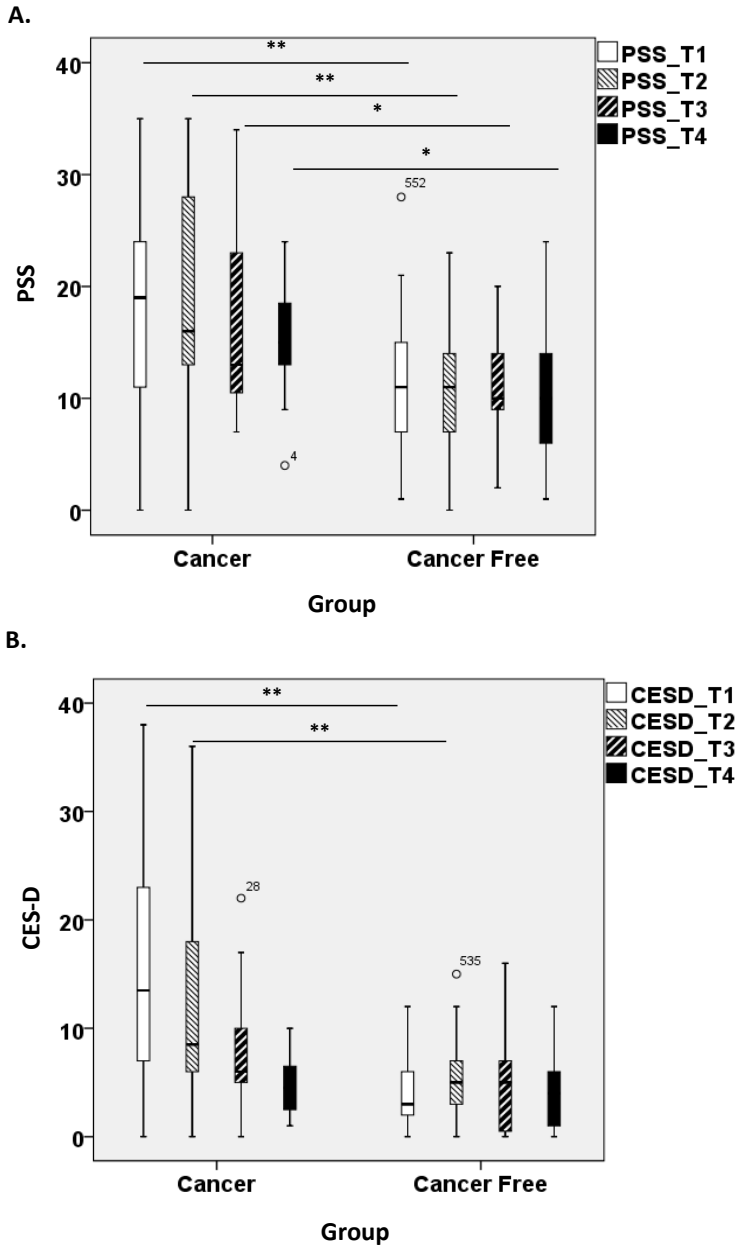


Figure 20. Psychological Assessments across timepoints. Global appraisal of (A) stress as measured by the perceived stressor scale (PSS) and (B) depressive mood as measured by the Centers for Epidemiologic Studies-Depression Scale (CES-D) at T1-T4 for Cancer and Control groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (quartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Open circles represent outliers. *=p,0.05, **=p<0.01, Cancer vs. Cancer Free. T1= T2= T3= T4=.

Table 6. Comparison of psychological parameters across timepoints.

	Cancer	Cancer Free	p value	n Cancer	n Cancer Free
PSS_T1	18.37 ± 1.37	11.35 ± 0.76	0.0001	41	54
PSS_T2	17.94 ± 1.61	10.45 ± 0.79	0.0001	33	53
PSS_T3	16.7 ± 1.91	10.86 ± 1.07	0.012	20	22
PSS_T4	15.18 ±1.81	10.56 ± 1.15	0.045	11	25
CES-D_T1	15.52 ± 1.65	3.67 ± 0.40	0.0001	38	48
CES-D_T2	12.27 ± 1.68	5.37 ± 0.54	0.0001	30	43
CES-D_T3	7.94 ± 1.41	4.75 ± 0.95	0.063	17	20
CES-D_T4	4.75 ± 1.06	4.17 ± 0.75	0.689	8	24

Data are mean values ± S.E.M. p value for Cancer v. Cancer Free.

and 8 months after diagnosis, T3 and T4, respectively. Women diagnosed with breast cancer also exhibited increased levels of depressive mood as measure by CES-D at T1 (Figure 20B), which continued to T2 but returned to levels equivalent to the Cancer Free group at both T3 and T4 (Figure 20B). The range (0-35) and normal distribution (black line) in PSS scores at T1 of all individuals enrolled in the study is shown in histogram form (Figure 21A). Separation of the Cancer and Cancer Free groups demonstrates that the Cancer group alone exhibits a large range in PSS scores (0-35) and normal Gaussian distribution (black line)(Figure 21B, *top panel*). The Cancer Free group exhibits a smaller range in PSS scores (0-29) and a positively skewed distribution (Figure 21B, *bottom panel*). Although PSS scores were significantly different between the cancer and cancer free groups at T1, the distribution demonstrated that 37% of the women in the cancer free group reported perceived stressor scores above a score of 14 indicated by vertical black line (Figure 21B, *bottom panel*). CES-D scores for all individuals recruited are present in histogram form (Figure 21C). CES-D scores exhibited a large range (0-38) but a positively skewed distribution (Figure 21C). Separation of the Cancer and Cancer Free groups demonstrates that the Cancer group alone exhibits a large range in CES-D scores (0-38) and normal Gaussian distribution (black line)(Figure 21D, *top panel*). The Cancer Free group exhibits a smaller range in CES-D scores (0-26) and a positively skewed distribution (Figure 21D, *bottom panel*). The mean difference in perceived stress and depressive mood was greatest at T1; therefore, timepoint 1 (T1) was used to evaluate the stress induced immune alterations and potential epigenetic mechanism(s) involved.

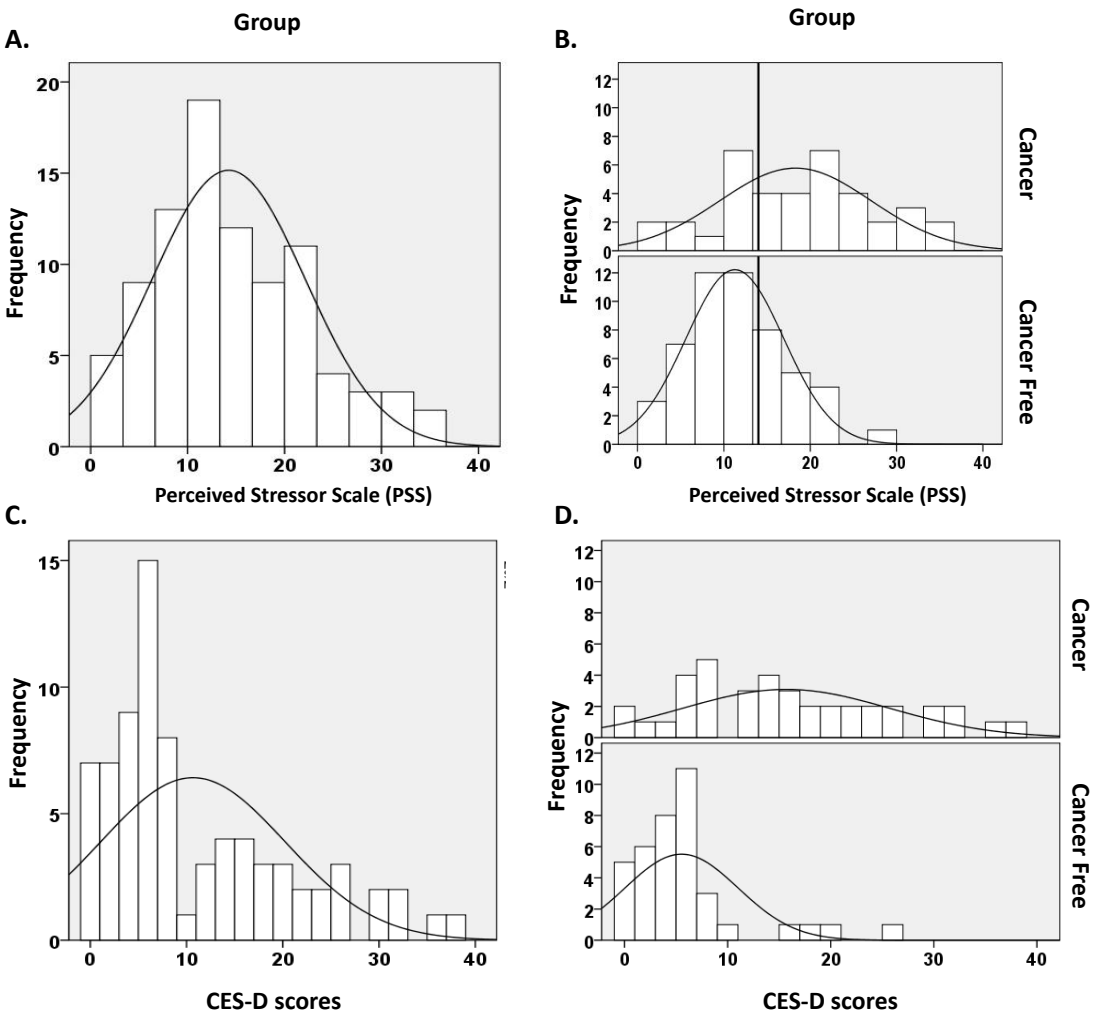


Figure 21. Distribution analysis of psychological parameters at T1. (A) Histogram representing the range and distribution of PSS scores among all individuals at T1. (B) Histograms representing the range and distribution of PSS scores within the Cancer (upper panel) and Cancer Free (lower panel) groups. (C) Histogram representing the range and distribution of CES-D scores within all individual at T1. (D) Histograms representing the range and distribution of CES-D scores within the Cancer (upper panel) and Cancer Free (lower panel) groups.

Comparison of the Cancer and Cancer Free Groups at T1

We have published that the diagnosis of breast cancer results in significant psychosocial distress in women which is accompanied by altered NKCA and cytokine production [202,203]. In this study we reconfirm our previous findings demonstrating that the diagnosis of breast cancer results in significant psychological distress as measure by elevated PSS and CES-D scores (Figure 20A and B). The differences in immune function between the cancer and cancer free groupings at T1 were investigated using a Student's two-tailed T-test. Analysis of NKCA from *ex vivo* derived PBMC standardized to the percent of CD56+ NK cells exhibited no significant differences between the cancer and cancer free groups (Figure 22A). Analysis of IFN-g and IL-6 production from *ex vivo* stimulated PBMC yielded no significant differences between the cancer and cancer free groups (Figures 22B and C). The effect of psychological distress on NK cells has been particularly attributed to stress-induced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis [245]. Thus comparison of the immune regulation by stress could not be attained through comparison of the cancer and cancer free groups in part due to the large percentage (37%) of women in our cancer free group with elevated perceived stressor scale (PSS) scores.

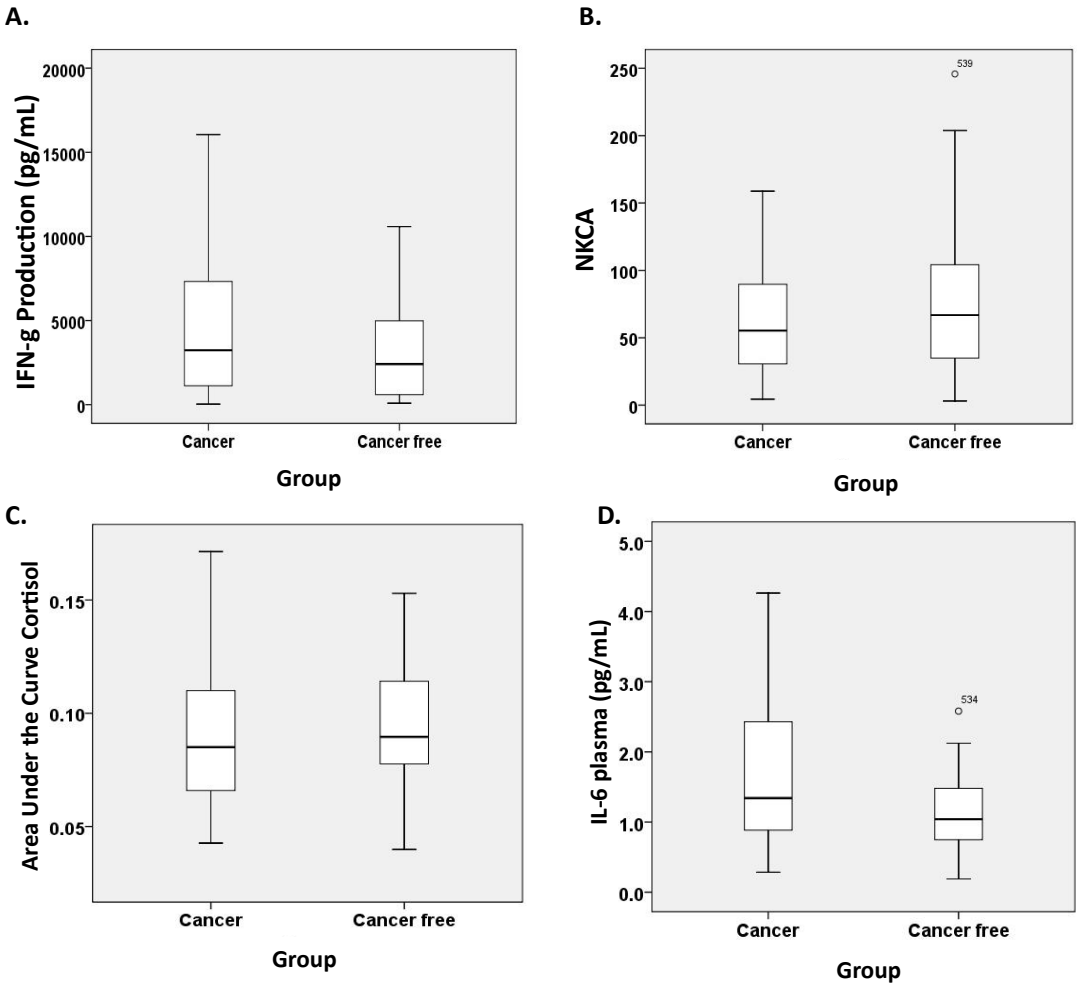


Figure 22. Analysis of biological parameters between Cancer and Cancer Free groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (quartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Open circles represent outliers. *=p,0.05, **=p<0.01, Cancer vs. Cancer Free.

Comparison of high, intermediate, and low stress groups

In order to investigate the immune phenotypes associated with psychological distress and the mechanisms mediating these immune changes, a tertile split on the entire cohort of women enrolled at T1 was performed, creating equal groups based on their PSS scores, independent of cancer diagnosis. Binning produced three groups; PSS<=14 (low stress), PSS 15-21 (intermediate stress), PSS>=22 (high stress) (Figure 23A). Individuals experiencing psychological distress exhibit HPA dysregulation resulting in altered glucocorticoid (GC) levels[202,203] and disruption of the diurnal cortisol rhythm [162,179]. Comparison of diurnal cortisol levels for these three groups demonstrated those individuals with higher perceived stress to have significantly higher daily cortisol levels than those with lower perceived stress (Figure 23B). Individuals with intermediate stress exhibited numerically elevated cortisol levels compared to those with low perceived stress but less than those with higher perceived stress (Figure 23B). Thus perceived stress is associated with activation of the HPA axis.

Abundant evidence demonstrates that psychological stress precipitates alterations in immune function. Therefore, a comparison of immune function between individuals who exhibit high and low perceived stress was performed using a Student's two-tailed t-test. Analysis of supernatants from *ex vivo* derived PBMC stimulated for 48 hour with PMA/PHA by ELISA revealed increased IFN-g production in individuals who exhibit high stress compared to low stress (Figure 23C). Individuals who exhibited high stress also displayed increased circulating IL-6 levels in plasma compared to individuals with low stress as measured by ELISA (Figure 23D). Analysis of NK cell lytic

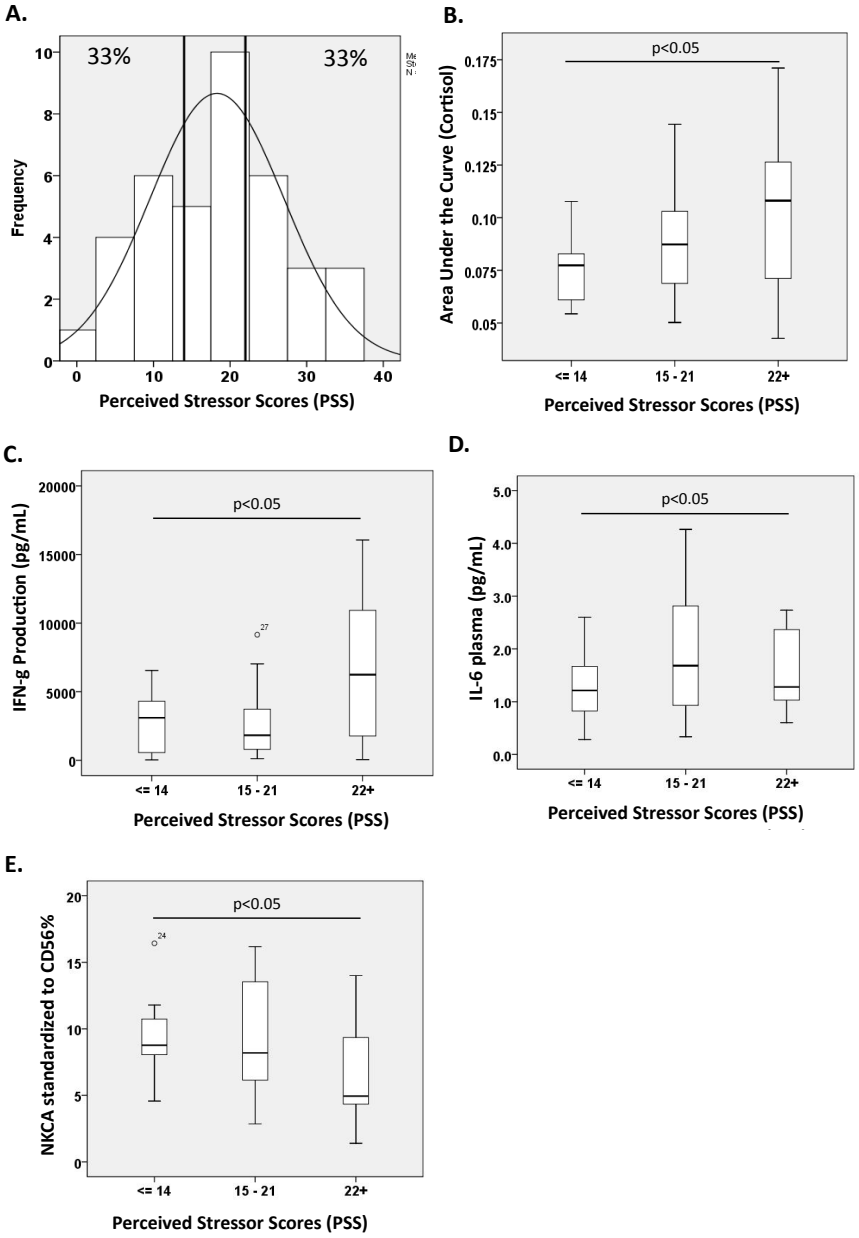


Figure 23. Analysis of biological parameters between high, intermediate, and low stress groups. (A) Histogram representing the distribution of PSS scores indicating cutoffs (vertical lines) for tertile splits of low, intermediate, and high scores. Boxplots representing differences in (B) AUC, (C) IFN-g production, (D) plasma IL-6 levels, and (E) NKCA standardized to CD56% in the PBMC from individuals in the low, intermediate, and high PSS groups. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (quartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Open circles represent outliers. A student's two-tailed t-test was used for comparison. *=$p<0.05$.

function demonstrated that individuals with high stress exhibited decreased NKCA standardized to the percent CD56+ in *ex vivo* derived PBMC compared to low stress individuals (Figure 23E).

These observations demonstrate that individuals grouped by their perceived stress scores exhibit increased daily cortisol production. Increased daily cortisol production indicates that individuals with increased perceived stress exhibit a corresponding increase in HPA activity. Individuals with increased perceived stress also exhibited altered immune function characterized by increased proinflammatory cytokine production and decreased NKCA. We hypothesize that this dichotomous immune phenotype occurs via the overactivity of the HPA activity observed. Therefore to more clearly delineate that the observed immune phenotypes result from the biological manifestation of stress of increased activity of the HPA axis individuals were reclustered by their perception of stress and biological output.

Categorization of Individuals based on PSS scores and Daily Cortisol Levels

Individual responses to the diagnosis of breast cancer are varied and although individuals report increased perceived stress many physiological and social factors can impact the biological output of this psychological stress. Wards Method of Cluster Analysis was used to statistically classify the women based on their perceived stress (PSS) and daily cortisol levels (AUC) into distinct clusters based on the Euclidean mean squared distance. This classification allowed categorization of the women based on both their perception of the stress as well as the associated biological response to stress, i.e.

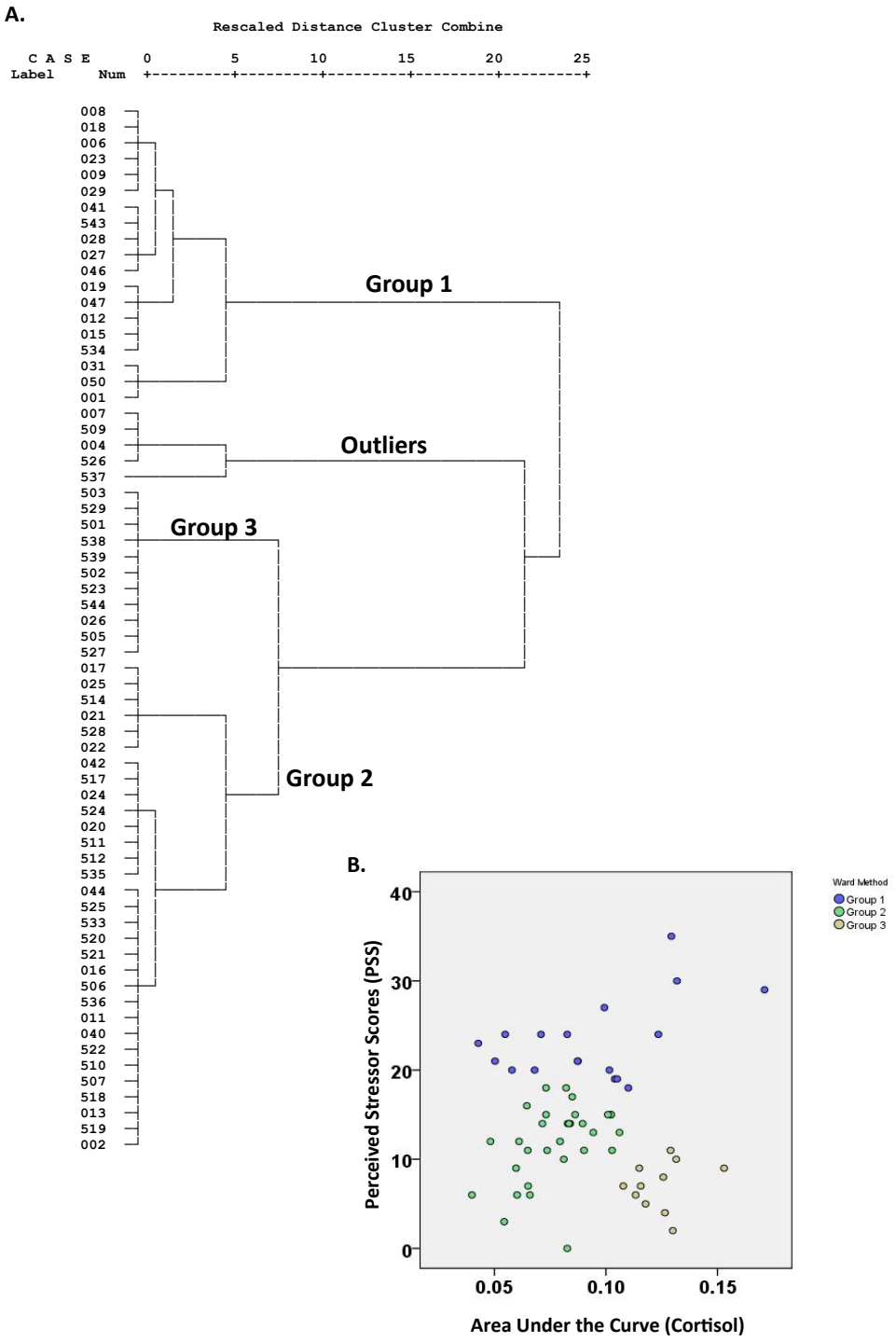


Figure 24. Wards Method of Classification by PSS and Cortisol Rhythms. (A) A dendogram representing the Euclidean mean squared distance between patients classified by their PSS scores and AUC levels. (B) Scatterplot of PSS scores and AUC levels representing the groups created using the Wards Method of cluster analysis

cortisol production, into groups with similar phenotypes. Figure 24A shows the dendogram representation of the Ward Method of Clustering. Three groups emerged from this classification methodology and are shown in a scatterplot of PSS vs. AUC (Figure 24B). The first cluster was of individuals exhibited low PSS scores and low AUC (Figure 24B, *green circles*), the second cluster of individuals exhibited high PSS scores and high AUC values (Figure 24B, *blue circles*), the third and final cluster had low PSS yet high AUC (Figure 24B, *brown circles*). I investigated this third cluster and found that they were individuals within the cancer free group who had low PSS but high CES-D scores (data not shown). Thus this group of women were statistically different from the other groups had the biological alterations most likely due to their depressive mood. Immune parameters were compared between individuals who exhibited both the perception (i.e. high PSS) and biological output (i.e. high cortisol by AUC) of psychological stress (high stress) with the individuals of group 2 (i.e. low PSS and low cortisol by AUC).

Comparison of PSS and AUC between the high stress and low stress groupings demonstrated that indeed both were statistically elevated compared to the low stress group (Figure 25A and B). IFN-g production from *ex vivo* stimulated PBMC as well as circulating IL-6 plasma levels were increased within individuals with increased daily cortisol and PSS compared to low daily cortisol and low PSS (Figure 25C and D). Additionally, NKCA standardized to the percent CD56+ from *ex vivo* derived PBMC was decreased in the high stress group (Figure 25E). These results highlight that individuals who report increased perceived stress and exhibit the biological output of

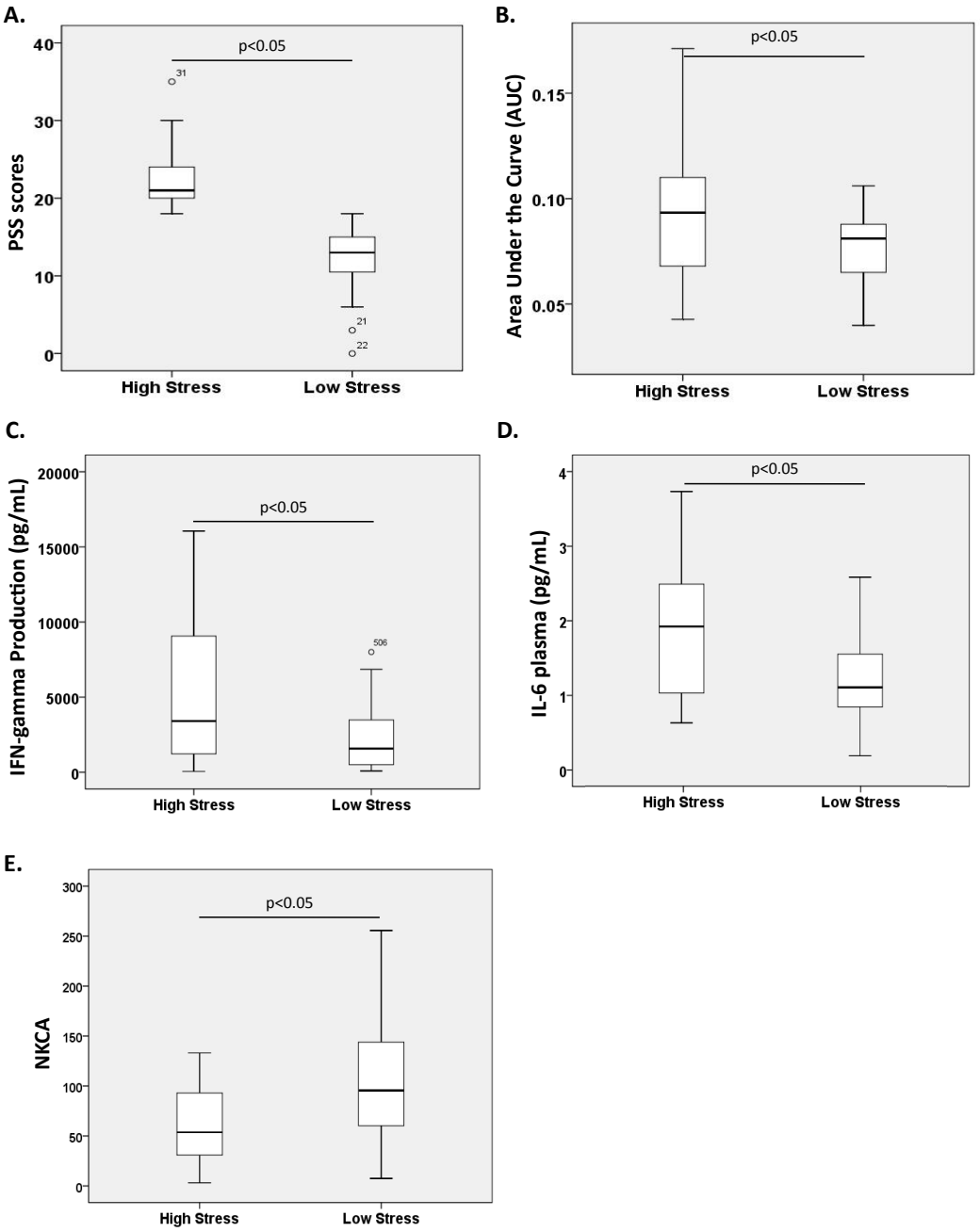


Figure 25. Comparison of immune phenotypes within selected groups. Boxplots representing the differences in (A) PSS scores, (B) AUC cortisol, (C) IFN-g production, (D) NKCA, and (E) IL-6 plasma levels between individuals exhibiting high and low biological stress. Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (quartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Open circles represent outliers. *=p,0.05, **=p<0.01.

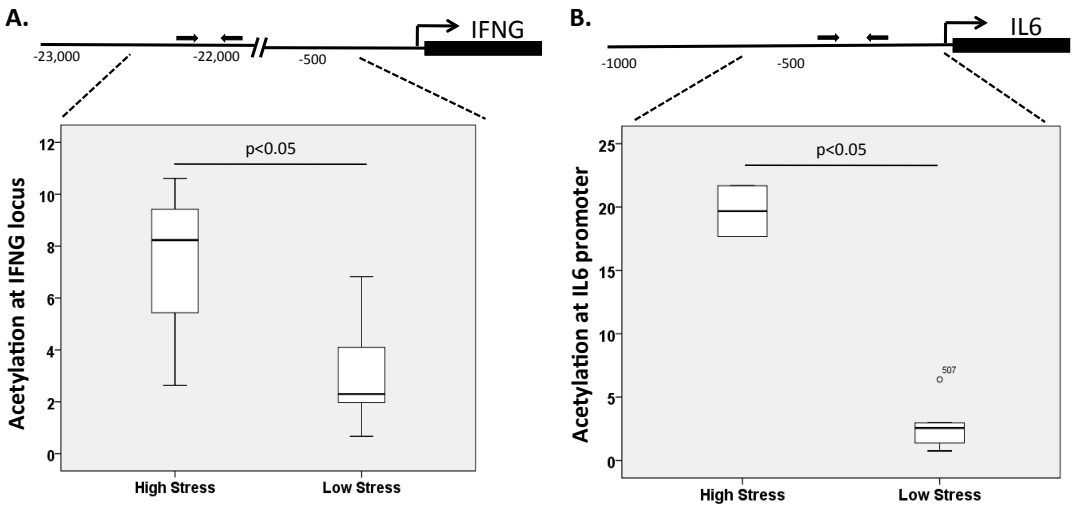


Figure 26. Comparison of promoter specific acetylation pattern between selected groups. Boxplots representing the differences in the ratio of acetylated H3K9 to trimethylated H3K9 at the (A) IFNG enhancer and (B) IL6 promoter between individuals exhibiting high and low biological stress.Values are presented in box plots where the black line represents the median and the tinted area box represents 50% (quartile) of the data set. The whiskers represent the upper 25% and lower 25% of the data set. Open circles represent outliers. *=p,0.05, **=p<0.01, High Stress vs. Low Stress.

stress, altered cortisol rhythms, display a dichotomous regulation of immune function (i.e. increased pro-inflammatory cytokine production and reduced NKCA).

Data presented in this dissertation as well as our previously published data indicated that these immune phenotypes result from transcriptional changes in NK cell effector genes [117,118]. As transcriptional rates are regulated via post-translational modifications on histone tails, the histone acetylation and methylation patterns were investigated within proinflammatory cytokine regulatory regions. Chromatin immunoprecipitation (ChIP) was used to quantify the levels of acetylated H4K8, acetylated H3K9, and tri-methylated H3K9 at the *IFNG* enhancer and *IL6* promoter from $5.0x10^6$ PBMCs. I then quantified the ratio of acetylated H3K9 to tri-methylated H3K9. This was used as a measure of *ex vivo* derived PBMC containing acetylated histones (open chromatin) compared to methylated histones (closed chromatin) at proinflammatory cytokine regulatory regions. Comparison of the ratio of acetylated to trimethylated H3K9 at the *IFNG* enhancer region demonstrated that individuals with high stress exhibited a statistically significant increase compared to individuals with low stress (Figure 26A). An increase in the ratio of acetylated to trimethylated H3K9 was also evident at the *IL6* promoter between individuals exhibiting high stress and low stress (Figure 26B). No difference in the presence of acetylated H4K8 was observed at either the *IFNG* locus or *IL6* promoter (data not shown).

These data in total demonstrate that individuals in our study who report increased perceived stress exhibit the biological output of this stress as increased daily salivary levels of cortisol. Individuals with increased stress exhibit immune alterations characterized by increased proinflammatory cytokine production as well as reduced

NKCA. Lastly, increased histone acetylation can be observed in the regulatory regions of proinflammatory cytokine genes in individuals with increased stress and increased proinflammatory cytokine production. The above data demonstrate that differences are evident between high and low stress groups but do not provide insight into the relationships between these variables and possible mediators of these effects.

Relationships between psychological and immunological variables

Bivariate Pearson correlations were used to evaluate the relationships between psychological stress, daily cortisol, and immune function in the population of individuals at T1. A significant positive correlation was observed between PSS scores and AUC in all individuals including both the cancer and cancer free groups (Figure 27A). The relationship between perceived stress and cortisol production demonstrates that not only were cortisol levels difference between high and low stressed individuals but the greater stress an individual perceives corresponds to an increase in their daily cortisol production. Additionally, both proinflammatory cytokines (IL-6 and IFN-g) exhibited a significant positive correlation with AUC (Figure 27D-E). Indicating that increased HPA activity results in increased proinflammatory cytokine production. A positive correlation was also observed between IFN-g and IL-6 production from *ex vivo* stimulated PBMC indicating a coordination in proinflammatory cytokine regulation in response to increased HPA activity (Figure 27C). Results from this body of work demonstrate that NK cell effector functions are dichotomously

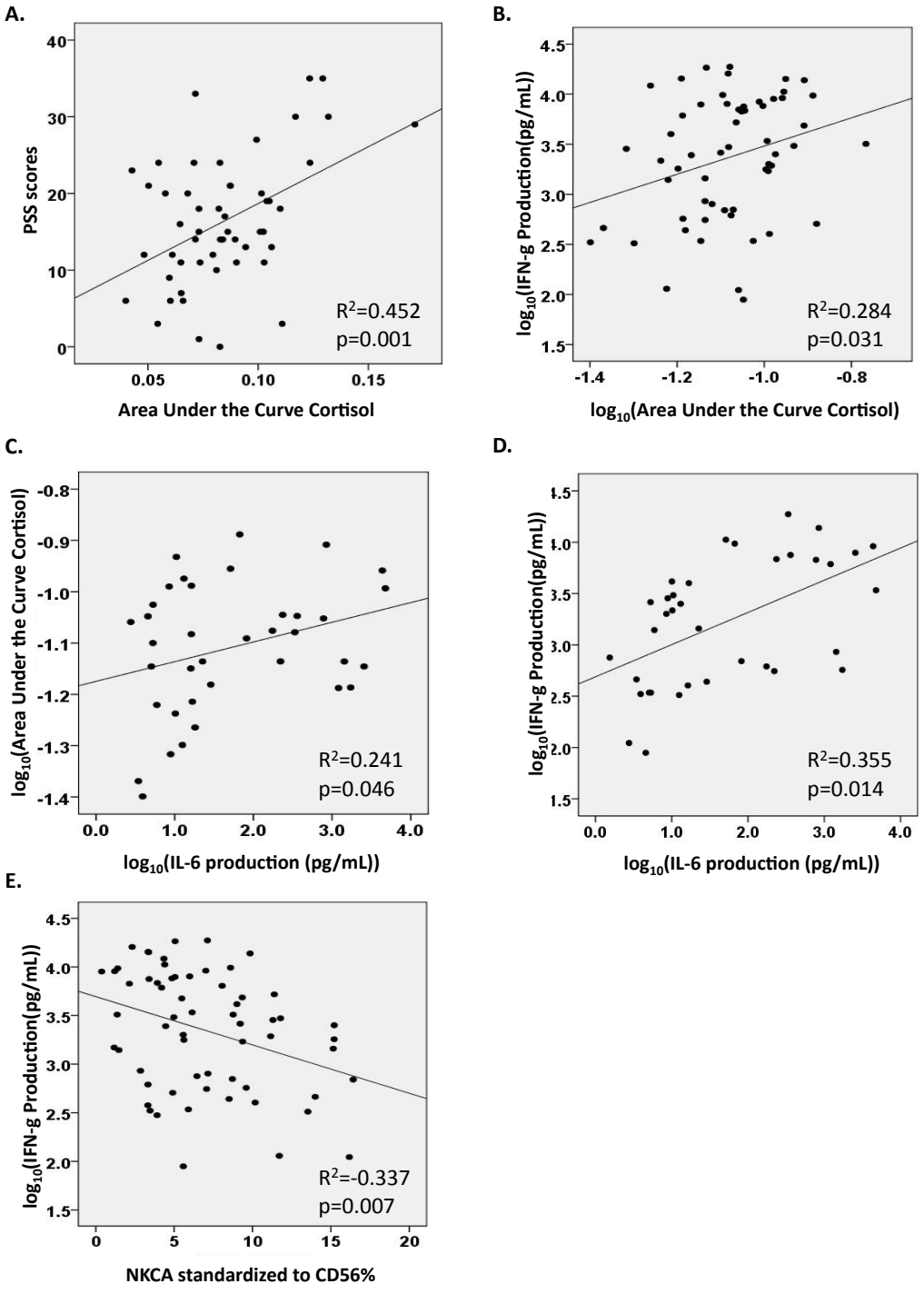


Figure 27. Pearson correlations at T1. Scatterplots representing the relationship between (A) PSS scores and AUC levels at T1. (B) IFN-g production versus NKCA. (C) IFN-g versus IL-6 production. (D) AUC levels versus IL-6 production. (E) AUC levels versus IFN-g production.

regulated following chronic GC treatment. In support of this dichotomous regulation *ex vivo,* a negative relationship between IFN-g production from *ex vivo* stimulated PBMC and NKCA standardized to the percent of CD56+ NK cells per individual was observed (Figure 27B). These relationships demonstrate that as perceived stress increases HPA activity is increased and a subsequent dichotomy in immune function is observed.

As histone acetylation corresponds with transcription, the relationship between promoter specific epigenetic patterns and proinflammatory cytokine production was investigated. The ratio of acetylated to tri-methylated H3K9 was positively related to IFN-g production at T1 (Figure 28B). Furthermore, PSS scores were also positively related to the ratio of acetylated to tri-methylated H3K9 (Figure 28C). These three relationships allowed statistical testing of mediation, through a mediator model (Figure 28E) whereby the increases in IFN-g associated with PSS are mediated by histone acetylation patterns.

Statistical testing of a Mediator Model

The mediation pathway from perceived stress (PSS) to IFN-g production through histone acetylation at the IFNG enhancer was tested using the Sobel mediation equation (Figure 29). The multistage linear regression model of testing mediation depicted in Figure 29 includes four tests. 1) PSS is associated with IFN-g production (path c). 2) PSS is associated with histone acetylation at the *IFNG* enhancer (path a). 3) When controlling for PSS, histone acetylation at the *IFNG* enhancer is associated with

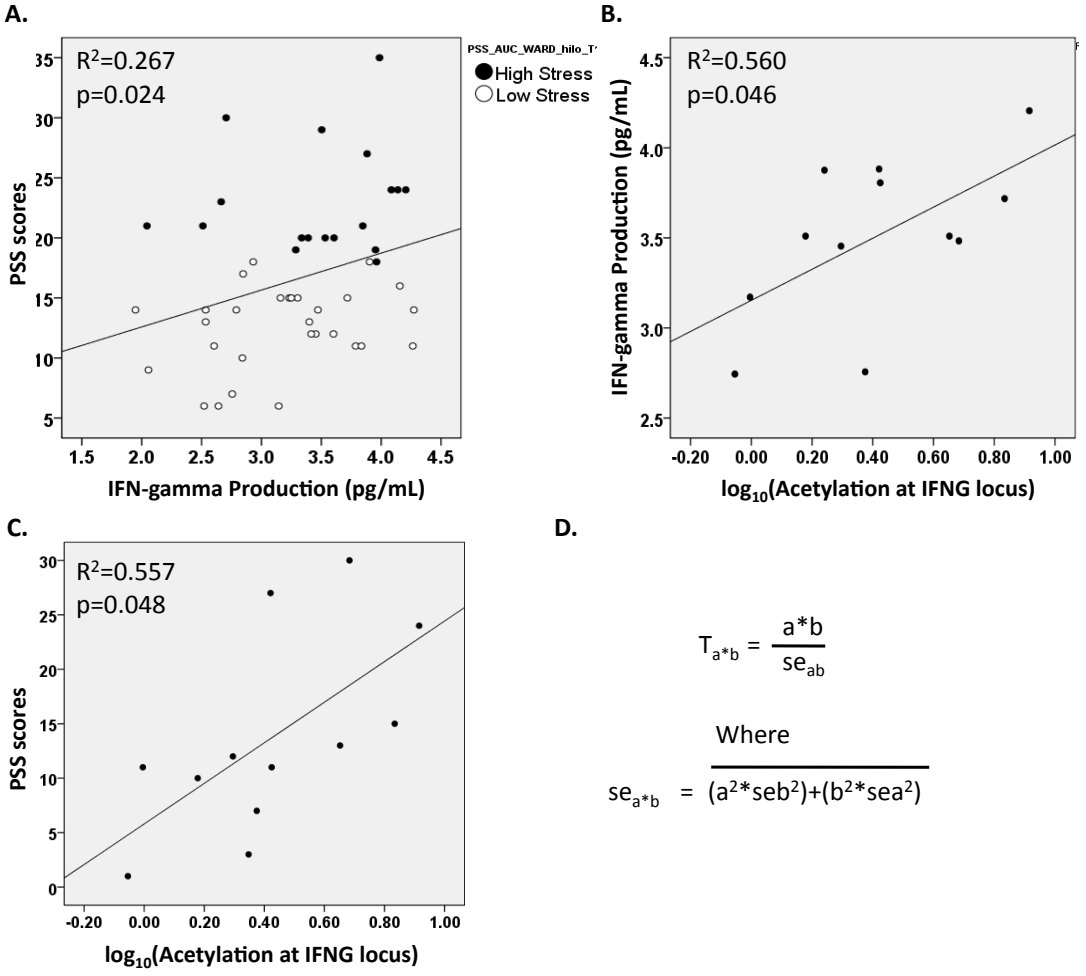


Figure 28. Relationships to test mediation model. Scatterplots representing the relationships between (A) PSS scores and IFN-g production, (B) IFN-g production and the ratio of H3K9 acetylation and H3K9 trimethylation at the IFNG locus and (C) PSS scores versus the ratio of H3K9 acetylation and H3K9 trimethylation at the IFNG locus (D) Sobel test equation.

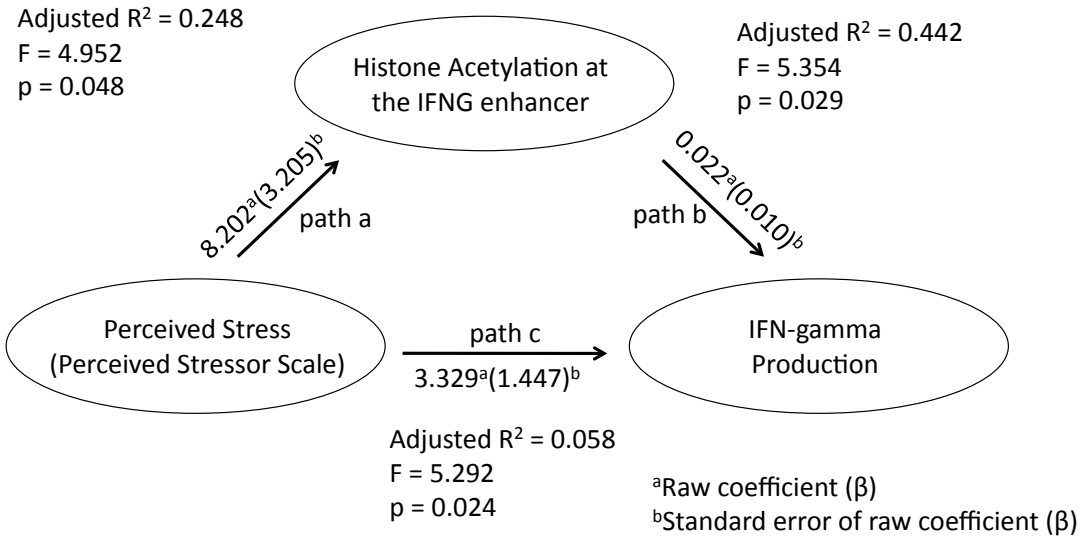


Figure 29. Diagram of Mediation Model. The multistage linear regression model of testing mediation is depicted. 1) PSS is associated with IFN-g production (path c). 2) PSS is associated with histone acetylation at the *IFNG* enhancer (path a). 3) When controlling for PSS, histone acetylation at the *IFNG* enhancer is associated with IFN-g production (path b), and the association between PSS and IFN-g production (path c) is either no longer statistically significant (full mediation) or reduced significantly (partial mediation).

IFN-g production (path b), and the association between PSS and IFN-g production (path c) is either no longer statistically significant (full mediation) or reduced significantly (partial mediation). 4) A statistically significant indirect path exists between perceived stress and IFN-g production through histone acetylation at the *IFNG* enhancer. The Sorbel mediation test (Figure 29D) demonstrated that the indirect effect of PSS on IFN-g production through the mediator, histone acetylation, is significant ($p=0.029$). These analyses were confirmed using two additional variations of the Sobel test, the Aroian and Goodman tests both reaching significance ($p<0.05$) (data not shown). CES-D, another psychological parameter, also correlated with both PSS and IFN-g production and met all the requirements to run a mediation analysis. CES-D did not mediate the changes PSS driven increases in IFN-g production ($p=0.419$) (data not shown). These data demonstrate that within a population stress is associated with an increase in daily cortisol production and an increase in IFN-g production which is partially mediated by the acetylation of histones at the *IFNG* locus.

Categorization of individuals by immune phenotypes

The above date demonstrates that individuals grouped by their stress scores or clustered by stress and the biological phenotype of stress exhibit altered immune function characterized by decreased NKCA and increased IFN-g production. The Ward Method of Cluster Analysis was used to reclassify the women based on Natural Killer cell activity (NKCA) and IFN-g production from *ex vivo* derived PBMC at T1 independent of cancer or cancer free groupings. I questioned whether the reverse

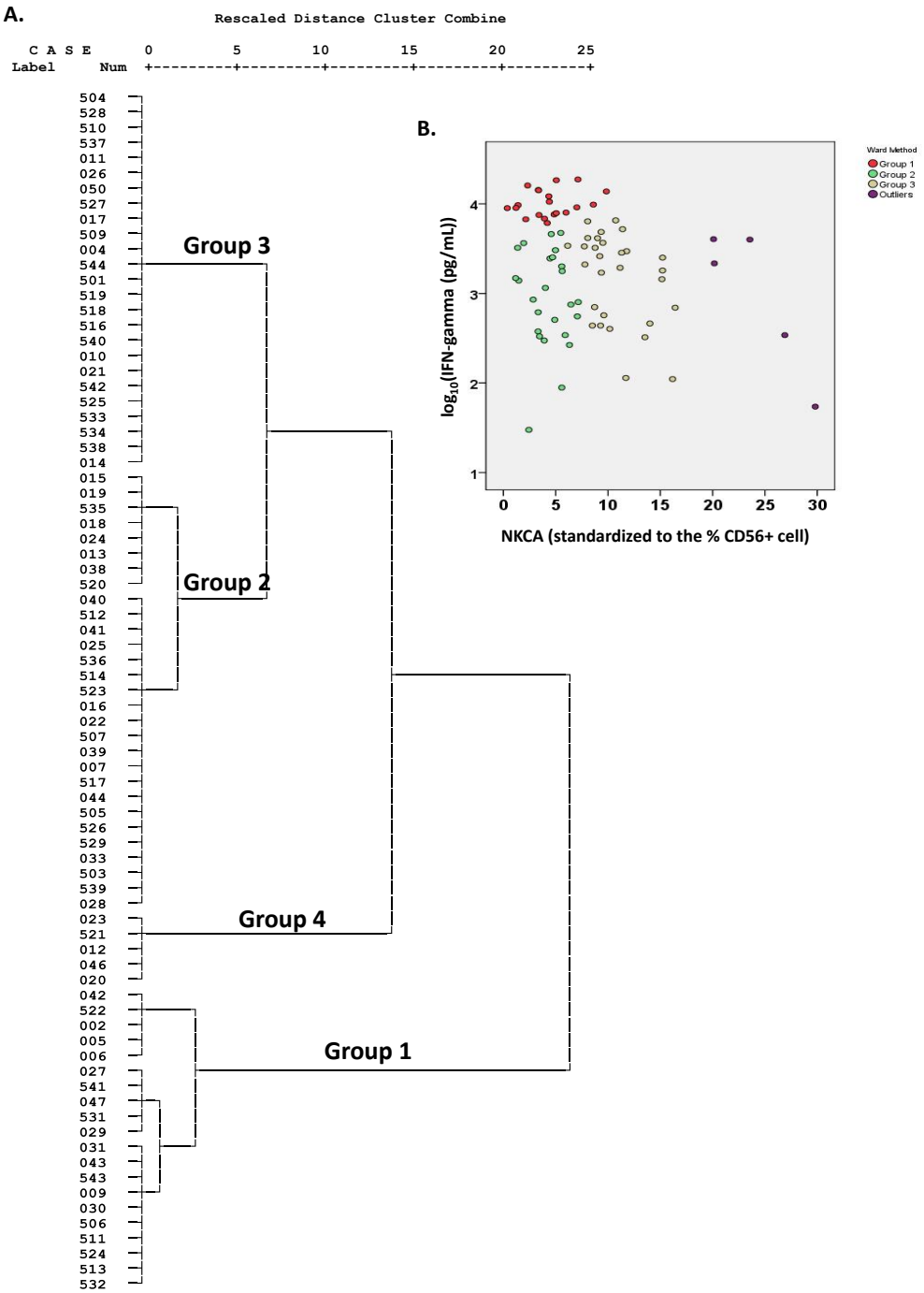


Figure 30. Wards Method of Classification by NKCA and IFN-g production. (A) A dendogram representing the Euclidean mean squared distance between patients classified by their NKCA and IFN-g production. (B) Scatterplot of NKCA and IFN-g representing the groups created using the Wards Method of cluster analysis

also held try, in that I investigated whether women grouped by their immune function (NKCA and IFN-g production) would exhibit differences in daily cortisol production and stress levels. A dendogram of the Ward Method classification is shown in Figure 30A. Four groups emerged from this analysis and are shown on a scatterplot of NKCA standardized to CD56 percentages vs. IFN-g production (Figure 30B). Group 1 consisted of women who exhibited high IFN-g production and low Natural Killer cell lytic activity. Group 2 consisted of women who exhibited both low IFN-g production and NKCA. Group 3 consisted of women who exhibited high NKCA and low IFN-g production. Group 4 consisted of 5 women who were statistical outliers. This analysis allowed us to determine whether women who exhibit alterations in immune parameters differ in their psychological parameters, diurnal cortisol rhythms, and epigenetic patterns. The PSS scores for women in group 1 were increased compared to women in group 3 (Figure 31A). Stress has been attributed to alterations in diurnal cortisol rhythms. Figure 31B shows that women in group 1 exhibited a reduced decline in cortisol throughout the day as measure by the slope from 30min post awakening to bedtime compared to group 3. These results imply that women who exhibit decreased NKCA and increased IFN-g production also exhibit increased stress and changes in cortisol diurnal rhythms.

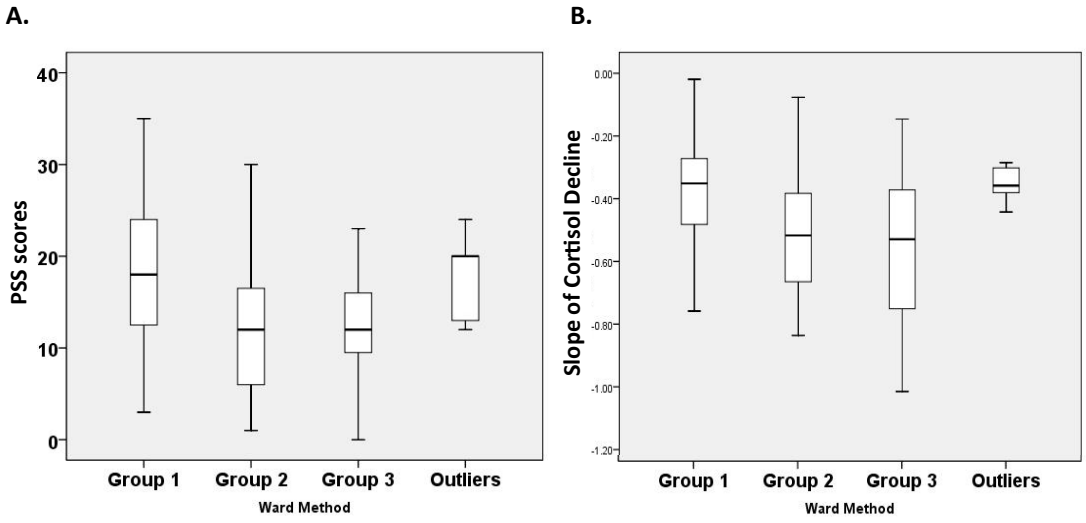


Figure 31. Analysis of stress parameters within individuals clustered by immune parameters. Boxplots representing the (A) PSS scores and (B) cortisol levels between individuals clusterd using Wards method of classification based on NKCA and IFN-g production.

Conclusion

In summary, our analysis of *ex vivo* derived PBMC from women diagnosed with DCIS and a matched control group demonstrated that increases in the level of perceived stress is associated with increases in daily cortisol production. Classification of these individuals by perceived stress and the biological manifestation of stress, cortisol production, demonstrated that analysis of immune function within these groups show a stress induced immune phenotype of increased proinflammatory cytokine production and reduced NKCA. This dichotomous phenotype within stress individuals could also be used as a classification scheme in that individuals grouped by this dichotomous immune phenotype also exhibited differences in stress and cortisol production. Mediation model testing demonstrated that histone acetylation patterns within proinflammatory cytokine regulatory regions partially mediated the stress induced increases in cytokine production. These results not only characterize the immune phenotypes associated with stress but also demonstrate that an epigenetic mechanism is at play and partially mediates these effects.

CHAPTER FOUR

DISCUSSION

Analysis of an *in vitro* system to investigate the effects of chronic glucocorticoid treatment on NK cell effector function

Glucocorticoid induced dichotomy in NK cell effector function

It is well-established that stress is associated with a reduction in NK cell functional activity, which may be mediated by altered adrenergic as well as glucocorticoid activity [200,247,248,249]. This investigation focused on chronic exposure to the synthetic glucocorticoid, dexamethasone, with the observation that chronic GC treatment suppressed NKCA and increased stimulated proinflammatory cytokine production. We conclude this is within a single cell population as 85-98% of NK92 cells were perforin, granzyme B, and IFN-g positive independent of treatment. Additionally, chronic GC treatment was associated with changes in MFI but not the percent of cells positive for each individual effector protein. The gene products (mRNA) for NK cell lytic granules were quantitatively reduced by GC treatment, and such reductions related to reduced functional capacity of NK92 to mediate lysis of K562 cells. These observations are in agreement with a number of systems investigating the effects of GC on NK cell function both from *ex vivo* derived CD56+ cells as well as multiple NK cell lines including; NK92, NK3.3, and YT [117,118,119]. All demonstrate that GC treatment reduced NKCA, in part, through reductions in effector molecule production.

In contrast, upon cellular activation, the production of the IFN-g and IL-6 gene products were quantitatively increased by chronic GC treatment. Chronic GC treatment primed NK92 cells to produce more proinflammatory cytokine when stimulated, regardless of the stimulant tested (PMA/ionomycin, IL-12, or IL-2), each of which signals through a different cellular pathway. These observations suggested that some common mechanism downstream of transcription factor activation mediated these effects.

The molecular mechanisms underlying the effects of GC remain incompletely understood. Many studies have focused on mechanisms upstream of GC binding to its receptor, including modified extracellular or intracellular GC concentrations or GR mutations, splice variants, or insufficient GR expression. Here we demonstrated that chronic GC did not alter steady state GR protein levels or the sensitivity of GR to a subsequent high anti-inflammatory concentration (10^{-7}M) of GC after chronic GC treatment (Figure 15B-C). From these studies we conclude that our observations are not the result of GR resistance, because no changes in GR levels or sensitivity were observed.

Additional studies also report mechanisms downstream of GC binding to GR that involve the GC signaling pathways which regulates the transcription of many genes involved in the immune response [250,251]. By interacting with JAK-STAT signaling pathway and with transcription factors such as NF-κB and activated protein-1 (AP-1), GR has been shown to alter the transcription of immune relevant genes [252,253]. Although activated glucocorticoid receptor (GR) can directly interact with proinflammatory transcription factors like AP-1 (Fos-Jun heterodimers) and NF-κB (p65-p50 heterodimers) [109,254,255], chronic GC treatment did not alter the steady

state levels of NF-κB or AP-1 nor did it impact the ability of cellular stimulation to induce NF-κB or AP-1 nuclear translocation in NK92 cells (Figure9 E-F). These data demonstrate that the enhanced cytokine production did not result from chronic GC treatment induced enhancement of nuclear NF-κB or AP-1 signaling. This suggested that some other mechanism primes NK cells for enhanced cytokine production with the likely possibility being changes at the epigenetic level.

Glucocorticoid induced promoter specific epigenetic modifications

Our lab has previously demonstrated that GC through GR can induce epigenetic modifications at NK cell effector genes. These epigenetic modifications are associated with altered gene transcription [117,118],when induced with high concentrations of GC (10^{-7}M) for 24 hours [117,118]. The effects of GC on cellular function are known to be dependent on both the dose and duration of exposure. Therefore, the epigenetic pattern associated with NK cell effector genes was assessed using chromatin immunoprecipitation during chronic GC treatment. The levels of H4K8Ac, H3K4me3, H3K9Ac, H3K9me3, H3K27Ac, and H3K27me3 were assessed at days 2 and 5 during chronic treatment and compared to the levels in untreated cells. H3K9me3, a marker of constitutive heterochromatin, was at or below the limit of detection for all genes investigated [125,126,127,128]. This was expected as NK92 are fully differentiated NK cells and have the potential to express both cytokine and lytic molecules which should be located in euchromatic regions. H3K27me3 (associated with silenced genes) was poorly represented at all regulatory regions investigated. H3K4me3 is typically present

at transcription start sites, is strongly correlated with transcription potential and was measurable at all regulatory regions investigated[129,130]. This pattern of H3K4me3 with no H3K9me3 or H3K27me3, indicates genes present in euchromatin with the potential for expression. H3K9Ac is typically represented at active promoters and enhancers and the level of H3K9Ac at a given region correlates with the level of transcription[127]. Chromatin immunoprecipitation demonstrated a quantitative reduction in the acetylation of H3K9 at the *PRF1* and *GZMB* promoters. The reduction in H3K9Ac at the *PRF1* and *GZMB* promoters is the likely epigenetic modification that likely contributes to the reduced production of transcripts for both of these effector proteins.

Chromatin immunoprecipitation demonstrated increased acetylation of H4K8 and H3K27 for both the *IFNG* enhancer and the *IL6* promoter. Conceptually, the histone proteins possesses highly basic amino-terminal tail domains located outside of the core nucleosome particle, which are accessible to multiple post-translational covalent modifications, including acetylation and methylation[256]. Lysine acetylation neutralizes the charge of histone proteins, whereas deacetylation restores the positive charge of lysines. Following acetylation, nucleosome mobility is enhanced, increasing accessibility of the promoter for transcription machinery[257]. Thus, acetylated lysine residues increase access of transcription factors for regulatory regions of immune response genes. By enhancing this downstream target (chromatin accessibility), GC can prime genes for altered transcription upon cellular activation and transcription factor nuclear localization. Hence, the data presented here demonstrate that GC enhanced cytokine production is in part due to increased proximal promoter and enhancer

accessibility. The increase in H4K8Ac and H3K27Ac at the –22kb *IFNG* enhancer and at the *IL6* promoter are the likely major epigenetic modifications that contribute to the increased production of transcripts for both of these effector proteins upon cellular stimulation.

The question becomes, how do these specific and independent histone tail modifications contribute to the control of the lytic molecule vs. cytokine regulatory regions? First, the cell line being used is a fully differentiated NK cell line, with the potential to express both lytic and cytokine genes and thus the expression potential of all genes investigated are equivalent, in that, they are all located within euchromatic regions, indicated by the lack of H3K9me3. What separates these lytic and cytokine genes are the transcriptional profiles prior to GC treatment. Real-time analysis demonstrated that the relative number of transcripts for the lytic molecules is roughly 10,000 times that of the cytokine genes in resting cells, as the mRNA isolated from NK92 cells in culture had to be diluted 1:10,000 to obtain the same C(t) value as those of the cytokine genes from unstimulated cells (data not shown). *PRF1* and *GZMB* are constitutively expressed genes in NK cells, whose protein products accumulate and are stored in cytoplasmic granules. The cytokine genes on the other hand exhibited a small, almost undetectable, level of basal expression and are held poised until cellular stimulation (Figure 7C). Thus *PRF1* and *GZMB* were highly transcribed and exhibited histone tail modifications (high H4K8Ac, high H3K9Ac, and high H3K27Ac), which marked them as such. In contrast, the cytokine genes exhibited low H4K8Ac, low H3K9Ac, and low H3K27Ac which suggested lower transcription than the lytic

molecules and potentially less accessible chromatin, and possibly lacking the docking sites for transcriptional machinery.

Specifically, H4K8Ac is a docking site for the bromodomain containing protein complex SWI/SNF [258]. Recruitment of SWI/SNF, via the increased H4K8Ac after chronic GC treatment, would make the cytokine regions more accessible to transcription factors and other transcriptional regulators through the nucleosome remodeling functions of the SWI/SNF complex[259]. This is indeed true as the *IFNG* enhancer and *IL6* promoter exhibited a 37% and 18% increase in accessibility after chronic GC treatment, respectively (Figure 12A). These observations are in agreement with experiments testing the histone code hypothesis investigating the contribution of individual histone marks to the step-by-step activation of transcription. These experiments demonstrated that H4 acetylation occured first, which creates a docking site for the SWI/SNF complex[258]. The H4K8 modification cannot induce transcription but requires GCN5 or other histone acetyltransferase mediated acetylation of H3K9 and H3K14 that provides a docking site for transcription factor II D (TFIID)[258]. TFIID is required for the formation of the transcription initiation complex and thus a critical step in transcriptional regulation[260]. I speculate that prior to cellular stimulation H3K9Ac is limited, most likely due to the actions of resident HDACs and lack of histone acetyltransferase activity/recruitment at cytokine regulatory regions. Without TF activation, nuclear translocation, and recruitment of a histone acetyltransferases such as GCN5, TFIID association and thus transcription is limited. This scenario explains why basal transcription was unaffected even with the observed increase in H4K8Ac. Therefore, the GC dependent loss of HDAC1 and HDAC3 from proinflammatory

cytokine loci and subsequent increase in H4K8Ac primes these regions for enhanced transcription upon stimulation, due to the increased acetylation of H4K8 and increased chromatin accessibility due to the hypothesized recruitment of the SWI/SNF complex.

In contrast to the cytokine loci, H3K9Ac is reduced at *PRF1* and *GZMB* most likely due to the recruitment of HDAC2 during chronic GC treatment. HDAC2 has been shown to directly deacetylate the amino terminal tail of H3 and has been shown to inhibit the activity of histone acetyltransferase-containing complexes. In either senario, HDAC2 recruitment could reduce the availability of docking sites required for TFIID recruitment and therefore decrease the transcriptional rate of the lytic genes.

Preliminary ChIP analysis with anti-RNApol II antibody demonstrates that chronic GC treatment resulted in increased RNApol II at the *PRF1* and *IL6* promoters, as well as, at the *IFNG* enhancer (Figure 12B). The increase in H4K8Ac at cytokine loci and decrease in H3K9Ac at lytic molecule loci could exhibit a similar effect on RNApol II recruitment. That is, the presence of H4K8Ac could allow for the binding of the SWI/SNF complex which would allow access of the RNApol II holoenzyme[261], and poise the cytokine gene loci for transcription. No change in RNApol II recruitment was observed at the *TNF* promoter which also displayed no change in histone tail modifications following chronic GC treatment (Figure 12B). We hypothesize that the increase in RNApol II is stalled RNA polymerase as no concomitant changes in basal transcription were observed. Furthermore, no increase in H3K9Ac was observed at the cytokine loci and therefore suggest no change in transcriptional rates.

As described *PRF1* and *GZMB* undergo high rates of transcription and would continually recruit RNApol II during normal cell function due to the high levels of

H4K8Ac, H3K9Ac, and H3K27Ac. The higher levels of RNApol II in untreated cells at the loci which code for lytic molecules compared to the cytokine regulatory regions is indicative of this higher level of transcription (Figure 12B). The decreased levels of H3K9Ac seen in at the *PRF1* and *GZMB* promoters following chronic GC treatment could decrease the docking site for TFIID. The increased presence of RNApol II after chronic GC treatment at the PRF1 promoter is likely stalled RNApol II, as transcripts from *PRF1* were decreased 50% after chronic GC treatment. Interestingly, the GZMB promoter exhibited decreased RNApol II binding after chronic treatment. Why the *GZMB* promoter is regulated differently than the other NK cell effector genes tested remains to be determined. These experiments were performed using total anti-RNApol II antibody and was not specific for the stalled or active forms which can be differentiated by the presence of specific serine phosphorylation on the RNApol II enzyme. These observations clearly delineate the contribution of the GC dependent histone tail modifications in the epigenetic control of NK cell effector gene transcription.

In summary, chronic GC treatment imparts a dichotomous regulation of NK cell effector function through promoter specific epigenetic modifications. These promoter specific changes in histone acetylation patterns are congruent with the transcriptional profile of NK cells after chronic GC treatment. These changes in histone acetylation are associated with not only changes in the chromatin structure but also changes in the RNApol II deposition. The next question becomes what is the mechanism by which GC mediates these changes in promoter specific acetylation patterns.

Glucocorticoid regulation of histone modifying enzymes

Activated GR can alter histone acetylation and thus gene expression by directing the interaction of chromatin with histone acetyl transferase complexes and/or interaction with histone deacetylase (HDAC) complexes[118,146]. In accordance with the observed decrease in H3K9 acetylation, 2 days of GC exposure exhibited increased levels of HDAC2 at both the perforin and granzyme B promoters. These promoter specific increases in HDAC2 corresponded with global increases in the level of cellular phosphorylated HDAC2. Phosphorylated HDAC2 has been shown to be located in the nucleus and enzymatically active[242]. These observations were confirmed using multispectral flow cytometry and co-localization (similarity dilate) of HDAC2 with nuclear DAPI staining after chronic GC treatment (Figure 14F-G). The data presented for the NK cell lytic molecules are consistent with the described transrepression mechanism for glucocorticoid action mediated by the HDAC2 / co-repressor complex[118,146] and is in full agreement with many observations for the decreased immune function in the presence of GC.

The cytokine genes; 1) were regulated in the opposite manner, 2) with different histone modifications associated with transcription potential, and 3) with chromatin accessibility altered at the cytokine loci and not the lytic molecule promoters. These observations suggested that these dichotomous effects were regulated by two different epigenetic mechanisms. One possible basis for this difference is differential enrichment/absence of HDACs.

Chronic GC treatment reduced the levels of resident HDAC1 and HDAC3 bound at the proinflammatory cytokine regulatory regions in NK92 cells, which mechanistically explains the increases in histone acetylation and chromatin accessibility observed. The role of HDAC1 and HDAC3 in the control of proinflammatory cytokine regulation has been clearly demonstrated previously for both *IFNG* and *IL6* [262]. Herein, loss of HDAC3 from these regions corresponded to a total cellular decrease in HDAC3 protein level (Figure 14B). It has been previously shown that GR can interact with HDAC3[147] and HDAC1[118,145]. We previously confirmed the interaction of GR and HDAC1 and demonstrate here that in NK92 cells, GR and HDAC3 co-immunoprecipitate (Figure 15F). Furthermore, the cellular levels of both GR[263,264,265] and HDAC3[266,267] are regulated by the proteasome pathway. I speculate that the time dependent decrease in total HDAC3 protein and HDAC3 at cytokine regulatory regions likely result from a direct interaction of HDAC3 with ligand activated GR and subsequent targeting for proteasomal degradation.

Reports demonstrate that although RU-486 can antagonize the transrepression of genes by GR[268], RU-486 can also act as a GR agonist in a gene specific manner[269]. We demonstrate that GC and RU-486 synergistically decrease HDAC3 protein levels in NK92 cells (Figure 15E). Dual staining for IFN-g and HDAC3 in GC and RU-486 treated cells revealed that the level of IFN-g in NK92 cells related inversely to the level of HDAC3 (Figure 15E). We hypothesize that both GC and RU-486 induce nuclear localization of GR, interaction with HDAC3, and its subsequent proteosomal degradation, relieving HDAC3 mediated inhibition (deacetylation) of the IFNG and IL6 loci. These data together demonstrate that GC can dichotomously regulate NK cell

effector function through two independent mechanisms. The first involves the classical repression mechanism via HDAC2-mediated decreases in H3K9Ac which can be inhibited by RU-486; the second involves the epigenetic priming of proinflammatory cytokine loci through reductions in HDAC1 and HDAC3, increasing H4K8Ac.

We hypothesize that direct interactions between GR and HDACs are mediating these changes in the association and loss of specific HDACs from the regulatory regions of NK cell effector genes. Our data suggests that these promoter specific alterations in HDAC recruitment are gene specific, as HDAC2 was detected at both the IL6 promoter and IFNG enhancer but was unchanged at day 2 or day 5. This was in contrast to the dramatic increase in HDAC2 at PRF1 and GZMB promoters (Figures 13A-D). GR recruitment to these regulatory regions was assessed by ChIP but quantification of GR levels at genomic loci was unsuccessful. We hypothesize that quantification of GR recruitment activated with such a low concentration of ligand was hindered by the limit of detection for the ChIP assay. Evidence indicates that the binding of ligand activated GR to chromatin in living cells is extremely rapid with off and on rates of seconds to minutes [270,271]. Further studies investigating GR binding and occupancy to genomic loci using photobleach microscopy experiments highlight that once ligand bound GR is recruited to regulatory regions it only remains there for a few seconds before new unbleached fluorophore conjugated GR replaces it[272]. Finally, GR rapidly engages and disengages the chromatin template, during which chromatin remodeling undergoes dynamic exchange [273,274]. Thus, I hypothesize that the effect of GC through GR is a cumulative effect of ligand binding over days and

thus changes in the occupancy of GR at these regions could not be detected. As such, it is unlikely that it is possible to detect promoter specific recruitment of GR in our chronic low level GC treatment system using ChIP.

Phospho-HDAC2 was increased as early day 2 of chronic glucocorticoid treatment (Figure 14E). The nuclear translocation, enzymatic activity, and degradation of HDACs are regulated by post-translational modifications[275]. Phosphorylation of HDAC2 on Ser394 results in nuclear translocation and enzymatic activity[242,275,276]. This increase in phospho-HDAC2 is evident throughout treatment and corresponds to increased nuclear HDAC2 localization and recruitment to the PRF1 and GZMB promoters. Further, decreased HDAC3 protein was observed after chronic GC treatment (Figure 14A-B). The phosphorylation of Ser394 on HDAC2 is regulated by casein-kinase-2 (CK2)[242]. Interestingly, a recent report demonstrates that CK2 can also phosphorylate HDAC3[267]. In contrast to HDAC2, the phosphorylation of HDAC3 by CK2 precedes its degradation[267]. These studies demonstrate CK2 to have opposing functions on HDAC2 and HDAC3 regulation. The results presented herein demonstrate that HDAC2 and HDAC3 are dichotomously regulated during chronic GC exposure; these observations could be explained by activation or increased CK2 mediated phosphorylation of HDAC2 and HDAC3 precipitating the dichotomous regulation of NK cell function. The implications for these findings are interesting as a specific CK2 inhibitor is available. Further investigation using this specific CK2 inhibitor could provide data supporting this hypothesis. Preliminary experiments using this CK2 inhibitor proved ineffective as CK2 is involved in a number of cellular processes including cell cycle regulation. NK92 cells treated with an inhibitor of CK2

demonstrated a significant decrease in both cellular proliferation and viability and thus no conclusions could be drawn from these experiments (data not shown).

In unpublished data we screened for potential changes in HDAC and HAT gene expression to investigate whether chronic GC treatment altered the expression of other histone modifying enzymes besides Class I HDACs described in this work. Quantification of HDAC1, 2, 3, and 4 as well as SIRT1 mRNA levels following chronic GC treatment demonstrated that HDAC1, 2, and 4 mRNA levels were unchanged throughout treatment (Figure 32A). However, HDAC3 mRNA levels were decreased at day 5 of treatment as demonstrated previously (Figure 32A). Furthermore, SIRT1 mRNA levels were increased after chronic GC treatment (Figure 32A). Whether the minimal but significant increase in SIRT1 contributes to the dichotomous phenotype observed remains to be investigated. Whether the increase in mRNA levels corresponded to changes in SIRT1 protein levels after chronic GC treatment NK92 cells was not investigated. The role of SIRT1 in the regulation of immune function has not been investigated and might provide an additional pathway by which GC regulate NK cell function.

Additionally, GR has been shown to interact with a number of HATs including CBP, PCAF, and p300. Analysis of HAT mRNA levels demonstrated that chronic GC treatment slightly but significantly increased P/CAF and CBP transcript levels (Figure 32B). Although global increases in P/CAF and CBP transcript levels were observed in Figure 32B concomitant increases in cellular protein levels were undetected (data not shown, the thesis work of Karen Krukowski). Additionally, preliminary ChIP experiments revealed no change in the recruitment of CBP, P/CAF, or p300 at any

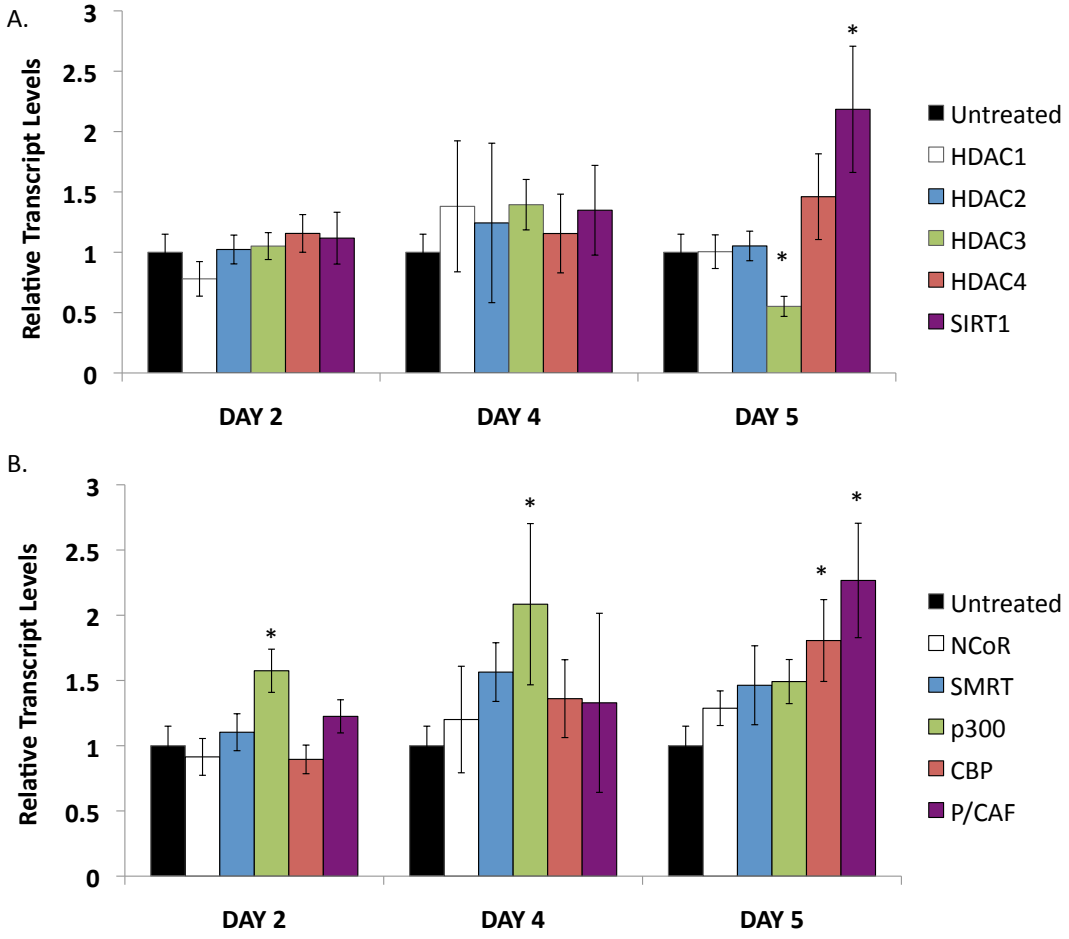


Figure 32. Quantitative expression analysis of coactivator and corepressor complexes during chronic GC treatment. (A) Relative transcript levels of HDAC1, 2, 3, 4 and SIRT1 in NK92 cells harvested at indicated days during treatment and standardized to transcript levels in untreated (black bars) cells set to 1. **(B)** Relative transcript levels of N-CoR, SMRT, p300, CBP, and P/CAF in NK92 cells harvested at indicated days during treatment and standardized to transcript levels in untreated (black bars) cells set to 1.

regulatory region investigated (data not shown). The role of these or other histone acetyltransferases still remains to be investigated. These preliminary results suggest that acetyltransferases are not recruited to the cytokine regulatory regions following chronic GC treatment, therefore it is believed that the concomitant increase in histone acetylation results from the loss of HDAC mediated inhibition and resident HAT activity rather than recruitment of additional HATs. Additionally, there was not a decrease in HAT levels at the lytic molecule regulatory regions. These data in total suggest that the recruitment of HDAC2 to these regions mediates the decrease in H3K9Ac directly or inhibits the activity of a resident HAT activity at these regions.

These data demonstrate that the GC induced promoter specific alterations at NK cell effector genes are the result of altered deposition of histone modifying enzymes. These promoter specific changes in HDAC recruitment occur concomitantly with global changes in HDAC subcellular localization. Therefore we investigated whether these changes in HDAC distribution imparted global changes across the epigenome.

Glucocorticoid induced global epigenetic phenotypes

In addition to the promoter specific epigenetic changes associated with GC treatment, we previously published that NK lytic function, NKCA, correlates with global histone acetylation patterns as well[117]. In the data presented by that study, nuclear localization of phosphorylated enzymatically active HDAC2 was increased after chronic GC treatment and thus might exert genome wide changes in histone tail modification in addition to the promoter specific changes described herein. HDAC2 has been shown to

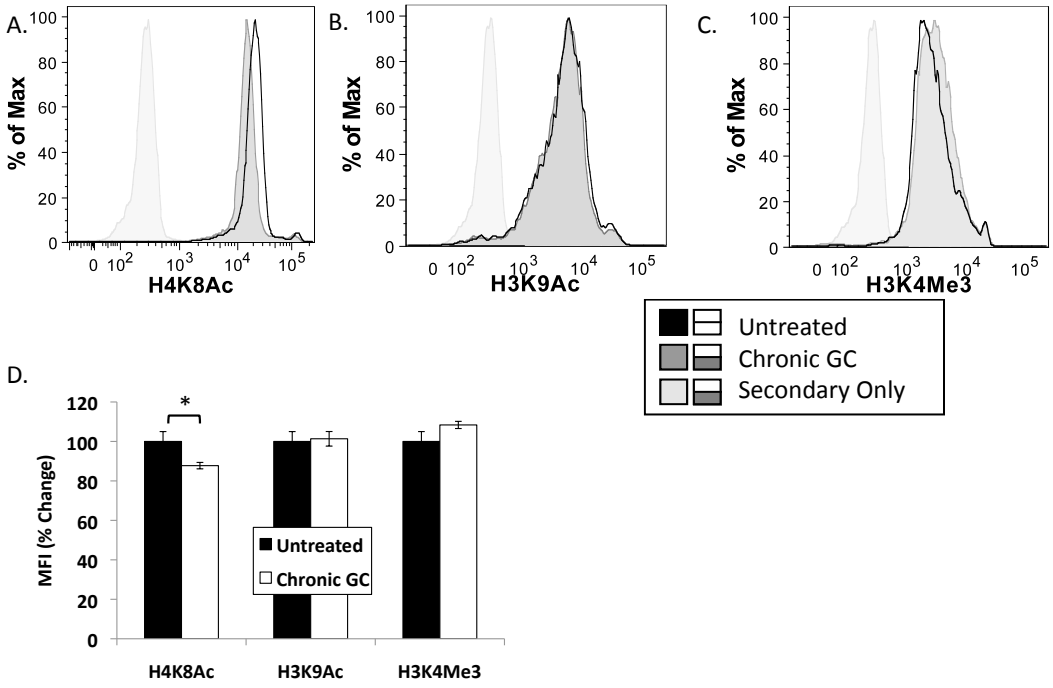


Figure 33. Global histone modifications after chronic GC treatment in NK92s. Representative histograms of global (A) H4K8 acetylation, (B) H3K9 acetylation, and (C) H3K4 trimethylation by flow cytometry in untreated (black line) and chronic GC treated (shaded) NK92 cells. (D) Quantification of MFI from flow cytometric analysis of global histone modifications in untreated (black bars) and chronic GC treated cultures (white bars) where the MFI of untreated cells is set to 100%.

deacetylate both H3 and H4 histone proteins. After chronic GC treatment a significant decrease in global H4K8Ac as measured by flow cytometry was observed, compared to untreated cells (Figure 33A and D). I hypothesize that HDAC2 recruitment in a gene specific manner directly regulates transcription and that the nuclear accumulation of HDAC2 also results in the loss of H4K8 acetylation at susceptible loci across the entire genome. These susceptible loci are most likely a combination of regulatory as well as non-regulatory intergenic regions to account for a greater than 10% decrease in total H4K8 acetylation across the genome after chronic GC treatment (Appendix Figure 33D).

Conclusions

To our knowledge this is the only demonstration that a dichotomous NK phenotype is demonstrable in a cell line. Furthermore, we demonstrate that GC alone can impart this dichotomous phenotype. Chronic GC treatment mediated these effects through changes in the transcription of NK cell effector genes. These transcriptional changes occurred via specific histone acetylation patterns imparted by GC induced HDAC redistribution. Cellular HDAC redistribution also imparted a global change in H4K8 acetylation. These pathways were then used to investigate changes in immune cell function during periods of psychological stress, known to result in dysregulation of glucocorticoid production *in vivo*.

Ex vivo analysis of human derived PBMC

Introduction

These *in vitro* observations may relate to the chronic effects of GC dysregulation; in humans. A large number of studies have shown psychological distress to impact immune function[277,278,279,280]. The stress response results from internal or external stimuli that activate ‘‘fight or flight’’ and “defeat/withdrawal” responses associated with the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenocortical (HPA) axis activation. It is well established that specific central nervous system pathways act to translate social stimuli into peripheral biological signals that regulate inflammatory responses [281]. For instance, stress activates neuroendocrine and autonomic pathways such as the HPA axis and the SNS, resulting in the release of GC, catecholamines, and pro-inflammatory cytokines such as IL-1, IL-6, and TNF alpha. The release of these sympathetic, neuroendocrine, and immune factors has a profound influence on immunity, behavior, and physiology in both humans and rodents; and triggers peripheral biological responses that, in turn, signal back to the CNS to complete a bi-directional communication circuit[282,283,284,285]. The effect of psychological distress on NK cells has been attributed to stress-induced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis [245]. Individuals experiencing chronic psychological distress exhibit HPA dysregulation resulting in altered GC levels[202,203] and disruption of the diurnal cortisol rhythm [162,179].

Effects of Perceived Stress on HPA axis regulation

Preliminary analysis between cancer and cancer-free groups in our study yielded no significant differences in diurnal cortisol rhythms or immune function (Figure 22). Furthermore, analysis of the cancer-free group demonstrated that 37% of these individuals exhibited PSS scores greater than 14 (Figure 21). Literature demonstrates that within the cancer-free population the average score for healthy "non-stressed" individuals is 12, while a score of 14 is the standard cutoff for stress. Thus investigating the impact of stress on hormonal and immune function was precluded by this relatively high number of individuals in the cancer free group exhibiting intermediate levels of perceived stress. In order to gain insight into how the perception of stress alters HPA activity, individuals were regrouped by dividing them into low, intermediate, and high stress groups using a tertile split based on the scores obtained from the PSS. Analysis of total cortisol levels as judged by area under the curve (AUC) demonstrated a quantitative increase in AUC values as stress increased from low, to intermediate, to high levels (Figure 20B). Pearson correlations confirmed that psychological stress, as measured by the perceived stressor scale, positively related with the AUC values (Figure 23A). These data demonstrate that as perceived stress increased, altered HPA axis regulation was observed, which was characterized by an increase in HPA activity.

Analysis of Immune Function between high and low stress

Abundant evidence indicates that psychological stress precipitates immune alterations and additionally the effects of GC have been readily demonstrable in peripheral blood mononuclear cells (PBMC)[246]. An additional layer of categorization

was applied by regrouping individuals based on their perception of stress as well the biological manifestation of stress (cortisol production) using Wards method of cluster analysis (Figure 21). This grouping allowed investigation into the immune phenotypes associated with both the perception of stress as well as the biological manifestations of HPA activation.

It has been demonstrated previously, that prior adverse events in which GC is dysregulated can "prime" inflammatory responses, promoting excessive cytokine production in response to subsequent stressors or stimuli [286-294]. In experimental animals, stressors have been shown to potentiate neuro-inflammation [295-299]. Our analysis demonstrates that circulating plasma IL-6 levels as well as IFN-g production from stimulated PBMC were quantitatively increased in individuals who exhibit high stress (Figure 22). Pearson correlations were used to determine how HPA activity was related to this apparent increase in proinflammatory cytokine production. AUC values positively correlated with both IFN-g and IL-6 production from stimulated PBMC (Figure 23D-E). Additionally, IL-6 and IFN-g production were positively correlated (Figure 23C). These data suggest that the proinflammatory cytokines, IFN-g and IL-6, are coordinately regulated in the PBMC of individuals and that increased perceived stress and the accompanying increase in HPA activity results in a proinflammatory phenotype.

Natural killer cells are especially responsive to GC and NKCA is especially responsive to psychological stress, with ample evidence demonstrating psychological stress to reduce NKCA[199,300]. Analysis of *ex vivo* derived PBMCs yielded reduced NKCA standardized to percentage CD56+ in the PBMC of individuals exhibiting both

the psychological and biological phenotypes of stress (Figure 22). These observations demonstrate that psychological stress, as classified by the phenotype of increased perceived stressor and increased daily cortisol production, corresponded with reduced NKCA and increased proinflammatory cytokine production. This dichotomous phenotype was confirmed using Pearson correlations demonstrating a significant negative correlation between NKCA standardized to CD56+ cell percentages and IFN-g production from stimulated PBMC (Figure 23B).

There are examples in which an apparent similar dichotomous phenotype has been demonstrated. For example Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease characterized by fatigue, pain and muscle aches, as well as cognitive impairment [301]. Inflammatory pathways play a key role in the pathophysiology of ME/CFS [302] and encompass low-grade inflammation, with increased circulating levels of cytokines, including IL-1alpha and beta and IL-6 [303,304] as well as immunosuppression characterized as lowered NK cell lytic activity and decreased lymphocyte expression of activation markers, such as CD69[305,306,307]. Of particular relevance, the dichotomous phenotype was demonstrated in CD56+ lymphocytes derived from these patients.

Many studies have investigated the regulation of NK cell effector function during periods of psychological distress[199,202,203,300]. Some studies suggest that the expression levels of CD56 on human NK cells is related to effector function, in that, $CD56^{bright}$ NK cells are more proinflammatory while $CD56^{dim}$ NK cells are more cytolytic. No significant differences in the percentages of CD56+ cells were observed between our cancer and cancer free groups or any grouping investigated. Furthermore,

no change in the ratio of $CD56^{bright}$ vs $CD56^{dim}$ were observed within individuals in any of the grouping or cluster strategies I used (data not shown). From these observations we conclude that chronic exposure to GC *in vitro* or psychological stress did not alter the expression of CD56 or the ratio of $CD56^{bright}$ vs. $CD56^{dim}$ populations.

The above data demonstrate that clustering individuals based on their perceived stress as well as daily cortisol production yielded significant differences in immune function (i.e. enhanced proinflammatory cytokine production and reduced NKCA). In the reverse, classification of individuals based on the immune phenotypes of NKCA and IFN-g production from *ex vivo* derived PBMC yielded significant differences in both perceived stress and cortisol production (Figure 26A-B). These observations in total demonstrate that stress imparts altered immune function within individuals who exhibit increased HPA activity. Secondly, this dichotomous immune phenotype of enhanced proinflammatory cytokine production and reduced NKCA within PBMC can be used to characterize individuals with both increased perceived stress and HPA activity.

Perceived stress and global epigenetic patterns

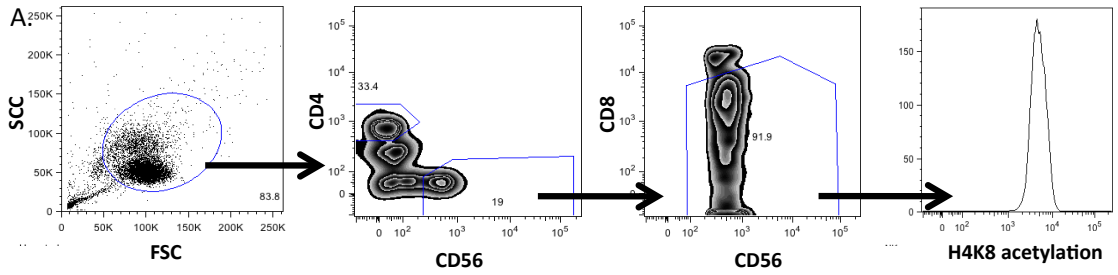
We have previously published that stress is associated with a reduction in the global acetylation of H4K8 within CD56+ cells[308]. In that study, individuals with low, medium, and high stress exhibited high, medium, and low H4K8Ac MFI in the CD56+ NK cell population, respectively. In the current study, NK cells were gated as the CD4-CD8-CD56+ cell population and the MFI of acetylated H4K8 was calculated from this gate. A representative schematic of the gating parameters to determine the MFI

of histone acetylation is presented in Figure 34A. A significant negative correlation was observed between PSS scores and the global levels of H4K8 acetylation in CD56+ NK cells (Figure 34). A representative histogram containing two individuals who exhibit high PSS scores (average global MFI = 5719), two individuals who exhibit intermediate PSS scores (average global MFI = 16259), and two individuals who exhibit low PSS scores (average global MFI = 25548) (Figure 34B and D). These observations confirm our previous findings and suggest that epigenetic mechanisms may mediate immune dysregulation within the NK cell population.

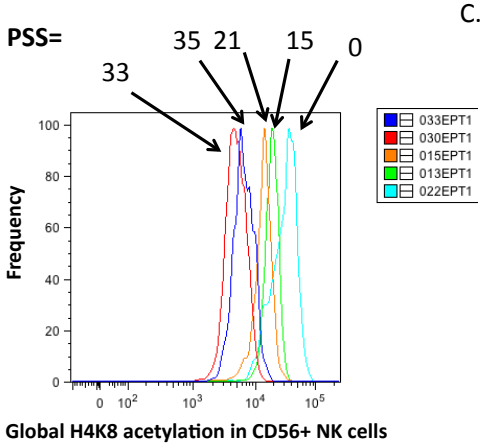
Histone acetylation as a meditor of stress induced IFN-g production

To determine if epigenetic mechanisms could explain the enhanced cytokine production after PBMC stimulation, promoter specific epigenetic changes were investigated within the regulatory loci of proinflammatory cytokine genes. H3K9 can be acetylated as well as methylated. The acetylation versus methylation of H3K9 are present in distinct chromatin regions. Acetylation of H3K9 is enriched in euchromatic, actively transcribed regions while tri-methylation of H3K9 is enriched in silenced heterochromatic regions. H3K9 modifications were used as a tool to investigate the epigenetic patterns at proinflammatory cytokine loci to gain further insight into their regulation during periods of stress. By calculating the ratio of H3K9Ac/H3K9me3 a relative index of active (turned on) to inactive (turned off) proinflammatory cytokine loci could be obtained. The ratio of acetylated H3K9 to trimethylated H3K9 was quantitatively increased at both the IFNG and IL6 loci when compared between

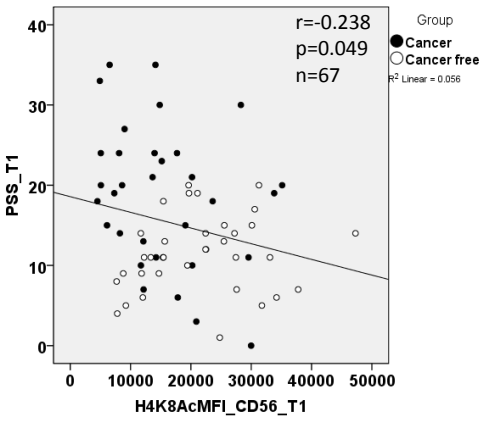
A.



B.



C.



D.

Patient	PSS	H4K8Ac MFI
013EPT1	15	19063
015EPT1	21	13455
021EPT1	3	20773
022EPT1	0	30322
030EPT1	33	4950
033EPT1	35	6489

Figure 34. Global H4K8 acetylation levels with CD56+ cells at T1. (A) Gating scheme for assessment of global H4K8 acetylation MFI within CD4-CD8-CD56+ cells by flow cytometry. (B) Representative histogram depicting 5 patients from cancer group who exhibit low (PSS<14), intermediate (15-21), and high (22+) stress. PSS scores of individual patients are depicted above arrows. (C) Scatterplot representing the relationship between PSS scores and global H4K8 acetylation at T1. (D) Table depicting patient identification, PSS score, and global H4K8 acetylation MFI within CD56+ cell population at T1.

high and low stressed individuals (Figure 22E-F). These results demonstrate that individuals with increased perceived stress and increased HPA activity also have a a higher percentage of PBMC with a signature of more open chromatin structure at the proinflammatory regulatory loci and thus a higher potential for cytokine gene expression. This relationship was apparent in the population indicated by a significant positive correlation between the ratio of histone acetylation to histone methylation at the *IFNG* locus and stimulated IFN-g production from PBMC in this study (Figure 24B). In total these data demonstrate that as stress increases the biological manifestation of this stress is increased HPA activity and an epigenetic priming of cytokine loci for expression. Furthermore, this priming was related to expression from these loci and constitutes a potential mechanism for the proinflammatory phenotype of psychological stress.

In order to determine whether the stress-associated increase in IFN-g production was mediated by changes in histone acetylation a mediation model was investigated. The results of the mediation analysis indicate that PSS directly influences IFN-g production and indirectly influences *IFNG* expression by its effects on histone acetylation. In a full mediation model, the direct effect of PSS on IFN-g production would have become insignificant when the role of histone acetylation was added to the model. The model demonstrates only partial mediation because the path between PSS and IFN-g production (path c) stays significant (Figure 29). These data demonstrate that the enhanced proinflammatory cytokine production observed during stress is partially mediated by the epigenetic priming of cytokine regulatory loci via histone acetylation but additional mediators are present.

Conclusion

A stressor constitutes a challenge to the individual which manifests as physiologic alterations [309], and the results observed herein suggests that these effects can be integrated at an epigenetic level. The interplay between the environment and the epigenetic landscape remains poorly understood; this is particularly true with regard to the endogenous signals that alert the immune system to danger and/or threat. The ability of GR, when ligand activated by GC, to sense environmental signals of danger or stress due to changes in GC, appears to actively control or sense the environment affecting the epigenetic landscape and consequently alter gene expression programs essential to immune function[310]. The overlapping yet specialized physiological contribution of GC is to perform essential functions. For NK cells, which migrate to diverse sites within the body and continually survey for both infectious and malignant challenges, such a scenario may be functionally important in protection of the host. The findings presented herein indicate that GC are a means by which immune functions can be epigenetically primed for altered activity in response to environmental signals such as psychological stress.

Comparative analysis of *ex vivo* and *in vitro* systems

In this body of work *in vitro* chronic low level glucocorticoid exposure was used as a model to understand the mechanisms of GC regulation of immune function *in vivo* during periods of psychological stress. In this study, the psychological stress attributed to breast cancer diagnosis resulted in increased daily cortisol production. These observations were made at or within two weeks of diagnosis at timepoint 1 (T1). Chronic GC treatment *in vitro* lasted for 5 days and would fall within the timeframe window of our T1 timepoint. In unpublished data, when chronic GC treatment was extended to 10 days a similar dichotomous phenotype was observed but maintained its effects and was not exacerbated by the extended time (data not shown).

Both chronic low level GC exposure *in vitro* and increased daily cortisol levels *in vivo* corresponded with enhanced production of IFN-g and IL-6 protein upon cellular stimulation. These results highlight that the effects of GC on cytokine transcription occurs via a priming mechanism, as both systems exhibit no change in basal cytokine production but only after cellular stimulation. In both systems promoter specific histone acetylation patterns were associated with enhanced cytokine production. We did observe differences in the specific acetylation marks which regulate cytokine production between our two system. *In vitro* H4K8 and H3K27Ac were markers IFNG and IL6 epigenetic priming while H3K9Ac was a marker in *ex vivo* derived PBMC. Both observations involve changes in histone acetylation and thus correspond with transcriptional activation. These differences could be explained by a number of mechanisms. Our *in vitro* system uses an immortalized clonal cell line; how cellular

transformation and continued proliferation impacts the epigenetic regulation of NK cell effector genes is unknown and could mask changes in specific histone acetylation marks which are more maliable than DNA and histone methylation. This is in contrast to the heterogenous pool of NK cells in the circulation, which exhibit different levels of activation, expression of CD56, and differentiation states. Additionally, our *ex vivo* observations use whole PBMC which include B cells, T cells, and NK cells. The ratio of H3K9Ac to H3K9me3 presented would represent the relative number of PBMC acetylated at the IFNG enhancer and thus would indicate that these immunological effects are not confined to the NK cell subset as they only represent 5-15% of circulating PBMC. Additionally, our *in vitro* culture system cannot test the interaction of the many immune cell populations present in whole PBMC. Furthermore, circulating immune cells experience constant signaling from the host in addition to GC. In that humans are continually exposed to foreign agents from commensal bacteria, diet, as well as pathogens. How these constant normal commensal relationships and constant low level exposure to pathogens effects and interacts with GR signaling in circulating immune cells is beyond the scope of this project. Although the specific histone mark is different the overall conclusions are the same, that increased daily exposure to GC increases histone acetylation at proinflammatory regulatory regions.

A number of studies suggest that the enhanced proinflammatory cytokine production associated with psychological distress occurs via a mechanism involving glucocorticoid resistance. These hypotheses are based on observations, in a model of depression, where the EC50 of dexamethasone inhibition of stimulated cytokine production was increased in *ex vivo* derived PBMC. The authors hypothesize that this

results from GC resistance via changes at the level of the GR, although no direct evidence of any such changes in GR was demonstrated. Additional studies demonstrate that enhanced transcription of the NF-κB regulon occurs during psychological stress[311,312]. Authors of these studies suggest that this results from alterations in GR and/or NF-κB functional activity. The results from both our systems are parsimonious with the latter as IFN-g and IL-6 investigated here are part of the NF-κB regulon. However, neither of the previous studies directly test the mechanism for enhanced production of proinflammatory cytokine. We propose that these observations can be explained by the epigenetic priming of specific genes within the NF-κB regulon as demonstrated in this body of work. Epigenetic priming would produce the same phenotype without any direct changes in the functional capacity of either GR or NF-κB. We did not directly test the functional capacity of GR or NF-κB within *ex vivo* derived PBMC, but we did directly test both *in vitro* during chronic GC treatment. Chronic GC treatment exhibited no effect on GR or NF-κB levels nor the functional capacity of either transcription factor.

This body of work focused on the mechanisms governing the enhanced cytokine production associated with psychological stress. We were also interested in the mechanisms regulating the reduced lytic potential of NK cells after chronic GC treatment and during periods of psychological stress. Our *in vitro* data demonstrates that NK cell lytic molecules are also regulated epigenetically after chronic GC treatment. This is characterized by reduced H3K9 acetylation at both the *PRF1* and *GZMB* promoters (Figure 11A-B). Although reduced NK cell lytic activity was observed in individuals exhibiting increased perceived stress; relationships between NKCA and lytic

molecule production, as measured by flow cytometry, exhibited a trend towards a positive relationships but did not reach significance (data not shown). As described in the introduction, NKCA is a complex process and is regulated at multiple stages. Furthermore, perforin and granzymeB are constitutively produced and stored in lytic granules until degranulation occurs. The timeframe in which reductions in lytic molecule transcription would correspondingly exhibit reductions in the protein levels of these molecules *in vivo* remains unclear, as the exact half-life of lytic molecules within CD56+ granules *in vivo* has not been investigated. Thus further analysis of lytic molecule transcription as well as protein production would yield the most insights into the regulation of these molecules in regards to psychological stress.

The lytic activity of NK cells is also regulated by the surface expression of KIRs. A number of reports suggest that psychological stress can alter the expression of both NK cell activing and inhibitory receptors[121,313,314]. An additional complication is the variability of NK cell activating and inhibitory receptor expression between individuals[314]. The expression of KIR expression was not assessed in this study and we cannot exclude the contribution of receptor expression on NK cell functional activity during periods of stress. Investigation of NKp30 and NKp46 expression on NK92 cells following chronic GC treatment exhibited no significant changes in surface expression (Table 4).

Our lab has previous published that PSS is also associated with global changes in histone modifications[308]. Specifically the acetylation of H4K8 is reduced across the entire genome in individuals with high stress[308]. These observations were validated in this study demonstrating that PSS scores negatively relate with global H4K8

acetylation in the CD56+ cell population (Figure 34C). Global reductions in H4K8-acetylation were also observed in our *in vitro* system and suggest that this phenotype can be used as a marker of NK cell function activity. Our *in vitro* observations demonstrate that as early as day 2, phospho-HDAC2 levels are increased in the nucleus and remain elevated throughout treatment. I would speculate that individuals would exhibit increased PSS and AUC would have increased nuclear localization compared to low PSS and AUC individuals. Prelimary results from 3 high stress and 3 low stress individuals show this trend (Appendix Figure 31). Thus global H4K8 acetylation seems to be a marker for the immune phenotypes observed during stress and possibly could be used as a diagnostic tool.

Chronic GC treatment resulted in alterations in both HDAC2 and HDAC3 that regulated the transcription of lytic molecules and cytokines, respectively. These *in vitro* observations suggest the HDAC2 and HDAC3 are dysregulated within the CD56+ cell population during periods of psychological stress. The results from *in vitro* chronic GC treatment would suggest that HDAC3 is involved in the regulation of these inflammatory processes. Total HDAC3 protein levels were decreased following chronic GC treatment, additionally HDAC3 levels were decreased at the IFNG and IL6 loci corresponding with the increased histone acetylation. I would speculate that individuals who exhibit increased PSS and AUC would exhibit decreased HDAC3 protein levels within the CD56+ cell population.

In conclusion, *in vitro* chronic GC treatment of an NK cell line was used to investigate the molecule mechanism(s) which mediate altered immune function during periods of psychological stress. This body of work characterized a dichotomous

phenotype of reduced NKCA and enhanced proinflammatory cytokine production after chronic low level GC treatment. These observations were continued using *ex vivo* derived PBMC from individuals diagnosed with breast cancer as a model of psychological stress. Analysis revealed that the perceived stress of cancer diagnosis corresponded with increased daily cortisol production. Increased perceived stress, with the accompanying increases in cortisol production, corresponded with decreased NKCA and enhanced cytokine production, a dichotomous phenotype similar to that observed following chronic GC treatment *in vitro*. Lastly, this enhanced cytokine production was partially mediated by the acetylation of proinflammatory cytokine loci. These observations demonstrate that GC through epigenetic pathways mediate changes in immune function.

Stress induced immune changes: A benefit or detriment to the host

With the observations that psychological stress results in altered immune function, the question becomes whether this is a dysregulation of the immune system and is thus detrimental or somehow beneficial to the host. Acute stress such as the flight or flight response are scenarios in which the host has a higher probability of injury and subsequent infection. Acute stress, accompanied by a rapid increase in cortisol and other adrenocorticoids, leads to an influx of newly differentiated immune cells into the circulation from the bone marrow. Additonally, these factors redistribute circulating innate immune cells, such as NK cells, to migrate from the circulation into the peripheral tissues to potentially detect injury or infection. These changes in surface adhesion

molecule expression by NK cells have been shown in acute stress models in both mice and humans, and have been shown to involve both GC and adrenergic receptor mediated mechanisms. Thus it is hypothesized that acute stress prepares the immune system for the potential of injury or infection leading to a more rapid immune response that is evolutionarily beneficial for host survival.

Whether the immune regulation associated with chronic on-going stress as demonstrated in this work is evolutionarily beneficial or not is of intense debate. Most literature regards the immune alterations associated with chronic stress as detrimental to host health and quality of life. Chronic stress has been repeatedly associated with decreased NKCA. The decreased NKCA associated with stress is of particular relevance to breast cancer because epithelial tumors are responsive to the immune protective effects of NK cells and IFN-g[206,207,208,209]. The importance of NK cell activity is highlighted by findings involving the relationships between cancer and NKCA. First, high levels of NKCA in cancer patients correlate with a good prognosis[214,215,216,217]. Impaired NKCA also correlates with invasiveness of human malignancy[218]. NK cell infiltration into primary tumors is associated with fewer metastases to the lymph nodes and less lymphatic invasion[214]. Lastly, overall survival for patients with high and low NKCA was 71 vs. 30 weeks, respectively and NKCA was an independent prognostic factor for overall survival [215].

The second phenotype highly associated with chronic stress is a low grade inflammatory phenotype. Over the last decade inflammation has been attributed as a leading factor in the progression of a number of diseases. Low grade inflammation has been associated with multiple disease states including atherosclorosis, heart disease,

activation of autoimmune diseases, and arthritis. Thus both of the immune phenoypes associated with chronic stress are implicated in poor health outcomes and reduced quality of life. So why would these detrimental phenotypes persist evolutionarily?

One newly developed view, does not ask the question why the inflammatory state of chronic stress detrimental but when would these effects be beneficial in the life of a host. More specifically when, evolutionarily, would this proinflammatory state be beneficial to the host thus promoting survival and such alleles would be passed on to future generations. Termed the Pathogen Host Defense (PATHOS-D) hypothesis, it suggests that in infancy and early childhood the adaptive immune response is not at fully potency and does not contain the full arsenal of pathogen specific immune cells (i.e memory T and B cells) primed and ready to fight infection[315]. Thus a robust innate proinflammatory response to infection early in life would be beneficial. This is in contrast to adulthood, most relevant to this study in which the adaptive immune system is fully functional and able to mount a quick and pathogen specific response. These hypotheses imply that perhaps in childhood these effects are beneficial and the resilience of childhood homeostatic mechanisms permits eventual resolution of these detrimental effects but in adulthood they are maladaptive to the host[315].

These hypotheses are obviously in their infancy but provide interesting concepts for future investigation. Here in I have uncovered a potential mechanism by which chronic stress imparts these immune phenotypes. Epigenetics provides a means by which the activity of the immune system can be modulated in response to the environment without direct changes to the DNA sequence. Furthermore, epigenetic changes allow for the transcriptional priming of genes, in essense, a preparing of the

immune system. The characterization of these epigenetic mechanisms provides researchers with potential avenues for therapies to overcome the effects of stress on the immune system but also avenues in which to modulate the activity of NK cells for other immune based disorders.

Clinical Significance and Future Work

Research into the alterated immune function during periods of stress have also illucidated genetic, social, and intervention mechanisms that can ameliorate or dampen the effects of stress on these biological outcomes and quality of life. Of particular relavence to our lab are the results from our published work regarding the Mindfulness Based Stress Reductions (MBSR) paradigm[202]. MBSR stems from contemplative Eastern spiritual practices that use meditation to cultivate conscious awareness (i.e., mindfulness) of one's experience in a non-judgmental or accepting manner (Kabat-Zinn, et al., 1990). In this study enrollment into the MBSR program could ameliorate some aspects of the immune dysregulation associated with the stress of breast cancer diagnosis compared to a group of women who were similarly diagnosed but enrolled in a control eduction group[202]. These observations involving the positive aspects of MBSR can now be expanded to investigate the mechanisms uncovered in this body of work. Does MBSR mediate these effects through restoration of HPA activity or through a secondary pathway which can overcome these epigenetic mechanisms?

Conclusions

To our knowledge this is the only demonstration that dichotomous NK phenotype is demonstrable in a cell line. Here, we demonstrate the effect of chronic GC on the active promoter and enhancer landscape of NK effector genes. Chronic GC treatment regulates 2 members of the class I HDAC family imparting both promoter specific and genome wide changes in histone acetylation patterns. The ability of the immune system to sense and interpret environmental stimuli and appropriately modify gene expression is a fundamental tenet of immune responsiveness. Whether the stimulus is exogenous or endogenous (as in the case of GC), the final decoding of the message occurs at the genome level, resulting in either activation or repression of transcription. Taking part in the epigenetic landscape and chromatin structure, which determines accessibility of regulatory elements such as promoters and enhancers for transcription factors that carry out cell-type specific gene expression programs. This may be of particular relevance to understanding the effect of GC on immune responsiveness during stressful events. We further demonstrate that cancer diagnosis, a stressful event, culminates in the overactivity of the HPA axis resulting in immune cells exposed to increased levels of cortisol for a period of days. Furthermore, stress was also associated with a dichotomous regulation of immune function characterized by decreased NKCA yet enhanced proinflammatory cytokine production. Mechanistically, the increased IFN-g production associated with stress was mediated in part by promoter specific acetylation patterns within the IFNG locus. Lastly, stress is associated with genome wide changes in acetylation patterns. These observations provide mechanistic insights

and diagnostic tools to more fully characterize the immune regulation during psychological stress.

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VITA

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