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Glucocorticoid Dysregulation of Natural Killer Cell Function through Epigenetic Modification

Karen Krukowski[a], **Justin Eddy**[a], **Kelly Loster Kosik**[a], **Teresa Konley**[a], **Linda Witek Janusek**[b], and **Herbert L. Mathews**[a,*]

[a] Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University of Chicago, Maywood, IL 60153 USA

[b] Marcella Niehoff School of Nursing, Loyola University of Chicago, Maywood, IL 60153 USA

Abstract

It is well-established that psychological distress reduces natural killer cell activity (NKCA) and dysregulates cytokine balance. This maybe mediated by stress-induced release of glucocorticoids, which have broad effects on the immune system, including the suppression of NKCA and alteration of cytokine production. The purpose of this study was to evaluate epigenetic mechanisms that may underlie the effect of glucocorticoids on NK cells, using the human NK cell line, NK92. Treatment of NK92 cells with the synthetic glucocorticoid, dexamethasone, at a concentration of 10^{-7}M, produced a significant reduction in NKCA. Glucocorticoid inhibition was a consequence of not only a reduced capacity of the NK cells to bind to tumor targets but also a reduced production of granule constituents (perforin and granzyme B) with no detectable effect on granule exocytosis. Glucocorticoids also reduced the constitutive and the stimulated production of the cytokines, IL-6, TNF alpha and IFN gamma, and reduced the surface expression of LFA-1. Glucocorticoid treatment also reduced global histone acetylation, the acetylation of histone 4 lysine position 8, and the accessibility of the proximal promoters of perforin, interferon gamma and granzyme B. Histone acetylation was recovered by treatment of the NK cells with a histone deacetylase inhibitor, which also restored NKCA and IFN gamma production. These results demonstrate glucocorticoids to dysregulate NK cell function at least in part through an epigenetic mechanism, which reduces promoter accessibility through modification of histone acetylation status. This epigenetic modification decreases the expression of effector proteins necessary to the full functional activity of NK cells.

Keywords

Natural killer cell; Epigenetic; Histone Acetylation; Cytokines; Glucocorticoid

[*]Correspondence author. Herbert L. Mathews, Department of Microbiology and Immunology, Loyola University of Chicago, 2160 South First Ave., Maywood, IL 60153 USA. Tel. (708) 216-4586, Fax. (708) 216-9574, hmathew@lumc.edu.
Karen Krukowski and Justin Eddy contributed equally to this manuscript.

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1. Introduction

Natural killer (NK) cells are multi-functional lymphocytes that do not require a pre-activation process and are not restricted to antigen presentation by major histocompatibility complex molecules. Their rapid action places NK cells as a front line of defense against cancer and infectious agents (Biron, 1999; Gumperz and Parham, 1995; Trinchieri, 1989). Considerable attention has been paid to the direct anti-tumor activities of NK cells (Alexandroff et al., 1998) and evidence has existed for some time for their role in tumor protection. However, more recent direct evidence has shown these cells to be involved in immune surveillance, controlling the initiation and growth of tumors (Dighe et al., 1994; Kagi et al., 1994; Kaplan et al., 1998; Smyth et al., 1998; Smyth et al., 1999; Street et al., 2001; Van Den Broek et al., 1996; Seki et al., 2003). NK cells produce their effects against tumor cells by first engaging the tumor target, followed by exocytosis of NK cytolytic granule constituents against the tumor cell, disengagement of the tumor cell by the NK cell and lysis of the tumor targets.

A large number of studies have shown psychological distress to negatively impact immune function (Witek-Janusek and Mathews, 2000; Kemeny and Schedlowski, 2007; Glaser and Kiecolt-Glaser, 2005; Segerstrom and Miller, 2004). Reciprocal neuro-chemical pathways and shared receptor systems link the brain and the immune system and provide biological pathways whereby one's emotions influence immune function. NK cell activity (NKCA) is especially responsive to the impact of psychological stress and ample evidence demonstrates that psychological distress or negative affective states reduce NKCA (Biondi, 2001; Kiecolt-Glaser et al., 1987). Psychological distress also results in an altered pattern of cytokine production (Maes et al., 1999; Marshall et al., 1998; Witek-Janusek et al., 2007), including reductions in the production of interferon (IFN) gamma (Witek-Janusek et al., 2007; Glaser et al., 1986). Meta-analytical review (Herbert and Cohen, 1993) demonstrates life stressors to be reliably associated with immunological alterations. The effect of psychological distress on NKCA and cytokine production has been, in part, attributed to elevations in glucocorticoids produced by stress-induced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis (Schoneveld and Cidlowski, 2007). Individuals experiencing psychological distress exhibit HPA activation resulting in cortisol dysregulation (Witek-Janusek et al., 2007; Witek-Janusek et al., 2008) with disruption of the diurnal cortisol rhythm (Sephton et al., 2000; Bower et al., 2005). An altered cortisol rhythm predicts NKCA and cancer survival (Bower et al., 2005) with levels of distress correlated inversely with NKCA (Thornton et al., 2007). Glucocorticoid effects at the cellular level are multiple but previous work has demonstrated that reduced transcription factor (AP-1 and NFkB) nuclear localization does not contribute to continuous stress-induced immune dysregulation (Nagabhushan et al., 2001). However, epigenetic modifications are known to regulate gene transcription and physiologically elevated glucocorticoid levels are known to result in deacetylation of histones with reduced expression of immune response genes (Cippitelli et al., 1995; Santourlidis et al., 2002; Chan et al., 2005). In particular, glucocorticoids affect acetylation of histones through direct inhibition of histone acetyl transferase activity and by active recruitment of histone deacetylase (HDAC) complexes (Ito et al., 2000), which results in deacetylation of histones, increased tightening of DNA, and reduced gene expression (Cippitelli et al., 1995). This effect is most dramatic with selective deacetylation at histone 4 lysine position 8 (H4-K8), resulting in suppressed gene transcription (Ito et al., 2000; Matthews et al., 2004; Tsaprouni et al., 2002). Histone acetylation plays a significant role in gene transcription. Acetylation results in local chromatin structural unraveling, making DNA accessible for binding by transcription factors and basal transcriptional machinery (Y. J. Li et al., 2004). Acetylation is reversible and reflects a dynamic balance between the activities of histone acetyltransferases (HATs) and HDACs. Chromatin structure is known to regulate expression of molecules that mediate NKCA (e.g. perforin, granzymes, and NK cell

Ig-like receptors) (Araki et al., 2008; Lu et al., 2003; Santourlidis et al., 2002; Chan et al., 2005) and to control IFN gamma production (Hatton et al., 2006; Chang and Aune, 2005; Yano et al., 2003; Jones and Chen, 2006). The effects of glucocorticoids on chromatin structure and on immune response genes have been shown to be recovered by HDAC inhibitors, like Trichostatin A (TSA) (Kagoshima et al., 2001; Mishra et al., 2001; Plesko et al., 1983).

This study sought to determine whether glucocorticoids reduce NK functional activity and alter cytokine production through epigenetic modification of histone residues. The human NK92 cell line was used to identify those functional activities of NK cells that are affected by dexamethasone. Once established, the effect of dexamethasone on proteins known to carry out those functional activities was determined. Promoter accessibility for the genes of a subset of those identified proteins were then evaluated at the epigenetic level to determine not only the accessibility of the proximal promoters of those genes but also to determine the contribution of histone acetylation status of H4-K8 to that accessibility. The results demonstrate reduced acetylation of H4-K8 to be associated with reduced functional activity of NK cells. The reduced functional activity is a consequence of the reduced accessibility of relevant immune response gene promoters. The result is reduced expression of proteins necessary to the full functional capacity of NK cells to lyse tumor targets and to produce cytokines. Epigenetic modification, induced by dexamethasone, appears to be one mechanism by which natural killer cell function is dysregulated by glucocorticoids.

2. Materials and Methods

2.1. Cell Culture and Treatment

The human erythroleukemic like cell line, K562, was obtained from the American Type Culture Collection, Rockville, MD. K562 cells were maintained in suspension cultures *in vitro* in Corning 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) low LPS; (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100ug/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY). NK92 cells (established from a patient with non-Hodgkin's lymphoma with the capacity to lyse a broad range of leukemia, lymphoma and myeloma cell lines at low effector to target ratio *in vitro*) were obtained from the American Type Culture Collection, Rockville, MD and maintained in alpha MEM with 12.5% horse serum (Gibco Laboratories, Grand Island, NY), 12.5% fetal bovine serum (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100ug/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.2 mM inositol (Sigma Aldrich, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Gibco Laboratories, Grand Island, NY) and 0.02 mM folic acid (Sigma Aldrich, St. Louis, MO). NK92 cell cultures were also supplemented with IL-2 (100 units/ml).

2.2. Cellular Treatment

NK92s, cultured at 2.5×10^5 cells/ml, were treated with dexamethasone (Sigma Aldrich, St. Louis, MO) for most experiments at 10^{-7} M for 24 hrs in the absence of IL-2. This concentration of dexamethasone was chosen because it did not influence the viability of the cell line and is a concentration demonstrated previously to differentially regulate dexamethasone responsive genes (Reddy et al., 2009). This concentration of dexamethasone approximates physiologic concentrations (M. Schmidt et al., 1999). Other concentrations of dexamethasone examined ranged from 10^{-6} M to 10^{-10} M. After treatment with dexamethasone, NK92s were washed with media and resuspended to 1×10^6 cells/ml for assay. In a similar manner, NK92s were treated with dexamethasone (10^{-7} M) for 24 hours

and then Trichostatin A (Cell Signaling Technology, Danvers, MA) (100 nM) (TSA) was added to cultures for 1 to 6 hrs. One hr TSA treatments were used for NKCA, histone analysis (western and flow cytometric) and Chromatin Immunoprecipitation (ChIP) assays and 6 hr TSA treatments were used for intracellular IFN gamma analysis. NK92s were washed and resuspended with media to 1×10^6 cells/ml. Cell number and viability were determined by exclusion using 0.1% Trypan blue.

2.3 Immunofluorescent Flow Cytometric Analysis of Intra-Nuclear Epigenetic Pattern, Intracellular Proteins and Surface Proteins

NK92 cells (1×10^5/assessment) 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 then probed with antibodies specific for molecules of interest. For histone residues, cells were probed with antibodies specific for the acetylated form of lysine position 8 of histone 4 (H4-K8 Ac) (Millipore, Temecula, CA) (unconjugated) at 1:200 dilution. The cells were then washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA), after which secondary anti-IgG (FITC conjugated) (Millipore, Temecula, CA) was added for 1 hr at 4°C. Following this treatment, the cells were again 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 hr at 4°C. Following antibody staining the cells were washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA) in 0.1% BSA (Sigma Aldrich, St. Louis, MO) PBS (Gibco, Grand Island, NY). Antibodies for intracellular staining were anti-interferon (IFN) gamma (PE conjugated) (BD Biosciences, San Jose, CA), anti-granzyme B (Alexa Fluor 647 conjugated) (BD Biosciences, San Jose, CA) and anti-tumor necrosis factor (TNF) alpha (PE-conjugated) (BD Biosciences, San Jose, CA). For intracellular cytokine analysis of IFN gamma and TNF alpha, cells were incubated in leukocyte activation cocktail (BD PharMingen, San Jose, CA) at 37°C for 4 hrs prior to permeabilization and antibody staining. For surface molecule staining, cells were not permeabilized. Instead, cells 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-CD335 (NKp46) (PE conjugated) (BD Biosciences, San Jose, CA) and anti-LFA-1 (CD11a) (FITC conjugated) (BD Biosciences, San Jose, CA). After staining samples were analyzed by flow cytometry with a FACSCanto Fluorescence-Activated Cell Sorter equipped with a 15mW argon-ion laser and a red diode laser using FACSDiva software for data acquisition (Beno et al., 1995; Yamamura et al., 1995; Nagabhushan et al., 2001). 10,000–30,000 events were recorded and analyzed with FlowJo v8.4.1. Flow cytometric analysis was confirmed by fluorescence microscopy.

2.4 Cell Conjugation Assay

K562 were suspended in 1 ml of 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) at a final concentration of 5×10^6/ml, then labeled for 10 min at 37 °C with CFSE (2 uM, Sigma Aldrich, St. Louis, MO). The cells were washed three times with ice-cold 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY). NK92 cells were labeled with CD56 (APC-conjugated) (BD Biosciences, San Jose, CA) reactive antibodies for 30 minutes on ice and agitated after 15 minutes. Following antibody staining the cells were washed twice with 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) and resuspended in alpha-MEM media (Gibco Laboratories, Grand Island, NY). NK92 cells at a concentration of 5×10^6/ml were mixed with 5×10^6/ml

CFSE-labeled K562 target cells at an effector to target ratio of 1:1, allowing the formation of effector-target conjugates. The NK92-K562 cell mixture was centrifuged at 1,000 rpm for 2 seconds and incubated at 37 °C for 10 min. Then the cell mixture was gently resuspended in 0.1% BSA in PBS and fixed with 1% PFA in PBS (Sigma Aldrich, St. Louis, MO). The conjugation ratio was calculated as the portion of CSFE/APC double-positive events (Cavarec et al., 1990).

2.4 CD107a degranulation assay

NK92 cells were co-cultured for 3 hr with K562 at a ratio of 10:1 as previously described (Alter et al., 2004). After 1 hr, 1 ul of Golgi Stop (BD Bioscience, San Jose, CA) was added to the cells. Following the 3 hr incubation, cells were labeled with APC-conjugated anti-CD56 and PE-conjugated anti-CD107a antibodies (both from BD Biosciences, San Jose, CA) for 30 minutes at 4°C, followed by flow cytometric analysis. Isotype-matched antibodies from the same manufacturer were used to assess background fluorescence.

2.6. Natural killer cell activity (NKCA)

NK92 cell lytic activity against tumor targets was assessed using a standard chromium release assay, as previously described (Witek-Janusek et al., 2007). K562 tumor cells were radioactively labeled with 100 uCi of [^{51}Cr] (New England Nuclear, Boston, MA). Radiolabeled K562 cells were incubated for 3 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 were 3, 2, and 1:1.

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.

2.7 Evaluation of cytokine production

Cytokines were measured from bulk NK92 culture supernatant fluids as described previously (Witek-Janusek and Mathews, 1999). Briefly, NK92 (2.5×10^5 cells/ml) were incubated in 24 well plates for 24 hrs at 37 °C. Aliquots of the culture supernatants were stored at −80 °C for subsequent cytokine analysis.

2.8 Cytokine and perforin measurement (ELISA)

All molecules were measured using quantitative sandwich enzyme immunoassay techniques (Quantikine kits, R & D Systems, Minneapolis, MN). Sensitivities were; (IL-6 <0.7 pg/ml, IFN gamma <3 pg/ml, 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.

2.9 Western Blot

For Western Blot analysis, nuclei of NK92 cells were extracted from 1–3 × 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) and resuspended in Laemmelis SDS-Sample Buffer (4x) (Boston Bioproducts, Boston, MA). Samples were boiled for 5 minutes and proteins separated by electrophoresis with a 15% agarose gel and transferred to a nitrocellulose membrane for blotting. Proteins were visualized with anti- H4-K8 Ac antibodies or anti- H4-K12 Ac antibodies (Millipore, Temecula, CA), horseradish peroxidase (HRP) conjugated anti-IgG secondary antibody (Millipore, Temecula, CA) and chemiluminescence reagent (ThermoScientific, Rockford, IL). Blots were standardized to total H3 protein (Cell Signaling, Danvers, MA) and blot density quantified using Image J software.

2.10 Chromatin Immunoprecipitation (ChIP) Assay

NK92 cells (1×10^6) 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 and lysed with 750 μL of lysis buffer (Upstate, Temecula, CA). The samples were sonicated using a Branson Sonifier 250 on ice for a total of 5, 15-second cycles per sample. Sonicated samples were diluted and aliquoted into 100μl volumes. Supernatants were pre-cleared with ChIP-Grade Protein G Magnetic Beads (Cell Signaling, Danvers, MA) and immunoprecipitated overnight at 4°C with the following antibodies: anti-RNAP (Upstate), anti-histone H4-K8 Ac (Upstate, Temecula, CA, Cell Signaling, Danvers, MA), anti-glucocorticoid receptor (GR) (Abcam, Cambridge, MA), control Ig, TE buffer alone and ChIP-Grade Protein G Magnetic Beads. The immune complexes were collected, washed, and eluted. Cross-linking was reversed by heating at 65°C overnight and 10 min at 95°C in elution buffer containing 250 mM NaCl. DNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 20 μg of glycogen. Pellets were resuspended in 50 μl of TE. Quantitative real-time PCR was performed using an Opticon 2 Real-Time Detector (BioRad, Hercules, CA) as described previously (Le et al., 2001; Ortman et al., 2002). PCR cycles denaturation was at 94°C (30 s) and annealing at 59°C (30 s). No extension phase was necessary. The DNA samples were amplified with primer pairs to IFN-gamma, Perforin, Granzyme B, TNF-alpha, and Beta globin (non-glucocorticoid regulated) for standardization. PCR data was analyzed using Opticon Monitor 3 Software (BioRad, Hercules, CA). Primers used are identified in Table 1. Fold change in promoter acetylation was based on the Pfaffle Method using the formula below.

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

Target = promoter region. Reference = Beta globin.

2.11 Statistical methods

Data are expressed as means with the standard error of the mean (SEM). Main study variables were analyzed using Student's t test when two groups were compared and one-way ANOVA followed by Tukey post hoc analysis when three groups were compared. Pearson's correlation coefficients were calculated for correlation analysis. A two-sided alpha of 0.05 was set for significance, except as noted. The Statistical Package for Social Sciences (SPSS: version 13.0) was used for data analysis.

3. Results

3.1. Effect of dexamethasone on Natural Killer Cell Activity (NKCA)

The ability of dexamethasone to reduce NK92 cell lysis of tumor cell targets was evaluated. Treatment of NK92 cells for 24 hr produced a dose-dependent reduction in NK92 lytic

activity, Figure 1. A concentration of (10^{-7}M) dexamethasone produced an approximate 50% reduction in NKCA with no impact on cell viability, as judged by Trypan blue exclusion. See Supplemental Table 1.

3.2. Effect of dexamethasone on NK92 adhesion and degranulation when engaged by tumor cells

NKCA requires first the adhesion of NK cells to a tumor cell target, followed by the exocytosis of lytic granules, which destroy the tumor cell target. The ability of NK92 cells to form conjugates with target cells after dexamethasone treatment was analyzed by flow cytometry. NK92 cells were labeled with APC-conjugated antibodies specific for the NK cell surface molecule CD56. K562 tumor targets were labeled with CFSE. NK92-K562 conjugates were measured as double positive (CD56+, CFSE+) populations. The ability of NK92 cells to form conjugates with K562 cells was significantly decreased in response to dexamethasone (10^{-7}M), as compared to untreated NK92 cells. ANOVA demonstrated a significant difference among the 4 groups, [$F=13.180$, $p<0.0001$ df= 3,23]. The histone deacetylase inhibitor, TSA, did not mofify the effect of dexamethasone. See Table 2. Exocytosis of lytic granules by NK92 cells was measured as surface expression of the degranulation marker, CD107a and was unchanged in NK92 cells treated with dexamethasone (10^{-7}M) compared to untreated cells. No statistically significant change was observed. These results indicate that dexamethasone reduced NKCA, in part, by reducing the capacity of NK92 cells to bind to K562 target cells. However, under these conditions dexamethasone had no effect upon the capacity of NK92 to exocytose lytic granules, as evidenced by no change in the surface expression of the degranulation marker, CD107a.

3.3. Effect of dexamethasone on production of IL-6, IFN gamma, TNF alpha and perforin

Experiments were conducted to assess the effect of dexamethasone on the capacity of NK92 cells to produce effector molecules: IL-6, IFN gamma and perforin. The effect of dexamethasone on the NK92 constitutive production of IFN-gamma, IL-6 and perforin production was assessed by ELISA. A dose dependent effect was observed and dexamethasone at 10^{-7}M significantly inhibited the constitutive production of IFN-gamma (30.6 ± 1.1 pg/ml vs. 52.5 ± 1.9 pg/ml; $p<0.001$), IL-6 (27.9 ± 1.4 pg/ml vs. 41.8 ± 2.1 pg/ml; $p<0.001$), and perforin (17.0 ng/ml vs. 20.5 ng/ml), Figure 2. NK92 cells did not constitutively produce TNF alpha.

Following cellular activation, dexamethasone also reduced NK92 production of intracellular IFN-gamma and TNF alpha. As shown in Figure 3, dexamethasone (10^{-7} M) decreased the detectable level of the cytokines on a cell-to-cell basis (32.4 ± 2.9% reduction, $p<0.0001$ for IFN gamma and a 39.7 ± 1.6% reduction, $p<0.002$ for TNF alpha) but also the percentage of cytokine positive cells (30.9 ± 1.5% reduction, $p<0.001$ for IFN gamma and a 16 ± 1.25% reduction, $p<0.002$ for TNF alpha). In Table 3 the mean fluorescent intensity (MFI) of these detected intracellular cytokines are presented and demonstrate a significant reduction in detectable cytokine MFI by dexamethasone treatment.

3.4. Effect of dexamethasone on NK92 surface and intracellular proteins

The effect of dexamethasone on the capacity of NK92 cells to produce NK adhesion, effector and activation molecules was next assessed by flow cytometry. There was a significant reduction in the expression of the lymphocyte adhesion molecule, LFA-1 ($p=0.0001$), which is known to mediate binding of NK92 cells to K562, as well as the granule constituent, granzyme B ($p=0.022$), and the NK cell activating receptor molecule, NKp30 ($p=0.0001$). In Table 3 the mean fluorescent intensity (MFI) of these surface markers and granule constituent are presented. Additional statistical data for Table 3 are presented in Supplemental Table 2. All NK92 cells stained positive for these molecules, and

no change in the percentage of positive cells was detected. Interestingly, these effects were not universal in that the adhesion molecule, CD2 (also know to mediate binding of NK92 cells to K562), and another NK activating receptor (known to be influenced by glucocorticoids), NKp46, did not have reduced expression after dexamethasone treatment. See Table 3.

3.5. Effect of dexamethasone on H4-K8 acetylation

To assess the epigenetic effect of dexamethasone, western blot analysis was performed on nuclear extracts derived from NK92 cells. See Figure 4. The blots were probed with antibodies specific for the acetylated form of histone 4 lysine 8 (H4-K8 Ac). NK92 cells treated with dexamethasone had decreased global acetylation of H4-K8 (~50% reduction) when compared to untreated cells. In Figure 4a, a representative western blot is presented and shows the visual reduction in H4-K8 acetylation. (Similar results were obtained when blots were probed with antibodies specific for the acetylated form of histone 4 lysine 12.) Figure 4b illustrates the quantification of western blots for 4 groups of NK92 cells: No Treatment, Dexamethasone treated, TSA treated and Dexamethasone plus TSA. ANOVA demonstrated a significant difference among the 4 groups, [$F_{(3,12)}$=99.526, $p<0.0001$], such that there was a significant reduction in acetylation of H4-K8 with dexamethasone, which was modified by treatment with TSA, a histone deacetylase (HDAC) inhibitor. Dexamethasone also reduced acetylation of H4-K8 as judged by flow cytometric analysis (~20%). See Table 4. ANOVA demonstrated a significant difference among the 4 groups, H4-K8 MFI [$F_{(3,11)}$=33.104, $p<0.0001$] and H4-K8 % NT [$F_{(3,24)}$=12.918 $p<0.0001$] such that there was a significant reduction in acetylation of H4-K8 with dexamethasone, which was modified by treatment with TSA Direct microscopic observation demonstrated immunofluorescence to be localized to nuclei of analyzed cell populations. Western blot analysis was performed on nuclear extracts from NK92 cells and standardized to total nuclear histone 3 (H3) protein. Flow cytometric analysis evaluated nuclear acetylation of H4-K8 and was standardized to cell number, rather than cellular H3 protein. These differences likely account for differential reductions in H4-K8 Ac.

3.6. Promoter specific changes associated with dexamethasone treatment

These global changes in acetylation suggest that the effect of dexamethasone on NK92s is a possible consequence of epigenetic modification to promoter regions associated with genes essential to NK cell lytic effector function (e.g. perforin and granzyme B) as well as promoter regions associated with cytokine genes (e.g. IFN gamma and TNF alpha). If this is true, then dexamethasone treatment should reduce accessibility for the promoter regions of these genes. To assess these epigenetic effects, chromatin immunoprecipitation (ChIP) with anti-H4-K8 Ac, followed by quantitative real time PCR, was used to calculate the fold change in accessibility after dexamethasone treatment, Figure 5a. ANOVA analysis showed significant differences among the 3 treatment groups [No Treatment (NT), Dexamethasone (Dex) and Dexamethasone plus TSA] for IFN gamma [$F_{(2,14)}$=41.644, $p<0.0001$], perforin [$F_{(2,14)}$=72.641, $p<0.0001$] and granzyme B [$F_{(2,12)}$=66.452, $p<0.0001$]. Post hoc testing demonstrated that dexamethasone treatment produced significant reductions in H4-K8 acetylation 0.53 ± 0.03, 0.48 ± 0.03 and 0.58 ± 0.02 for promoters for IFN gamma, perforin, and granzyme B, respectively. The TNF-alpha promoter exhibited no change in H4-K8 acetylation after dexamethasone treatment. Treatment with TSA significantly modified the effect of dexamethasone in each case.

To assess the association of the glucocorticoid receptor with these promoter regions, glucocorticoid receptor recruitment was also assessed by ChIP. The glucocorticoid receptor was enriched 2.7 ± 1.1 fold at the perforin promoter. Enrichment was also seen at both the IFN-gamma and TNF-alpha promoters, 1.6 ± 0.45 and 2.1 ± 0.95 respectively, Figure 5b.

3.7. Effect of histone deacetylase inhibitor (TSA) on interferon gamma production and NKCA by dexamethasone treated NK92 cells

The alteration in NK cell functional activity after dexamethasone treatment may result from promoter deacetylation at relevant effector genes. If so, then treatment with TSA would be expected to modify the effects of dexamethasone on the functional activity of the NK cells. NK92 cells were treated with dexamethasone, followed by incubation with TSA. TSA modified the effects of dexamethasone for the intracellular production of IFN gamma, Table 5. ANOVA analysis showed significant differences among the 4 treatment groups [No Treatment (NT), Dexamethasone (Dex), Trichostatin A (TSA) and Dexamethasone plus TSA] for IFN gamma MFI [$F_{(3,15)}$=46.515, p<0.001] and IFN gamma % NT [$F_{(3,15)}$=49.973, p<0.0001]. Dexamethasone significantly reduced detectable levels of IFN gamma, but with the addition of TSA, these levels returned to that of the untreated cell population. Likewise for NKCA, dexamethasone significantly reduced lytic activity of NK92 cells for tumor targets and treatment with TSA modified NKCA to the level of untreated NK92 cells, Table 6. ANOVA analysis showed significant differences among the 4 treatment groups [No Treatment (NT), Dexamethasone (Dex), Trichostatin A (TSA) and Dexamethasone plus TSA] NKCA lytic units [$F_{(3,27)}$=30.770, p<0.0001] and NKCA % NT [$F_{(3,36)}$=44.110, p<0.0001].

3.8. Correlation analysis

Exploratory Pearson correlation coefficients were calculated. A two-sided alpha of 0.01 was set for significance. Percent change in H4-K8Ac was found to be correlated with; NKCA (r = 0.710, p=0.0001), constitutive production of IFN gamma by ELISA (r = 0.652, p=0.001), induced production of IFN gamma by MFI (r = 0.783 p=0.0001) and % change of LFA-1 by MFI (0.813 p=0.0001). NKCA was correlated with granzyme B by MFI (r = 0.554, p=0.009), LFA-1 by MFI (0.889, p=0.0001), constitutive production of IFN gamma by ELISA (r = 0.713, p=0.001) and induced production of IFN gamma by MFI (r = 0.908, p=0.0001).

4. Discussion

Therapeutic doses of glucocorticoids depress the level of NKCA in the peripheral blood of treated individuals (Onsrud and Thorsby, 1981) and physiological concentrations of glucocorticoids suppress NKCA of peripheral blood mononuclear cells (PBMC). This inhibition of NKCA is time and dose dependent (Holbrook et al., 1983). It is well-established that acute stress is associated with a reduction in NK cell functional activity (Ben-Eliyahu et al., 1999; De Gucht et al., 1999; Esterling et al., 1994), which may be mediated by increased adrenergic activity (Gan et al., 2002), as well as by the glucocorticoid hormone, cortisol (Gatti et al., 1987). This investigation focused on the effects of the synthetic glucocorticoid, dexamethasone, on the cellular and molecular function of the NK cell line, NK92. The findings demonstrate reductions in; NKCA, adhesion to tumor targets, expression of activation molecules and cytokine production. Each of these reductions was associated with reduced production of effector molecules that mediate these cellular functions. Further, reductions in effector molecules were associated with changes in the accessibility of the proximal promoter regions of genes encoding those molecules, with concomitant reduction in gene transcription. It is well known that glucocorticoids can induce gene transcription, yet their major effect on the immune system is to suppress transcription. The number of immune response genes negatively regulated by glucocorticoids is conservatively estimated at more than 100 (Adcock, 2001; Almawi and Melemedjian, 2002; Wang et al., 2004). Glucocorticoids can decrease cytokine, chemokine, and receptor expression by PBMC (Franchimont, 2004). These effects are primarily due to the glucocorticoid action at the transcriptional level. Although activated glucocorticoid

receptors (GR) can directly interact with proinflammatory transcription factors like AP-1 (Fos-Jun heterodimers) and NFk-B (p65-p50 heterodimers) (Barnes and Adcock, 1998; Ray and Prefontaine, 1994; Truss and Beato, 1993), the primary effect of glucocorticoids is downstream of transcription factor binding to DNA with gene repression a consequence of the reversal of histone acetylation (Ito et al., 2000; Ito et al., 2001). Activated GR repress gene expression by direct inhibition of histone acetyl transferase activity and by active recruitment of histone deacetylase (HDAC) complexes (Ito et al., 2000). Most importantly, physiologically elevated glucocorticoid levels induce HDAC expression in a time and dose dependent manner (Ito et al., 2000) resulting in deacetylation of histones, increased tightening of DNA, and reduced expression of immune response genes (Cippitelli et al., 1995). Conceptually, the histone protein, H4, possesses highly basic amino-terminal tail domains located outside of the core nucleosome particle, which are accessible to multiple post-translational covalent modifications, especially acetylation and methylation (Spencer and Davie, 1999). Lysine acetylation neutralizes histone proteins, whereas deacetylation restores the positive charge of lysines. Following deacetylation, interactions between positively charged lysines and the negatively charged DNA phosphodiester backbone, stabilize the DNA/histone complex, restricting nucleosome mobility on the DNA, and hindering accessibility of the promoter to transcription machinery (Ura et al., 1997). Methylation further enhances this process by promoting histone deacetylation (Eden et al., 1998). Thus, deacetylated lysine residues restrict access of transcription factors for regulatory regions of immune response genes. By repressing a downstream target of transcription factor activation (chromatin accessibility), glucocorticoids reduce gene expression irrespective of the exact transcription factor requirement for an individual gene. Hence, the data presented herein suggest that one likely explanation for the glucocorticoid induced reduction in NKCA and in cytokine production is decreased transcription of immune effector genes due to reduced proximal promoter accessibility.

Reduced proximal promoter accessibility was demonstrated for IFN gamma, perforin and granzyme B when NK92 cells were treated with dexamethasone. Chromatin immunoprecipitation with antibodies specific for H4-K8 Ac demonstrated a quantitative reduction in the accessibility of these promoters as a consequence of reduced acetylation of H4-K8. Further, the gene products were quantitatively reduced by dexamethasone treatment, and such reductions related to reduced functional capacity of NK92 to mediate lysis of K562 and to produce cytokines. Quantitative reductions were also demonstrated for the lymphocyte adhesion molecule, LFA-1, which is known to mediate binding of NK92 cells to K562 (Zheng et al., 2009), as well as the NK cell activating receptor molecule, NKp30. Although not evaluated by chromatin immunoprecipitation and quantitative real time PCR, it is quite possible that dexamethasone induced epigenetic modification also results in reductions in the quantity of these effector molecules. This remains to be determined. These quantitative reductions were not universal in that the adhesion molecule, CD2 (also know to mediate binding of NK92 cells to K562) (Zheng et al., 2009; Orange and Ballas, 2006) was not affected, and neither was another NK activating receptor, NKp46, known to be influenced by glucocorticoids (Mavoungou et al., 2005), Further, the recruitment of the GR to these promoter regions is most likely a result of indirect recruitment via other DNA binding factors, such as transcription factors and/or coregulators, as neither the IFN gamma, perforin, nor granzyme B promoters contain glucocorticoid response elements (GRE). Their repression likely occurs through a transrepression mechanism (defined a**s** a process whereby one protein represses (i.e., inhibits) the activity of a second protein through a protein-protein interaction) via the GR recruitment of HDACs to these sites. In contrast, the TNF alpha promoter evaluated in this study does contain a GRE and the accessibility if its proximal promoter was not affected in this analysis.

Molecular mechanisms underlying the effects of glucocorticoids remain incompletely understood. Many studies have focused on mechanisms upstream of the binding of glucocorticoid to its receptor, including decreased extracellular or intracellular glucocorticoid concentrations or GR mutations, splice variants, or insufficient expression. Those studies also reported mechanisms downstream of the binding of glucocorticoid to its receptor that involve the glucocorticoid signaling pathways (S. Schmidt et al., 2004; Miller, 2008). However, after a glucocorticoid binds to the GR, conformational changes in the receptor expose nuclear localization signal domains, such that the glucocorticoid–GR complex can be translocated to the nucleus. Once in the nucleus, the complex may regulate the transactivation (defined as an increased rate of gene expression and in this case possibly via binding to glucocorticoid response elements, GRE, located in the promoter regions of target genes) of glucocorticoid-responsive genes. Or once in the nucleus, the complex may regulate the transrepression (in this case possibly via interactions with transcription factors such as AP-1 and/or coregulators like SMRT or NCoR) of glucocorticoid-responsive genes (Zhou and Cidlowski, 2005). Transrepression results in condensed chromatin structure, which inhibits gene transcription by blocking access of the transcriptional machinery to the DNA and by blocking the interaction between gene-specific transcription factors and their DNA recognition sequences (Percipalle and Farrants, 2006). Results presented herein demonstrate the later effect of dexamethasone in this human NK cell line. Further, dexamethasone at the examined concentration and period of treatment profoundly reduced the degree of H4-K8 acetylation as judged by western blot analysis. Such a global effect of this glucocorticoid suggests a profound reduction in overall functional NK cell activity. In actuality, reductions in functional activity, although significant, approximated 50%. This was true for NKCA, for cytokine production and for the accessibility of the promoters IFN gamma, perforin and granzyme B. This observation suggests that other effects in addition to transrepression may be ongoing as well and may influence the outcome of dexamethasone treatment and its impact on immune function. This may be the case for the reduced adhesion of NK92 cells to K562 cells treated with dexamethasone. TSA did not return the adhesion of NK92 to untreated levels. As such it is likely that the effects of this glucocorticoid are multiple or alternatively that TSA did not affect the HDAC(s) responsible for the observed reduction. This remains to be determined but also it may be that dose and time of treatment as described herein approximate the effect of short term or acute physiological effects of this glucocorticoid and that greater dose and/or more prolonged treatment may reveal a more profound outcome. Further, it may be that preformed proteins, prior to treatment with dexamethasone (e.g. perforin, LFA-1), are already expressed within granules or at the cell surface and as such may still carry out partial NK cell function.

With regard to this possibility, previous reports have demonstrated glucocorticoids to influence NK cell function as a consequence of reduced transcription and surface expression of natural cytotoxicity receptors. However, those effects were most dramatic when the NK cells were cultured for a period of days in high concentration IL-2 (Chiossone et al., 2007). This prolonged period of time and the IL-2 induced proliferation of NK cells may account for this differential effect. It is worth noting that there are a number of activating receptors involved in NK cell mediated cytotoxicity. Although other activating receptors exist, human NK-mediated lysis of tumor cells is primarily mediated by NKp46, NKp30, and NKp44 and by NKG2D (Moretta et al., 2001; Moretta et al., 2000; Bauer et al., 1999; Pende et al., 2001). Each of these three is expressed by NK92 cells (A. Li et al., 2007). Glucocorticoids have been demonstrated to inhibit the expression of NKp30 with a marginal effect on NKp46 and no effect on NKp44 (Mavoungou et al., 2005). Those observations are consistent with the results reported herein, although no effect on NKp46 was observed. NKG2D is not involved in the lysis of K562 cells by NK92 cells (Olcese et al., 1997) and since K562 cells are deficient in expression of MHC class I molecules, killer inhibitory receptors were not evaluated. It is acknowledged that an association between NK cells

derived from stressed individuals and the expression of the killer inhibitory marker, NKG2A, has been reported (Lutgendorf et al., 2005). However, it is unlikely that such an effect is operable in this human NK cell line.

Finally, it is worth noting that in contrast to the results described herein, previous work has shown that HDACi (valproic acid and suberoylanilide hydroxamic acid) reduce the cytotoxic activity of NK cells (Ogbomo et al., 2007). NK cell inhibitory effects of HDACi were associated with reduced surface expression and function of specific activating receptors (NKp46 and NKp30) responsible for the induction of NK cell-mediated cytotoxicity. This inhibitory effect was at the transcriptional level as judged by real time PCR. These effects were only seen with IL-2 activated NK cells, with no effect on fresh NK. No effect of these HDACi was observed for LFA-1 or for IL-2 receptors. Surface expression levels of inhibitory receptors including KIR and NKG2A were not influenced by HDACi treatment, indicating a specific effect of HDACi on NK cell activating receptors. These differences, with regard to the results of this study, may be explained in multiple ways. The likely difference is that TSA modified the effect of dexamethasone within a one to six hour period, while the effects of valproic acid and suberoylanilide hydroxamic acid were observed in NK cells cultured for 4 days with the HDACi. Further, TSA did not modify the effect of dexamethasone upon the adhesion of NK92 cells to K562 and the reason for this is not clear. As described above it is possible that differing periods of treatment or alternative HDACi(s) may be effective but this has not been determined.

In sum, these results associate glucocorticoid induced epigenetic histone modification with NK cell function. The acetylation status of H4-K8 was found to correlate with not only NKCA but also with cytokine production and with the expression of an adhesion molecule known to mediate the interaction of NK with tumor cells. The function of NK cells against tumor cells is carried out first by engagement of the tumor target, followed by exocytosis of NK cytolytic granule content and/or production of immune effector cytokines. The observation reported herein, demonstrate an effect of glucocorticoids to be at the epigenetic level with reduced transcription of immune effector gene products required for optimal functional activity of NK cells, leading to a reduction in NK cell functional activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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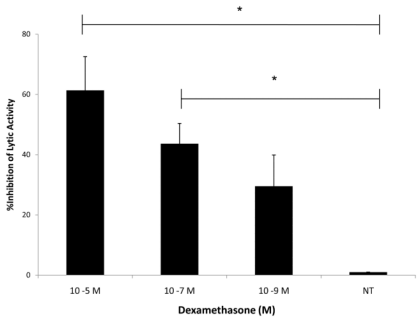

Figure 1.

The effect of dexamethasone on Natural Killer Cell Lytic Activity is illustrated. NK92 cells were treated with varying amounts of dexamethasone for 24 hrs. Percent (%) inhibition was calculated by: [(Lytic Units No Treatment)-(Lytic Units Dexamethasone Treatment)]/(Lytic Units No Treatment) × 100. Values are presented as the mean and standard error of the mean (SEM). *N* = at least three independent experiments. *, $P < 0.05$, Treatment with Dexamethasone vs. No Treatment (NT).

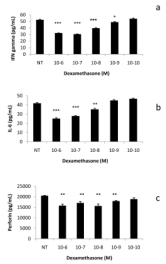

Figure 2.

The effect of dexamethasone on constitutive production of cytokines and perforin is illustrated. NK92 cells were treated for 24 hr with dexamethasone (10^{-7} M). Supernatants were collected and assayed for (a) IFN-gamma, (b) IL-6 and (c) perforin by ELISA. Results are expressed as mean ± SEM; N = at least three independent experiments. NT=No Treatment. *p* values represent statistically significant difference, No Treatment (NT) vs. Dexamethasone at indicated concentration,*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

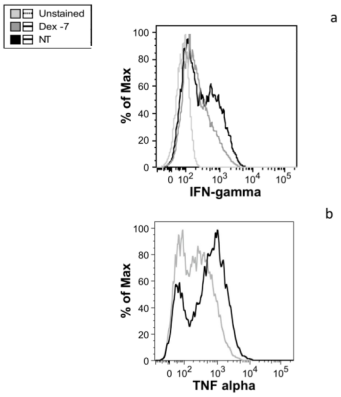

Figure 3.

The effect of dexamethasone on induced production of intracellular cytokines is illustrated. NK92 cells were treated with dexamethasone (Dex, 10^{-7} M) for 24 hr and then stimulated with leukocyte activation cocktail for 4 hrs. Intracellular cytokine staining was performed using anti-human IFN-gamma (a) and anti-human TNF-alpha (b) antibodies and assessed by flow cytometry. Representative results are depicted. NT=No Treatment.

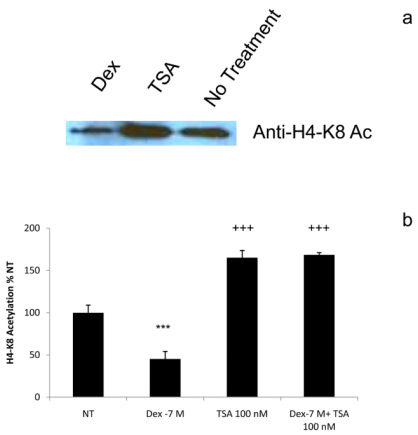

Figure 4.

The effect of dexamethasone and the histone deacetylase inhibitor (TSA) on acetylation status of H4-K8. **(a)** Representative western Blot analysis of nuclear extracts of NK92 cells probed with antibodies specific for the acetylated form of H4-K8. **(b)** Blots (n= 3) were standardized to total H3 protein (blots not shown) and blot density quantified using Image J software. NT = No Treatment, Dex= Dexamethasone, TSA = Trichostatin A (100 nM). % Change= [(Density Dex or Dex +TSA)/(Density NT)] × 100. Results are expressed as mean ± SEM (n = 3). Statistically significant difference, ***p <0.001, No Treatment (NT) vs. Dexamethasone (10^{-7}), +++ p <0.001, Dexamethasone vs. Dexamethasone + TSA.

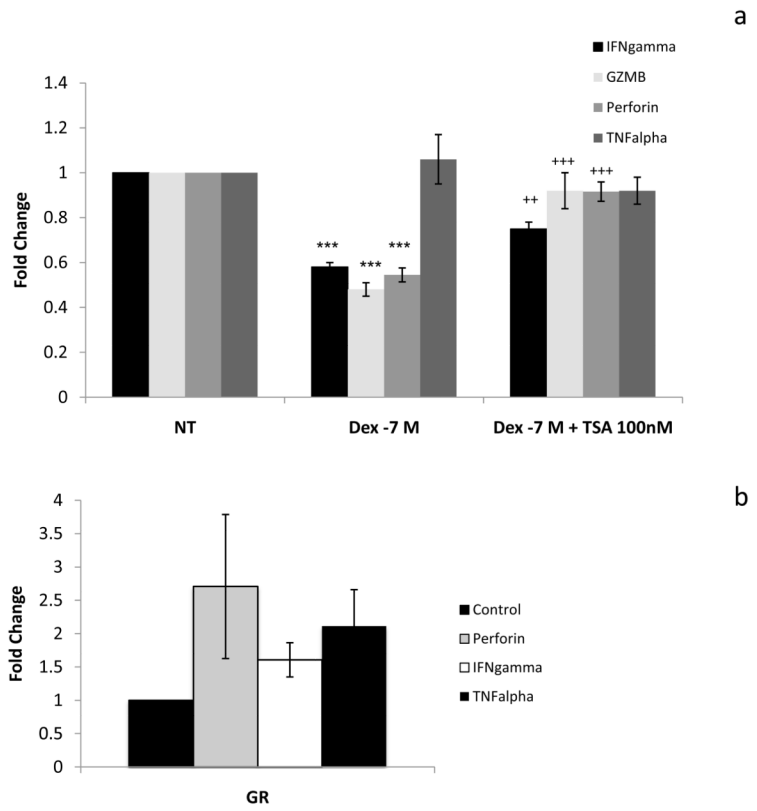

Figure 5.

Promoter specific changes in acetylation status and glucocorticoid receptor recruitment is shown. NK92 cells were treated with 10^{-7} M dexamethasone for 24 hr. Cells were collected, cross-linked, sheared, and lysed. Total sheared lysates were isolated with antibodies specific for **(a)** H4-K8 Ac or **(b)** glucocorticoid receptor (GR). Fold change in acetylation and GR recruitment at the granzyme B (GZMB), perforin, IFN-gamma, and TNF-alpha promoters as compared to beta globin and calculated using the Pfaffl Method. Results are expressed as mean ± SEM (N= 4). Statistically significant difference ***p <0.001, No Treatment vs. Dexamethasone (10^{-7}M), ++ p<0.01, +++ p <0.001, Dexamethasone vs. Dexamethasone + TSA.

Table 1

Real-time PCR information. Primer sequences (proximal promoter), PCR products length, and temperature of annealing.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′ – 3′)	PCR product length (base pairs)	Annealing temperature
β-globin	acg gct gtc atc act tag acc tca	aag caa tag atg gct ctg ccc tga	127	60
Perforin	ggc aca gtt cca agc act tca caa	agc ctc act gtg cct cag ttt ctt	92	59
IFN gamma	tca tcg tca aag gac cca agg agt	atg gtg aca gat agg cag gga tga	110	59
TNF alpha	cgc ttc ctc cag atg agc tc	tgc tct cct tgc tga ggg a	151	58
Granzyme B	act tca tag gct tgg gtt cct gct c	tcc tct ggg tgc ttg tgt gag aat c	111	61

Table 2

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

Treatment	% Conjugates	P value (compared to NT)
No Treatment (NT)	100	
Dex (10^{-7} M)	73.3 ± 3.3	0.0001
TSA (100 nM)	97.32 ± 6.0	0.975
Dex (10^{-7} M) + TSA (100 nM)	72.23 ± 6.11	0.001

Data are mean values ± S.E.M., n ≥ 7. Dex= dexamethasone. TSA= Trichostatin A. NT= No Treatment. % Conjugates = [(No Treatment (NT) - Dex or Dex + TSA)/(No Treatment (NT)] × 100. Statistically significant difference, NT vs. Dex, NT vs. Dex + TSA, Dex vs. Dex + TSA.

Table 3

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

Mean Fluorescent Intensity (MFI) of Surface and Intracellular Proteins					
Cellular Protein	**No Treatment**	**Dexamethasone Treatment (10^{-7} M)**	**P value**	**% Change**	**P value**
IFN gamma	376 ± 17	272 ± 4.7	0.004	−31.6 ± 2.8	0.0001
TNF alpha	692 ± 18.9	418 ± 11	0.0001	−39.6 ± 1.5	0.002
LFA-1	2,722 ± 50	2,377 ± 28	0.0001	−13.1 ± 0.7	0.0001
CD-2	783 ± 12	884 ± 9.0	0.0001	12.8 ± 1.2	0.0001
Granzyme B	63,156 ± 1178	57,522 ± 1706	0.022	−11.3 ± 2.7	0.001
NKp30	929 ± 5.7	816 ± 1.5	0.0001	−12.14 ± 0.16	0.0001
NKp46	279 ± 1.8	336 ± 3.4	0.001	20.6 ± 1.2	0.0001

Values are mean fluorescent intensity (MFI) of surface and intracellular proteins ± S.E.M., N= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI No Treatment - MFI Dexamethasone)/(MFI No Treatment) × 100. Statistically significant difference, No Treatment vs. Dexamethasone Treatment.

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Table 4

Effect of dexamethasone and Trichostatin A on H4-K8 nuclear acetylation of NK92 by flow cytometric analysis.

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

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

Table 5

Effect of dexamethasone and Trichostatin A on intracellular interferon gamma production by NK92 cells as judged by flow cytometric analysis.

Treatment	IFN gamma MFI	P value (compared to NT)	% NT	P value (compared to NT)	IFN gamma MFI P value (compared to Dex)
No Treatment	753 ± 22				0.039
Dex (10^{-7} M)	563 ± 12	0.039	74.8 ± 1.6	0.032	
TSA (100 nM)	1255 ± 25	0.0001	166.5 ± 3.3	0.0001	
Dex (10^{-7} M) + TSA (100 nM)	933 ± 78	0.051	123 ± 10	0.043	0.0001

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

Table 6

Effect of dexamethasone and Trichostatin A on NKCA by NK92 cells

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

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