Published in final edited form as:

Brain Behav Immun. 2018 January ; 67: 279–289. doi:10.1016/j.bbi.2017.09.004.

Chromatin organization as an indicator of glucocorticoid induced natural killer cell dysfunction

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Abstract

It is well-established that psychological distress reduces natural killer cell immune function and that this reduction can be due to the stress-induced release of glucocorticoids. Glucocorticoids are known to alter epigenetic marks associated with immune effector loci, and are also known to influence chromatin organization. The purpose of this investigation was to assess the effect of glucocorticoids on natural killer cell chromatin organization and to determine the relationship of chromatin organization to natural killer cell effector function, e.g. interferon gamma production. Interferon gamma production is the prototypic cytokine produced by natural killer cells and is known to modulate both innate and adaptive immunity. Glucocorticoid treatment of human peripheral blood mononuclear cells resulted in a significant reduction in interferon gamma production. Glucocorticoid treatment also resulted in a demonstrable natural killer cell nuclear phenotype. This phenotype was localization of the histone, post-translational epigenetic mark, H3K27me3, to the nuclear periphery. Peripheral nuclear localization of H3K27me3 was directly related to cellular levels of interferon gamma. This nuclear phenotype was determined by direct visual inspection and by use of an automated, high through-put technology, the Amnis ImageStream. This technology combines the per-cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry. Most importantly, this technology provides for a direct assessment of the localization of signal intensity within individual cells. The results demonstrate glucocorticoids to dysregulate natural killer cell function at least in part through altered H3K27me3 nuclear organization and demonstrate H3K27me3 chromatin organization to be a predictive indicator of glucocorticoid induced immune dysregulation of natural killer cells.

Keywords

Glucocorticoid; epigenetic; chromatin organization; natural killer cells; interferon gamma

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1. Introduction

Natural killer (NK) cells are group 1 innate lymphocytes (ILC1). Their frequency in peripheral blood approximates 10%. NK cells are functionally characterized by their cytotoxic activity and by their production of cytokines and chemokines [1]. Mature NK cell production of these molecules shapes both the innate and the adaptive immune response [2]. The best-characterized cytokine produced by NK cells is IFN gamma [3], which is quickly released within minutes to hours after NK cell stimulation [4]. NK cell produced IFN gamma has many effects on the immune response, including induction of MHC class II molecules on antigen-presenting cells, activation of myeloid cells, and the induction of T helper 1 (TH1) lymphocytes [5]. Macrophage activation by NK cell-derived IFN gamma has been shown to be essential for resistance to primary tumorigenesis [6] and to increased microbicidal activity [7]. In patients and animal models, impaired NK cells or NK cell deficiency have been associated not only with recurring viral infections, but also with an increased incidence of cancer [8]. Complete NK cell deficiency results in fatal infections during childhood [9]. As such, NK cells and the production of IFN gamma by NK cells are integral to an effective immune response.

Studies in humans and in animals demonstrate that the immune system, including NK cells, is influenced by psychological stress [10–12]. Psychological stress is associated with an increased risk for disease, which suggests a linkage among stress, the risk for disease, and the immune system [10, 12]. Cytokine production by the immune system is particularly sensitive to stress dysregulation [13–17], such dysregulation is a likely contributor to stress-related disease susceptibility. A range of psychological stressors result in the activation of the hypothalamic pituitary adrenal (HPA) axis, increasing circulating levels of glucocorticoids (GC) [18–21], reducing immune function, through activation of the glucocorticoid receptor (GR).

At the genomic level, GC:GR shuttle between the cytoplasm and GC-responsive elements (GRE) located in the promoter or enhancer regions of GC-responsive genes [22–24], controlling transcription of target genes through several mechanisms. Direct binding of GR to specific *cis*-acting GRE DNA sequences can repress gene transcription [25]. GR can also repress genes by tethering itself to other transcription factors, repressing their capacity to induce gene transcription [26, 27]. GR also binds to inverted palindromic sequences known as negative GREs (nGREs) [28] which also inhibit transcription of target genes. GR recruitment to nGREs promotes the assembly of corepressor complexes and the recruitment of histone deacetylases, which direct GC-dependent repression of specific genes by compaction of chromatin through lysine deacetylation of histone H3 or H4 [29].

At the global-genomic level, GR interacts with thousands of sites across the genome, influencing the expression of hundreds of genes [28, 30, 31]. GR binding sites potentiate transcription-factor clusters across the entire genome with interactions between distal sites that dramatically alter gene expression [32]. At this scale, the effects of GC are coordinated transcriptionally through alterations in chromatin organization. Alterations in chromatin organization determine which genes are repressed and which genes are transcribed. By genome-scale chromosome conformation capture, chromatin has been demonstrated to be

organized into large compartments that are “open” and highly transcribed or “closed” and less transcriptionally active [33]. Compartments are comprised of topologically-associated chromosomal domains (TADs), containing DNA segments ranging in length from hundreds of kilobases to many megabases [34, 35]. These DNA domains are organized by nuclear architectural proteins that regulate gene expression by constraining or permitting intra- and/or inter-DNA domain interactions [35]. Transcriptionally active chromatin domains are enriched for active epigenetic marks (e.g. H3K9Ac) while repressed chromatin domains are enriched for repressive epigenetic marks (e.g. H3K27me3) at gene regulatory regions [33, 36, 37]. Transcription factors like GR form epigenetically marked chromatin loops that contain multiple genes that permit interactions among gene promoter and enhancer regions. These loops regulate transcription locally [23, 38, 39], but GR can also act globally via chromatin loops, targeting active genes to transcription factories and repressed genes to heterochromatin and in that manner influence chromatin organization [40, 41]. We have previously demonstrated histone epigenetic, post-translational marks to localize to immune-response genes affected by GC and by psychological stress. GR was associated with these epigenetically marked genes and GC were shown to control transcription through local gene accessibility [42–45], influencing chromatin organization and immune effector function [46, 47]. Euchromatin is enriched for acetylated epigenetic marks that localize in the interior of the nucleus, while silent heterochromatin, enriched in histone tri-methylation marks, localizes toward the nuclear periphery [48–50].

The purpose of this investigation was to determine whether global chromatin organization can be used as a means to quantify the effects of GC on NK cells. The *in vitro* effect of GC on human PBMC immune function and upon the chromatin organization of NK cell nuclei was assessed. The effect of GC on nuclear-chromatin organization was measured as mean fluorescence intensity, density, and localization of the repressive, epigenetic mark H3K27me3. Measurement of chromatin organization in this manner was demonstrated to be related to NK cell function as measured by IFN gamma production. These data demonstrate that a quantitative measure of chromatin organization is directly related to intracellular levels of IFN gamma in NK cells and that this measure can be used as a means by which to detect GC induced immune dysregulation in NK cells.

2. Methods and Materials

2.1 Cell Lines and Media

The human erythroleukemic-like cell line, K-562 (ATCC® CCL-243™) was obtained from the American Type Culture Collection, certified under ISO 9001:2008 and manufactured under ISO/IEC 17025:2005 accreditation. The cell line was maintained as a low passage number with routine profiling. 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). For *in vitro* human peripheral blood mononuclear cell (PBMC) experiments, cells were maintained in complete RPMI media identical to that used for K562 cells.

2.2 Subject Recruitment

Healthy volunteers (two males and four females, ages ranging from 32–65 years) participated in this study and were excluded if they had an immune-based disease, were substance abusers, had a history of acute infection, or were taking corticosteroids. This study was approved by the Loyola University Medical Center Institutional Review Board for the Study of Human Subjects. All procedures were carried out with the understanding and written consent of the participants.

2.3 In vitro Cellular Treatment of Human PBMC

Whole blood was collected in sterile heparinized tubes and processed immediately. Heparinized peripheral blood was overlaid onto Ficoll/Hypaque and centrifuged at $400 \times g$ for 25 min. PBMC at the interface were extracted and washed twice in phosphate buffered saline (PBS) (Gibco, Grand Island, NY) prior to any treatment or phenotypic analysis. PBMC were then cultured at a concentration of 1×10^6 cells/ml in RPMI in the presence or absence of 10^{-6} M dexamethasone (Dex) (Sigma Aldrich, St. Louis, MO) or 10^{-6} M norepinephrine (NE) (Sigma Aldrich, St. Louis, MO) for 24 hr in 24 well plates. After treatment, cells were pooled, washed with RPMI and 1×10^6 cells were used for each analysis. Cell number and viability were determined by exclusion of 0.1% Trypan blue after isolation and after treatment. Viability was maintained between 90 and 95% in all experiments.

2.4 Natural killer cell activity (NKCA)

K562 tumor cells were radioactively labeled with 100 μ Ci of [51 Cr] (New England Nuclear, Boston, MA). Radiolabeled K562 cells were washed and then incubated for 4 hr with PBMC. 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 50, 30, 20, and 10.

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

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

All experimental means were calculated from triplicate values.

*DPM=disintegrations per minute.

2.5 Measurement of IFN gamma by ELISA

1×10^6 human PBMC were treated with Dex, NE, or left untreated. At the time of treatment, cells were activated with PMA/PHA (PMA @ 20ng/well; PHA @ 0.05%/well) and incubated for 48 hr at 37°C. Cell supernatants were then collected for analysis via ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.6 Intracellular staining of Human PBMC

After 24 hr, 1×10^6 Dex treated or untreated PBMC were aliquoted into fluorescent activated cell sorting (FACS) tubes. The cells were washed twice with phosphate buffered saline (PBS) (Gibco, Grand Island, NY), then activated with lymphocyte activation cocktail (LAC) (BD Pharmingen, San Jose, CA) for 4 hr at 37°C. LAC contains PMA, ionomycin and brefeldin A. After activation, surface antibodies were added for 30 min on ice and agitated every 15 min to identify PBMC sub-populations. Surface stain antibodies included anti-CD3 (APCCy7 conjugated) (BD Biosciences San Jose, CA) and anti-CD56 (BV421 conjugated), (BD Biosciences, San Jose, CA). PBMC were then washed twice in 0.1% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in PBS. PBMC were then fixed and permeabilized with Cytotfix/Cytoperm solution (BD Pharmingen, San Jose, CA) for 20 min at 4°C. Afterwards, PBMC were washed twice with Perm/Wash Buffer (BD Pharmingen, San Jose, CA), and then stained with antibodies specific for intracellular molecules of interest for 1 hr at 4°C. Intracellular antibodies included anti- IFN gamma (PE conjugated) (BD Biosciences, San Jose, CA) and anti- H3K27me3 (APC conjugated) (Cell Signaling, Beverly, MA). PBMC were then washed twice with Perm/Wash buffer. After wash the FACS tubes were then briefly vortexed and PBMC were transferred into 1.5 ml Eppendorf tubes. The tubes were then centrifuged, excess liquid was removed by pipet, and cells were resuspended in 50 μ l of 0.1% BSA in PBS to be analyzed with the Amnis ImageStream (EMD Millipore, Billerica, MA) equipped with Inspire software. Ten thousand events were collected and analyzed with ImageStream IDEAS software (EMD Millipore, Billerica, MA).

2.7 IDEAS Software analysis of H3K27me3 in PBMC

The Inspire software simultaneously collects six images from each cell that passes through the instrument. These images include 5 fluorescence images and one bright field image stored as raw image files (.rif). Single fluorochrome files were collected and then used to generate compensation matrices and compensated image files (.cif). From the compensated image files, data analysis files (.daf) were created and analyzed for H3K27me3 localization. All acquired cells were plotted as histograms of the gradient root mean square of the bright field image, which measured the sharpness of the bright field image. Focused cells were then analyzed by a scatter plot of the aspect ratio (roundness of the cells) and area (size of the cells) to insure individual lymphocyte analysis. CD56⁺ CD3⁻ cells were identified as NK cells, termed NK cells in this manuscript. NK cells from both the Dex treated and untreated cell populations were visually analyzed for H3K27me3 localization.

2.8 Automation of H3K27me3 localization with IDEAS Software

An automated technique was developed within the IDEAS software that allowed for analysis of H3K27me3 localization in every NK cell that passed through the flow cell. The automated technique provided a high throughput analysis of H3K27me3 localization within the nuclei of NK cells. Data analysis was performed with the Feature Finder program within the IDEAS software package, which provided multiple image-based parameters that included: size, location, shape, and texture (local intensity variations in the image) that enabled discrimination between cells based on their appearance. Additionally, the Feature Finder program calculated a statistic (termed Rd) that quantified the degree of discrimination

between Peripheral H3K27me3 nuclear localization and Non-peripheral H3K27me3 nuclear localization in the NK cell population (all NK cells were H3K27me3⁺). Two truth cell populations were then identified based on localization of H3K27me3. In this investigation, the truth populations were NK cells that had Peripheral H3K27me3 localization and NK cells that did not have Peripheral H3K27me3 localization (Non-peripheral H3K27me3 localization). Twenty-five representative cells were tagged for each truth population. The truth populations in this study were based on localization of H3K27me3 regardless of H3K27me3 intensity to insure that feature selection was based on the localization of H3K27me3 within NK cell nuclei. The truth populations were then analyzed by the Feature Finder program to identify features that discriminated Peripheral and Non-peripheral H3K27me3 localization within NK cell nuclei.

2.9 Confirmation that Modulation and Bright Detail Intensity (BDI) differentiate Peripheral and Non-peripheral H3K27me3 in NK cell nuclei

H3K27me3 Modulation as well as H3K27me3 Bright Detail Intensity (BDI) were the two identified features that discriminated Peripheral and Non-peripheral localization of H3K27me3. Modulation measures the intensity range of an image, normalized between 0 and 1. A numerically large Modulation value close to 1 identifies a nucleus with areas of bright fluorescence intensity and also areas of weak to no intensity of H3K27me3. Modulation of H3K27me3 served as a quantitative value that represented the contour of H3K27me3 staining in individual nuclei. Modulation was calculated as: $\text{Modulation} = \frac{\text{Max Pixel} - \text{Min Pixel}}{\text{Max Pixel} + \text{Min Pixel}}$. Bright Detail Intensity quantifies the local fluorescence density at a radius of 3 pixels (BDI). The BDI feature scans the nucleus for bright spots, areas of increased density of H3K27me3 staining, within a 3 pixel radius, then eliminates any background staining and reports the mean intensity of the bright spots within the nucleus. A numerically large BDI identified H3K27me3 with high density over a 3 pixel radius. These two features provided detailed information regarding H3K27me3 localization and density within NK cell nuclei.

H3K27me3 Modulation and H3K27me3 BDI were plotted for Dex treated and untreated NK cells. H3K27me3 Modulation Intervals (groups of individual NK cells with similar Modulation of H3K27me3) were analyzed in 10% increments of the maximum H3K27me3 Modulation present within the NK cell population. There are 10 H3K27me3 Modulation Intervals that each represent 10% of the maximum H3K27me3 Modulation value within each NK cell population. Confirmation that H3K27me3 Modulation and H3K27me3 BDI discriminated between Peripheral H3K27me3 localization and Non-peripheral H3K27me3 was accomplished by in-depth visual inspection (>2,000 NK cells analyzed) of H3K27me3 localization from untreated and Dex treated NK cells in each H3K27me3 Modulation Interval derived from six independent samples. Representative images of H3K27me3 localization from each Modulation Interval (taken at the Modulation midpoint of each interval) were visually assessed within H3K27me3 Modulation Intervals. Fifty cells from each Modulation Interval in both untreated and Dex treated NK cells from six independent samples were assessed. If less than 50 NK cells were detected in the H3K27me3 Modulation Interval, all were counted. The percentage of NK cells within each Modulation Interval that had H3K27me3 localization similar to the representative image was recorded.

2.10 IDEAS Analysis of H3K27me3 Modulation Interval IFN gamma production in NK cells

H3K27me3 Modulation Interval IFN gamma production in NK cells was calculated by the following equation: H3K27me3 Modulation Interval mean IFN gamma intensity $\times$ % of NK cells for each H3K27me3 Modulation Interval. This resulted in H3K27me3 Modulation Interval IFN gamma values that represented the level of IFN gamma within NK cells of each H3K27me3 Modulation Interval and served as an index of immune function for those NK cells.

2.11 Calculation of H3K27me3 Modulation Interval Chromatin Organization (CO)

H3K27me3 Modulation Interval CO was calculated with the following equation: mean H3K27me3 Modulation $\times$ mean H3K27me3 BDI $\times$ % of NK cells for each H3K27me3 Modulation Interval. Given that the H3K27me3 Modulation is normalized between 0 and 1, while the H3K27me3BDI score can range from 1– 100,000, other mathematical manipulation of the scores would have resulted in H3K27me3 BDI dominating the analysis. Multiplication of the values equalized the weight given to both H3K27me3 Modulation and H3K27me3 BDI. Multiplication of H3K27me3 Modulation and H3K27me3 BDI by the percentage of NK cells within each H3K27me3 Modulation Interval resulted in a H3K27me3 Modulation Interval CO value that was representative of the NK cells detected in each H3K27me3 Modulation Interval. H3K27me3 Modulation Interval CO was compared between Dex treated and untreated NK cells for each H3K27me3 Modulation Interval and served as index of the effect of Dex on H3K27me3 CO throughout the entire NK cell population.

2.12 Calculation of H3K27me3 Global CO and Global IFN gamma production

For each H3K27me3 Modulation Interval, an H3K27me3 Modulation Interval CO value was calculated as: H3K27me3 Modulation Interval CO = mean H3K27me3 Modulation $\times$ mean H3K27me3 BDI value $\times$ % NK cells in a particular H3K27me3 Modulation Interval. Global H3K27me3 CO for the entire cell population was calculated as a summation of the 10 H3K27me3 Modulation Interval CO values.

H3K27me3 Modulation Interval IFN gamma was calculated as: H3K27me3 Modulation Interval IFN gamma MFI = mean IFN gamma fluorescence intensity (MFI) $\times$ % NK cells in the H3K27me3 Modulation Interval. Global IFN gamma MFI for the entire population was calculated as the summation of the ten H3K27me3 Modulation Interval IFN gamma values.

Global H3K27me3 CO and Global IFN gamma MFI provided quantified values that permitted a direct assessment of the effects of Dex treatment on Chromatin Organization and IFN gamma production in an entire NK cell population. Such values provide an assessment measure by which to compare separate populations of NK cells for the effects of Dex on both H3K27me3 Chromatin Organization and immune function.

2.13 Statistical Analyses

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) software (IBM, Armonk, NY). Statistical analyses of all H3K27me3 Modulation Interval comparisons were by Student's t-test. NKCA was analyzed by group repeated

measure ANOVA with Tukey HSD *post hoc* tests to determine if differences existed among Dex treated, NE treated, and untreated PBMC. IFN gamma production by ELISA was analyzed by ANOVA with Tukey HSD *post hoc* test to determine if significant differences existed among Dex treated, NE treated, and untreated PBMC. Other analyses used either Student's t-test, or Paired student's t-test as designated. Relationships were analyzed by Pearson's r in SPSS. For all statistical tests $p < 0.05$ was set for significance.

3. Results

3.1 Effects of dexamethasone (Dex) and norepinephrine (NE) on PBMC natural killer cell activity (NKCA)

Both Dex and NE are reported to influence natural killer cell function. The two best characterized functional activities of human NK cells are NKCA and IFN gamma production. The effect of Dex and of NE on PBMC NKCA for tumor targets was assessed in Figure 1. PBMC were either untreated, or treated with Dex or NE (10^{-6} M) for 24 hr. (Varying Dex and NE concentrations (10^{-5} – 10^{-7} M) were evaluated, with maximum effect observed at 10^{-6} M for Dex with no effect for NE at 24 hr for any tested concentration. Data are not shown.) A one-way group repeated measures ANOVA of the % Cytotoxicity of the effector to target ratios identified a significant effect of treatment on NKCA at the $p < 0.05$ level for the three conditions [$F(1, 2) = 6.971$, $p = 0.011$]. *Post hoc* Tukey HSD tests indicated significant differences in the means of Dex treated and untreated PBMC at the 50:1 and 30:1 effector to target ratios. NE had no demonstrable effect on the NKCA of PBMC. These data demonstrate that Dex, but not NE, reduced PBMC NKCA.

3.2 Effects of Dex and NE on IFN gamma production by PBMC and CD3⁺CD56⁺ NK cells

The effect of Dex and of NE on NK cell IFN gamma production was assessed in two ways, ELISA measured PBMC production, or measurement of cytoplasmic mean fluorescence intensity levels (MFI) in NK cells. PBMC were either untreated or treated with Dex or NE (10^{-6} M) and activated for 48 hr. (As above, varying Dex and NE concentrations (10^{-5} – 10^{-7} M) were evaluated, with maximum effect observed at 10^{-6} M for Dex and no effect for NE at any tested concentration. Data are not shown.) There was a significant effect of treatment on IFN gamma production at the $p < 0.05$ level for the three conditions [$F(2, 18) = 5.039$, $p = 0.018$]. *Post hoc* Tukey HSD tests indicated a significant reduction in IFN gamma production in Dex treated PBMC when compared to untreated PBMC. No effect was observed for NE. See Table 1. Further, Dex treated CD3⁺CD56⁺ NK cells (NK) had significantly reduced cytoplasmic IFN gamma mean MFI when compared to untreated cells. Taken together, these data and those in Figure 1 demonstrate Dex to decrease PBMC NKCA and IFN gamma production. NE had no such effect. The effect of Dex on PBMC was likely due to NK cells in that NKCA in short term assay is mediated by such lymphocytes. IFN gamma can be produced by multiple lymphocyte populations, but reductions in IFN gamma MFI within the NK cell population demonstrates a clear effect on NK cells. Since no apparent effect on NK cell function was observed for NE, no further experimental analysis of NE was considered.

3.3 Effect of Dex on H3K27me3 levels in NK cells

Previous investigations of NK cells have demonstrated Dex to lead to changes in histone post-translational epigenetic marks. Those modified epigenetic marks were shown to be directly associated with NK cell function including NKCA and IFN gamma production [42–44]. One well characterized epigenetic mark is H3K27me3, which is associated with repressed gene expression and with the heterochromatic regions of the nucleus [51, 52]. Therefore, PBMC were either treated or not with Dex for 24 hr and H3K27me3 MFI was assessed in NK cells. Dex treatment significantly increased the intensity of NK cell H3K27me3 immunofluorescence (as judged by MFI) when compared to untreated cells ($p < 0.005$). See Table 2. Such analysis demonstrated that Dex increased detectable levels of the epigenetic mark, but did not provide specific information regarding the location of the increased immunofluorescent intensity within individual cells. To analyze the cellular location of the epigenetic mark, high through-put technology was employed, i.e., the Amnis ImageStream. With this instrument, fluorescence intensity measurements can be acquired as with a conventional flow cytometer; however, the system's imaging advantage is to locate and quantify the distribution of fluorescence intensity within a cell.

3.4 Localization of H3K27me3 within Dex treated NK cells

By Amnis ImageStream analysis, a population of lymphocytes within the Dex treated NK cells exhibited a distinct epigenetic pattern or phenotype. The cellular phenotype was H3K27me3 intense localization to the nuclear periphery, depicted in Figure 2A and termed Peripheral H3K27me3 localization. A cell that did not exhibit the phenotype is in Figure 2B in which H3K27me3 immunofluorescence appears to be diffusely distributed throughout the nucleus and termed Non-peripheral H3K27me3 localization. DAPI staining of the cells confirmed localization of the epigenetic mark to the nucleus. See Supplemental Figure 1 for color images and DAPI H3K27me3 co-localization. NK cells that exhibited Peripheral H3K27me3 localization were significantly more abundant in Dex treated NK cell populations than in untreated NK cell populations. See Table 3.

3.5 Localization and density quantification of NK cell nuclear H3K27me3

H3K27me3 localization and density were automated and quantified within a given cell population by use of the Amnis ImageStream, Feature Finder program, within the IDEAS software. The Feature Finder program does so by quantification of H3K27me3 Modulation as well as H3K27me3 Bright Detail Intensity (BDI). A numerically large Modulation value identifies a nucleus with areas of bright fluorescence intensity and areas of weak intensity. Values are normalized to a number between 0 and 1. Modulation serves as a quantitative value representing the contour of H3K27me3 staining within individual nuclei. Bright Detail Intensity quantifies the local fluorescence density at a radius of 3 pixels (BDI). A numerically large BDI value identifies a nucleus wherein the fluorescent signal is highly condensed locally. The relationship of these two Features, H3K27me3 Modulation and H3K27me3 BDI was assessed for an individual NK cell population (derived from a single individual) either Dex treated or not for 24 hr. See Figure 3 (See Supplemental Figure 2 for color image). Each individual NK cell is represented by a single plotted point, with the population as a whole separated into H3K27me3 Modulation Intervals that represent 10%

increments of the maximum H3K27me3 Modulation value of the NK cell population. Mean H3K27me3 Modulation and mean H3K27me3 BDI were determined for each H3K27me3 Modulation Interval. When NK cell populations were derived from six separate individuals and Dex treated or not, H3K27me3 Modulation and H3K27me3 BDI for each H3K27me3 Modulation Interval did not differ statistically between the untreated and Dex treated NK cells. See Supplemental Figure 3. Data from Supplemental Figure 3 and Figure 3 do indicate that as H3K27me3 Modulation increased there was a concomitant increase in H3K27me3 BDI. A significant positive relationship between H3K27me3 Modulation and H3K27me3 BDI in both Dex treated NK cells ($r = 0.669$, $p < 0.005$) and untreated NK cells ($r = 0.666$, $p < 0.005$) was observed (Figure 3). This relationship was observed in NK cells from a single individual, but is representative of relationships seen in NK cells from six individuals. See Supplemental Table 1. These data demonstrate that with increased H3K27me3 Modulation there is a concomitant increase in H3K27me3 BDI. In Supplemental Table 2 representative images for each H3K27me3 Modulation Interval are depicted. (The representative images were taken from the H3K27me3 Modulation midpoint of each Modulation Interval.) It is apparent that for Modulation Intervals 8–10 a majority of NK cells had Peripheral H3K27me3 localization in both Dex treated and untreated NK cells from six independent samples. These data indicate that with increased H3K27me3 Modulation and H3K27me3 BDI there is an increase in Peripheral nuclear localization of H3K27me3 for both Dex treated and untreated NK cells. The percentage of NK cells with similar H3K27me3 nuclear localization within the H3K27me3 Modulation Intervals did not differ between Dex treated and untreated cells from six individual samples ($p > 0.05$).

However, calculation of the percentage of NK cells in each H3K27me3 Modulation Interval demonstrated a significant increase in H3K27me3 Modulation Intervals 8, 9, and 10 for NK cells treated with Dex when compared to untreated cells. There was also a significant reduction in the percentage of NK cells in H3K27me3 Modulation Intervals 6 and 7 for NK cells treated with Dex when compared to untreated cells. See Figure 4A. Taken together these data demonstrate Dex to increase the percentage of cells in H3K27me3 Modulation Intervals 8–10, and decrease the percentage of cells in H3K27me3 Modulation Intervals 6 and 7. Further, increased H3K27me3 Modulation and increased H3K27me3 BDI directly related to nuclear Peripheral localization of H3K27me3.

3.6 Relationships of H3K27me3 Modulation and H3K27me3 BDI to cytoplasmic IFN gamma levels in NK cells

In Dex treated NK cells there was a significant relationship between H3K27me3 Modulation and cytoplasmic IFN gamma MFI ($r = -0.344$, $p < 0.005$). No significant relationship was observed for H3K27me3 Modulation and cytoplasmic IFN gamma in untreated NK cells ($r = -0.030$, $p = 0.783$). These relationships were for the individual NK cell population shown in Figure 3. For the relationships observed in all six separate individuals, see Supplemental Table 3. These data demonstrate a negative relationship between H3K27me3 Modulation and IFN gamma levels in Dex treated NK cells. There was also a significant relationship between H3K27me3 BDI and cytoplasmic IFN gamma within the Dex treated NK cell population ($r = -0.236$, $p < 0.005$). No significant relationship was observed within the untreated NK cell population ($r = -0.190$, $p = 0.09$). The relationships for all six separate

individuals are found in Supplemental Table 4. These data demonstrate that increased H3K27me3 Modulation and increased H3K27me3 BDI relate to decreased IFN gamma production in Dex treated NK cells.

3.7 Quantification of H3K27me3 Modulation Interval IFN gamma production in NK cells

IFN gamma production was calculated for each H3K27me3 Modulation Interval as: mean IFN gamma MFI $\times$ % NK cells detected in each H3K27me3 Modulation Interval. This resulted in a H3K27me3 Modulation Interval IFN gamma value that was representative of the NK cells detected within each H3K27me3 Modulation Interval. H3K27me3 Modulation Interval IFN gamma values were then compared in Dex treated and untreated NK cells. See Figure 4B. Dex significantly decreased the H3K27me3 Modulation Interval IFN gamma value for intervals 7, 8, and 9 when compared to untreated NK cells. There were no significant differences detected in H3K27me3 Modulation Intervals 1–6, or 10.

3.8 H3K27me3 Chromatin Organization (CO) of Dex treated and untreated NK cells

H3K27me3 Modulation and H3K27me3 BDI provide rapid and high throughput quantification of the localization and density of H3K27me3 within the nucleus of individual NK cells. When combined with the percentage of NK cells within a particular H3K27me3 Modulation Interval, the mean H3K27me3 Chromatin Organization (CO) value of the NK cells within each H3K27me3 Modulation Interval was calculated: mean H3K27me3 Modulation $\times$ mean H3K27me3 BDI $\times$ % NK cells in each H3K27me3 Modulation Interval. A comparison of H3K27me3 CO for Dex treated and untreated NK cells is shown in Figure 4C. For H3K27me3 Modulation Intervals 8–10, (H3K27me3 Modulation Intervals with Peripheral H3K27me3 localization) Dex treated cells had a significant increase in H3K27me3 CO when compared to untreated NK cells. These data demonstrate Dex to increase H3K27me3 CO, due to an increase in the percentage of NK cells with Peripheral H3K27me3 localization.

For six separate NK cell populations (derived from 6 separate individuals), relationships among H3K27me3 CO and H3K27me3 cytoplasmic IFN gamma were assessed. See Figure 5. A significant negative relationship ($r = -0.557$, $p = 0.004$) was found between H3K27me3 CO and IFN gamma MFI in Dex treated NK cells. No relationship was observed for untreated NK cells ($r = -0.097$, $p = 0.638$). Data for H3K27me3 Modulation Intervals 6–10 were analyzed in that no difference in H3K27me3 Modulation Interval IFN gamma 1–5 was noted. Further, H3K27me3 Modulation Intervals 6–10 contained the majority of NK cells within each subject. In Dex treated NK cells, Modulation Intervals 6–10 contained 95% $\pm$ 1.7% of the entire NK cell population. While in untreated NK cells, H3K27me3 Modulation Intervals 6–10 contained 89.5% $\pm$ 1.8% of the entire NK cell population.

3.9 H3K27me3 Global Chromatin Organization (CO) of Dex treated and untreated NK cells with regard to NK cell function (IFN gamma production)

In order to make a direct comparison of NK cell populations, a H3K27me3 Global CO value was derived and is the summation of the ten H3K27me3 Modulation Interval CO values. In this manner, a single value was assigned to an entire NK cell population and that value was then evaluated with regard to NK cell function (i.e. IFN gamma level for the entire NK cell

population). See Table 4A. Dex treated NK cells had a significantly larger H3K27me3 Global CO value, which coincided with a significantly reduced Global IFN gamma value for the entire population of cells. Likewise, when H3K27me3 Global CO and Global IFN gamma were compared for the summation of data from H3K27me3 Modulation Intervals 6–10, there was an increase in H3K27me3 Global CO with a subsequent decrease in Global IFN gamma production. See Table 4B. These data demonstrated that H3K27me3 Chromatin Organization was coincident with decreased NK cell IFN gamma production.

4. Discussion

In this investigation the GC, dexamethasone, was shown to alter the H3K27me3 global chromatin organization (CO) of NK cells. This alteration was evidenced by an increase in the intensity (as judged by mean fluorescent intensity, MFI) of the histone epigenetic, post-translational mark, H3K27me3. Increased H3K27me3 intensity was related to peripheral H3K27me3 localization in individual NK cells. Peripheral localization was first determined by visual inspection and then by automated analysis of H3K27me3 Modulation and H3K27me3 BDI within individual NK cells. Increased H3K27me3 Modulation and increased H3K27me3 BDI identified localization of H3K27me3 to the nuclear periphery. With GC treatment, the percentage of NK cells with peripheral H3K27me3 localization was markedly increased. This peripheral H3K27me3 localization is consistent with heterochromatic DNA and the repression of genes so localized [51, 52].

As well, peripheral H3K27me3 localization was noted in untreated NK cells and this was not unexpected in that many silenced genes are located to heterochromatin [50]. Further, environmental factors including but not limited to diet [53], age [54, 55], smoking [56, 57], previous psychological stressors [42, 43], and alcoholic intake [58] are known to effect epigenetic marks and may result in peripheral H3K27me3 localization in untreated NK cell. However, it is clear is that GC significantly increased the percentage of NK cells with peripheral H3K27me3 localization. GC treatment induced the localization, density, and intensity of H3K27me3 at the nuclear periphery, which is consistent with repressed, heterochromatic DNA and with transcriptionally repressed heterochromatic domains [59, 60]. To the best of our knowledge, this is the first visualization of GC induced global alterations in H3K27me3 chromatin organization. Such visualizations may be potentially useful for not only monitoring the effects of psychological stress but also for the evaluation of a patient's response to exogenous GC administration.

Moreover, increased peripheral H3K27me3 localization was directly related to reduced production of intracellular IFN gamma in GC treated cells. In GC treated NK cells, negative relationships were observed between CO and IFN gamma in H3K27me3 Modulation Intervals 6–10, which were not observed in untreated NK cells. H3K27me3 Modulation Intervals 6–10 were chosen because no differences were observed in Intervals 1–5 between GC treated and untreated NK cells. Further, the majority of NK cells were detected in H3K27me3 Modulation Intervals 6–10. Based on these intervals, GC treated NK cells demonstrated a marked increase in H3K27me3 global CO and a marked decrease in NK cell IFN gamma. These data demonstrate GC to alter H3K27me3 CO by increasing the peripheral localization and density of H3K27me3 and this alteration directly related to

reduced IFN gamma production in GC treated cells. Hence, the peripheral localization and density of H3K27me3 (H3K27me3 CO) within NK cells is an indicator of GC induced NK cell dysregulation. The finding that H3K27me3 CO is directly related to effector function is further substantiated by a recent carcinoma study in which increased H3K27me3 localization to the nuclear periphery was correlated to an aggressive tumor subgroup of Hepatocellular Colon Carcinoma [61].

This investigation was accomplished by coupling assessment of the effects of GC on NK cells with an efficient and innovative high through-put technology, the Amnis ImageStream. It achieves high through-put capacity as a multispectral imaging flow cytometer, combining microscopic imaging with flow cytometry. It can image up to 100 cells/sec, with simultaneous acquisition of 6 images per cell, including bright field and fluorescent intensities. It combines the per-cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry. Localization of H3K27me3 signal intensity was measured with the Amnis ImageStream IDEAS software Modulation feature, which quantifies signal intensity distribution within the nucleus, normalized to a number between 0 and 1. H3K27me3 Modulation served as a quantitative value representing the contour of H3K27me3 staining in individual nuclei. Increased H3K27me3 Modulation was correlated with increased peripheral localization of H3K27me3. H3K27me3 BDI identified increased density of H3K27me3 in individual nuclei. With increased H3K27me3 BDI there was an increase in density of H3K27me3 at the nuclear periphery. Hence, H3K27me3 Modulation and H3K27me3 BDI were utilized to develop an H3K27me3 CO value to measure the degree of peripheral localization and density of H3K27me3 within NK cell nuclei. These observations are consistent with the concept that CO both reflects and impacts transcriptional regulation and is responsive to physiological stimuli [60, 62].

At the nuclear periphery, CO affects gene transcription, as well as inter- and intra-chromosomal gene clustering [60, 62], and is an important site for repressed gene transcription [63, 64]. Each chromosome within the nucleus occupies a defined nuclear territory with spatial domains dependent upon distinct structural and functional states. These are; euchromatin, which is loosely packed and contains transcriptionally active loci, and heterochromatin, which is highly compact and transcriptionally silent [60]. At the nuclear periphery euchromatin tends to localize to the nuclear pore complex (NPC) while heterochromatin tends to localize to the lamina [65, 66].

Actively transcribed genes are tethered to the NPC [67–69], which facilitates mRNA export [70, 71]. In close proximity to the NPC and associated with the nuclear lamina is heterochromatin [72, 73], in which gene transcription is repressed [74]. Hence, the nuclear periphery is a platform in which the more abundant heterochromatin is interrupted by the euchromatin associated with the NPC [72–78]. DNA regions marked by H3K27me3 are nucleosome compacted and when associated with the nuclear periphery are heterochromatic. These regions contain inaccessible regulatory elements, reduced transcription factor binding, and are readily detected as a highly ordered structure within the three dimensional architecture of the nucleus [51, 52]. Regions of DNA containing H3K27me3 form large compartments of topologically associated domains (TADs) containing tracts of DNA

silenced by the polycomb-repressive complexes (PRC) [34, 79]. These TADs dictate transcription of the genes contained within the TAD. Tri-methylation of lysine 27 on the exposed N-terminal tail of histone H3 (H3K27me3) is catalyzed by the methyl transferase Enhancer of Zeste Homolog 2 (Ezh2), which is a subunit of PRC2 [80]. Histone trimethylation of H3K27 at the targeted nucleosome enhances the activity of PRC2 and with increased PRC2 methyl transferase activity, H3K27me3 marks propagate along the chromosome [81]. PRC2 and H3K27me3 recruit protein complexes that compact chromatin and epigenetically silence genes within that region of DNA [80, 82, 83]. These protein complexes include histone deacetylase 1 (HDAC1) [84, 85] that reduces DNA accessibility by histone deacetylation [86] and is known to interact with PRC2 through EZH2 [85].

We have previously demonstrated GC: GR to associate with HDAC1 and to repress IFN gamma production by NK cells [24, 42]. Reduction of IFN gamma production by GC is associated with reduced histone acetylation at the *IFNG* promoter and with a quantitative reduction in *IFNG* mRNA production [42]. In this investigation we extend those observations to analysis of GC effects on the repressive epigenetic mark, H3K27me3. We found GC to not only increase detectable H3K27me3 within nuclei but also to increase localization of the epigenetic mark to the heterochromatic nuclear periphery. Such localization correlated with reduced IFN gamma production. Upon stimulation, mature NK cells are poised to produce IFN gamma within minutes to hours after stimulation [4]. Hence, it is possible that the euchromatic localization of *IFNG* is likely at the NPC, and, if so, this would allow for rapid mRNA nuclear export and active transcription. It is also possible that with increased GC (e.g. during stress), GC: GR recruitment of PRC2 with tri-methylation of H3K27 and deacetylation by HDAC1, may condense the chromatin domain containing *IFNG*, associating it with the nuclear lamina. GR binds to >10,000 sites within the human genome and GC regulate the expression of hundreds of genes interacting over tens of kilobases [32, 47]. These long range interactions regulate multiple genes within TADS [46]. Hence, it can be speculated that the GC induced detection of H3K27me3 at the nuclear periphery likely reflects the heterochromatic localization of chromatin domains containing other repressed genes in addition to *IFNG* [87, 88].

The relationship of psychological stress and the HPA axis is well established [89]. It is known that GC, at therapeutic concentration, is anti-inflammatory and as an immunosuppressive agents is used for the treatment of numerous autoimmune and inflammatory diseases [90]. Endogenous GC also play a physiologic role in feedback inhibition of immune/inflammatory responses [91, 92]. Further, exogenous GC at physiological concentrations have repeatedly been shown to suppress human and animal NK immune function [42, 93–97]. Further, GC are known to suppress production of cytokines, e.g. IFN gamma, as well as natural killer cell cytotoxic activity via GR activation [98–100] [42, 43], and GR interaction with immune effector gene loci. As we have shown previously, GC reduced NKCA and IFN gamma production *in vitro*. This effect was shown to be mediated at least in part through an epigenetic mechanism [42, 43]. Based on the data presented herein it appears that in addition to these effects, peripheral localization of the repressive epigenetic mark H3K27me3 is also influenced by GC and such localization is related to suppression of cytokine production. In contrast to GC, the effect of norepinephrine (NE) on immune function is controversial. NE has been shown to increase [101–103],

decrease [104–106], or have no effect [89] on NK cell immune function. This investigation sought to determine if NE suppressed NK cell function. NE treatment did not suppress NK cell lysis of tumor cells (NKCA), or suppress production of IFN gamma.

There are limitations to this investigation and chief among these is the *ex vivo* treatment of human PBMCs with GC. It is unknown if the effects so observed *in vitro* would be similar in subjects undergoing a psychologically stressful event. Further, we have explored a single histone epigenetic mark and a single immune effector molecule. However, H3K27me3 is a well characterized post-translational histone epigenetic mark associated with gene repression and with heterochromatic localization within the nucleus. We have explored only IFN gamma. However, IFN gamma is a hallmark cytokine produced by NK cells and is central to the innate and adaptive immune response. As such, it is likely that results obtained with this epigenetic mark and this cytokine are generalizable to overall immune function and to the effects of GC on the CO of NK cells. It is worth noting that environmental factors that may influence peripheral H3K27me3 were not examined in this investigation, and as well that the GC concentration used in this *in vitro* study is not necessarily similar to GC concentrations that may be found *in vivo*. In conclusion, GC was demonstrated to alter H3K27me3 CO and this alteration directly related to the capacity of NK cells to optimally carry out immune function. Alterations in CO, as a downstream and more proximal indicator of immune function, may be an effective means by which to identify those at risk for GC induced immune dysregulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported in part by National Cancer Institute R01-CA134736 and R01-CA197005. The authors gratefully acknowledge the expertise of Patricia Simms Loyola University Health Systems Flow Cytometry Facility, as well as Karen Kotz and Deborah Sindewald for the enrollment of volunteers and procurement of blood samples.

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Highlights

The influence of glucocorticoids on natural killer cell function relates to chromatin organization.

Glucocorticoids increase localization of the repressive epigenetic mark, H3K27me3, to the nuclear periphery.

Nuclear peripheral localization of H3K27me3 is negatively related to IFN gamma production

Chromatin organization, as assessed by H3K27me3 peripheral nuclear localization, is an index of natural killer cell function.

Figure 1. Effect of Dexamethasone (Dex) and Norepinephrine (NE) on NKCA of PBMC
 NKCA expressed as percent cytotoxicity for effector to target ratios of 50:1, 30:1, 20:1, and 10:1. Untreated PBMC are depicted in the black boxes, NE treated PBMC are depicted in the gray boxes, and Dex treated PBMC are depicted in the open boxes. Data represents the mean cytotoxicity of six individuals $\pm$ SEM. Data were analyzed by one-way repeated measure ANOVA to determine differences among groups over all 4 effector to target ratios. *Post hoc* Tukey HSD analyses were then used to determine differences between Dex, NE, or untreated PBMC within each effector to target ratio. * = $p < 0.05$, *** = $p < 0.005$ comparison of Dex or NK treated and untreated PBMC.

Figure 2. Nuclear localization of H3K27me3 in NK cells

Representative images of Peripheral and Non-peripheral H3K27me3 localization within NK cells. A. Peripheral localization of H3K27me3 in NK cell nuclei. Note the localization of H3K27me3 at the nuclear periphery. B. Non-peripheral H3K27me3 localization in NK cell nuclei. Note that H3K27me3 is not localized at the nuclear periphery, but rather throughout the nucleus.

Figure 3.

Relationship of H3K27me3 Modulation to H3K27me3 Bright Detail Intensity (BDI) within NK cells. A. Relationship between H3K27me3 Modulation to H3K27me3 BDI in untreated NK cells. B. Relationship between H3K27me3 Modulation and H3K27me3 BDI in Dex treated NK cells. Definitions of H3K27me3 Modulation and H3K27me3 BDI are detailed in the Methods and Materials Section. H3K27me3 Modulation is plotted on the x-axis. While H3K27me3 BDI is plotted on the y-axis. Each dot represents an individual NK cell. H3K27me3 Modulation Intervals represent 10% increments of the maximum H3K27me3 Modulation value for an individual NK cell population derived from a single individual. A single NK cell was detected with a H3K27me3 Modulation value of 0.95 that was obscured by the vertical line of H3K27me3 Modulation Interval 10. NT = untreated.

Figure 4A. NK cell percentage within each H3K27me3 Modulation Interval

Percentage of NK cells present in each H3K27me3 Modulation Interval was determined for NK cells derived from six individuals. Untreated NK cells are depicted by the closed boxes. Dex treated NK cells are depicted by the open boxes. Data are means of six individuals $\pm$ SEM. The data were analyzed by Student's t-test for each H3K27me3 Modulation Interval. * $p < 0.05$, ** $p < 0.01$.

Figure 4B. NK cell H3K27me3 Modulation Interval IFN gamma levels

H3K27me3 Modulation Interval IFN gamma production for untreated NK cells is depicted in the closed boxes and for Dex treated NK cells, open boxes. H3K27me3 Modulation Interval IFN gamma was calculated as follows: H3K27me3 Modulation Interval mean IFN gamma MFI $\times$ % NK cells for each H3K27me3 Modulation Interval. Data are the mean of six individuals $\pm$ SEM. The data were analyzed by Student's t-test for each H3K27me3 Modulation Interval. * $p < 0.05$.

Figure 4C. H3K27me3 Modulation Interval Chromatin Organization (CO) of Dex treated and untreated NK cells

H3K27me3 Modulation Interval CO for untreated NK cells is depicted in the closed boxes and Dex treated NK cells, open boxes. H3K27me3 Modulation Interval CO for each H3K27me3 Modulation Interval was calculated as: mean H3K27me3 Modulation $\times$ mean H3K27me3 BDI $\times$ Percentage of NK cells within each H3K27me3 Modulation Interval. Data are the mean of six individuals $\pm$ SEM. The data were analyzed by Student's t-test for each H3K27me3 Modulation Interval. * = $p < 0.05$.

Figure 5. Relationship of H3K27me3 Modulation Interval Chromatin Organization (CO) to H3K27me3 Modulation Interval IFN gamma levels in Dex treated and untreated NK cells Untreated NK cells are depicted in the open circles and Dex treated NK cells are depicted in the closed circles. The relationship between H3K27me3 Modulation Interval CO and H3K27me3 Modulation Interval IFN gamma production for untreated cells is depicted by the hashed line ($r = .097$, $p = 0.638$). The relationship between H3K27me3 Modulation Interval CO and H3K27me3 Modulation Interval IFN gamma production of Dex treated NK cells is depicted by the solid line ($r = -0.557$, $p = 0.004$). Data for H3K27me3 Modulation Intervals 6–10 for six individuals, comprise the Figure.

Table 1

Effect of Dex and NE treatment on IFN gamma production by PBMC and by NK cells.

	Untreated	NE- ⁶ M	Dex- ⁶ M
IFN gamma (ng/ml)	4.631 +/- 1.368	3.198 +/- 1.797	0.128 +/- .087 *
IFN gamma (MFI)	3,498 +/- 1236	ND	2,303 +/- 814 *

PBMC IFN gamma production, as ng/ml was measured by ELISA. Data represent the mean IFN gamma production by PBMC from six individuals +/- SEM. Data were analyzed by one-way ANOVA with Tukey HSD *post hoc* analysis, * = p < 0.05, comparison of Dex treated and untreated PBMC. No difference was observed between untreated and NE treated PBMC. NK cell (CD3⁻CD56⁺) IFN gamma production was measured by mean fluorescence intensity (MFI) by Amnis ImageStream. Data represents the mean IFN gamma MFI from six individuals +/- SEM. Data were analyzed by Paired Student's t-test.

* = p < 0.05 Dex treated NK cells compared to untreated NK cells. ND = not determined.

Table 2

Nuclear H3K27me3 mean fluorescence intensity (MFI) within dexamethasone (Dex) treated and untreated NK cells.

	Untreated	Dex ⁻⁶ M	p
H3K27me3 Intensity	86,210+/- 30,480	100,000+/- 46,111	p < 0.005

Data represent the mean H3K27me3 MFI of NK cells from six individuals +/- SEM. Data were analyzed by Student's t-test.

Table 3

Localization of H3K27me3 within Dex treated and untreated NK cells.

Phenotype	% Dex Treated	% Untreated	p
Non-Peripheral H3K27me3	40 +/- 2	64 +/- 2	<0.005
Peripheral H3K27me3	60 +/- 2	36 +/- 2	<0.005

Visual inspection of 100 randomly selected NK cells for Peripheral and Non- Peripheral H3K27me3 localization in Dex treated and untreated cells. Data represent mean values of NK cells from six individuals +/- SEM. Data were analyzed by Student's t-test.

Global Chromatin Organization (CO) Score and Cytoplasmic IFN gamma levels for individual populations of NK cells.

Table 4

A.		B.	
Untreated	Dex ⁻⁶ M	p	p
H3K27me3 Global CO (H3K27me3 Modulation Interval 1–10)	5,071+/- 504	9,383 +/- 1121	0.006
Cytoplasmic IFN gamma (H3K27me3 Modulation Interval 1–10)	3,382+/- 571	2,240 +/- 446	0.007
Untreated	5,055+/- 636	9,696+/- 1360	0.005
Cytoplasmic IFN gamma (H3K27me3 Modulation Interval 6–10)	3,008+/- 550	2,046+/- 488	0.001

A. H3K27me3 Global CO value for the entire cell population was calculated as the summation of the ten H3K27me3 Modulation Interval H3K27me3 CO values. As well a Global IFN gamma value for the entire cell population was calculated as the summation of the 10 H3K27me3 Modulation Interval IFN gamma values. Modulation Intervals 1–10. B. H3K27me3 Global CO was calculated as the summation of H3K27me3 Modulation Intervals 6–10. Global IFN gamma value was calculated as the summation of H3K27me3 Modulation Interval IFN gamma values from H3K27me3 Modulation Intervals 6–10. NK cells were derived from 6 individuals +/- SEM. Dex and untreated NK cells were compared by Paired T-test.