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Glucocorticoids regulate natural killer cell function epigenetically

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Abstract

Although glucocorticoids are well known for their capacity to suppress the immune response, glucocorticoids can also promote immune responsiveness. It was the purpose of this investigation to evaluate the molecular basis for this apparent dichotomous immunologic effect. Glucocorticoid treatment of natural killer cells (NK) was shown to reduce NK cell cytolytic activity by reduction of histone promoter acetylation for perforin and granzyme B, which corresponded with reduced mRNA and protein for each. In contrast, glucocorticoid treatment increased histone acetylation at regulatory regions for interferon gamma and IL-6, as well as chromatin accessibility for each. This increase in histone acetylation was associated with increased proinflammatory cytokine mRNA and protein production upon cellular stimulation. These immunologic effects were evident at the level of the individual cell and demonstrate glucocorticoids to epigenetically reduce NK cell cytolytic activity while at the same time to prime NK cells for proinflammatory cytokine production.

Keywords

Natural killer cells; Glucocorticoids; Epigenetic; Proinflammatory cytokines; Granule constituents

1.Introduction

Glucocorticoids (GC) are primary stress hormones necessary for life that are synthesized in the adrenal cortex and released into the blood stream homeostatically and in response to environmental and physiological stress. GC orchestrate various physiological processes (e.g. metabolism, energy production, vascular tone, bone mineralization, central nervous system

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and immune function) by regulation of gene transcription, both induction and repression, of nearly 25% of the genome [1; 2; 3]. Because of their potent anti-inflammatory and immunosuppressive effects, synthetic GC are widely used to treat inflammatory and autoimmune diseases, and are known to suppress both innate and adaptive immune responses. GC suppress cell mediated lytic activities [4; 5] as well as cytokine production [6; 7; 8; 9; 10; 11] by many immune cell populations; including NK [4; 12], T lymphocyte[13], monocyte [11; 14; 15] and macrophage [16]. Of particular note, natural killer cells and NK cell cytolytic activity (NKCA) are especially responsive to GC [4; 12; 17; 18; 19]. Even though GC are known to suppress immune function under some conditions, GC under other conditions can promote immune responsiveness [20; 21; 22; 23; 24]. Physiological doses of GCs have been shown to enhance immunoglobulin production by mitogen-stimulated human lymphocytes[25] and GC have been shown to stimulate B lymphocyte number and antibody production *in vitro* and *in vivo* [26]. Further, during the early stages of T-cell activation, low levels of GC enhance T-cell receptor induced lymphocyte proliferation increase T-cell responsiveness to IL-2 and enhance proliferation of memory T cells [27; 28]. GC have been shown to synergistically enhance the induction of IL-1 beta and IL-6 [29] and the biological effects of IL-2, interferon (IFN) gamma, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and oncostatin M [30]. These immune modulating effects of GC are concentration and time dependent [31; 32] and it is clear that in addition to well-known immunosuppressive effects, GC are also able to exert modulating and enhancing effects upon the immune system [33; 34; 35]. In experimental models, both suppressive and immune enhancing effects of GC have been demonstrated experimentally for inflammatory cytokine mRNA and protein production by monocytes [11; 14; 15], phagocytosis by macrophages[16], acute-phase protein gene expression by hepatocytes[36], delayed-type hypersensitivity reactions [37] and wound healing[38]. In those studies, immune enhancing effects were observed at lower GC concentration and immune suppressive effects at higher GC concentration.

The timing of GC administration also affects immune outcomes in that a 24 hour pre-treatment of experimental animals with GC potentiated the proinflammatory response to subsequent endotoxin challenge; whereas, the administration of GC 1 hour after endotoxin challenge resulted in suppression of the proinflammatory response [20]. In human volunteers, a 2 week administration of dexamethasone (a synthetic GC) resulted in an attenuation of GC mediated inhibition of IL-6 and TNF-alpha production *in vitro* [39]. Further, experimentally increased plasma cortisol levels (concentrations of 75 to 85 μg/dL for a 6-hr period) ending 12–144 hours before injection of endotoxin, resulted in an increased proinflammatory response to the bacterial product[13]. In that study, time as well as GC concentration were important in determining the effect of GC. More recent work has demonstrated exposure of humans to cortisol concentrations of 35 to 45 μg/dL total plasma cortisol (approximately 80 nM free cortisol) enhanced inflammatory responses to a subsequent, stimulus with endotoxin [40]. Those results demonstrated a “preparative” or “priming” effect of GC on the immune proinflammatory response. A GC concentration of 35 to 45 μg/dL is similar to concentrations commonly observed during human systemic stress responses [40].

The basis for these effects of GC are not well understood, although various possibilities have been proposed [41; 42; 43; 44] including; mechanisms upstream of the binding of GC to its receptor, modified intracellular GC concentrations or insufficient GR expression. Those studies also reported mechanisms downstream of the binding of GC to GR that involve GC signaling pathways [45; 46]. In addition to these, another possibility is that GC influence epigenetic processes that result in the observed immunological effects. Histone tail post translational modifications (e.g. acetylation, methylation) regulate gene transcription [47; 48; 49] and GC have been shown to modify NK cell function epigenetically [50; 51]. In those reports, GC at a high concentration reduced NKCA; global histone acetylation, the acetylation of histone (H) 4 lysine (K) position 8, and promoter accessibility for perforin, interferon gamma and granzyme B. These epigenetics effects corresponded with reduced production of granule constituents (perforin and granzyme B) as well as reduced constitutive and stimulated production of IL-6, TNF alpha and IFN gamma. Histone acetylation was fully recovered by treatment of the NK cells with a histone deacetylase inhibitor, which also restored NKCA and proinflammatory cytokine production levels. Those results demonstrated GC to dysregulate NK cell function through an epigenetic mechanism that reduced histone tail acetylation status, immune effector gene transcription and levels of immune effector proteins necessary to the full functional activity of NK cells [50]. Those results are consistent with the known immune suppressive effects of a high GC concentration on NK cell effector function. In the experiments described herein, the effect of a low GC concentration on NK cells was evaluated with the hypothesis that a dichotomous phenotype would be revealed by treatment of NK cells with a lower GC concentration.

2.Materials and methods

2.1. Cell Culture

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

2.2. Cellular Treatment

NK92s, cultured at 2.5×10^5 cells/mL, were treated with or without dexamethasone (GC) (Sigma Aldrich, St. Louis, MO) (10^{-10} M) for 4 days in the presence of IL-2 (100 units/mL),

every 48 hours cells were collected, washed, and resuspended in fresh media to a concentration of 2.5×10^5 cells/mL supplemented with or without dexamethasone and IL-2 (100 units/mL). For the final 24 hours of treatment cells were collected, washed, and resuspended to a concentration of 2.5×10^5 cells/mL with or without dexamethasone in the absence of IL-2. Following this treatment NK92s were washed with media and resuspended to 1×10^6 cells/ml and used for analysis. In all cases, cell number and viability were determined by exclusion of 0.1% Trypan blue. Viability was maintained between 85 and 95% in all cases. In some experiments, cell cultures described above were subjected to an additional 24 hour treatment in the presence of GC (10^{-7}M) to determine the sensitivity of the cells to further GC treatment (switch to −7M).

2.3. Evaluation of cytokine production

Cytokines were measured in NK92 culture supernatants as described previously [50]. To assess stimulated cytokine production, NK92s (2.5×10^5 cells/ml) were treated as described above and then stimulated with IL-2 (5,000 U) or IL-12 (40 pg/mL) for an additional 4 hours. Cell culture supernatants were harvested and aliquots of the supernatants were stored at −80 °C for subsequent cytokine determination by ELISA (R&D Systems, Minneapolis, MN).

2.4. Analysis of Gene Expression

Messenger RNA from NK92 cells was obtained using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) was used to generate cDNA for quantitative real-time PCR from 500 ng total RNA. Negative reverse transcriptase samples confirmed absence of contaminating DNA. All reactions were performed in triplicate using SYBRgreen supermix (Biorad) with the following synthetic oligonucleotide primer pairs. IFN-gamma: forward 5’-TGG AAA GAG GAG AGT GAC AG-3’ and reverse 5’- ATT CAT GTC TTC CTT GAT GG-3’. IL-6: forward 5’- CAA CCT GAA CCT TCC AAA GAT G-3’ and reverse 5’- ACC TCA AAC TCC AAA AGA CCA G-3’. Perforin: forward 5’- GGA GTG CCG CTT CTA CAG-3’ and reverse 5’- CGT AGT TGG AGA TAA GCC TGA G-3’. Granzyme B: forward 5’- AAG ACG ACT TCG TGC TGA CA-3’ and reverse 5’- CCC CAA GGT GAC ATT TAT GG-3’. PCR cycles were denaturation at 94°C (30 s) and annealing at 55–61°C (30 s), no extension phase was necessary and performed using the Opticon 2 Real-Time Detector (Bio-Rad, Hercules, CA) and analyzed using the Opticon Monitor Software (Bio-Rad, Hercules, CA). The AAC(t) method was used to determine changes in transcript levels between untreated and chronic GC treated cells using beta-actin as a reference gene.

$$\text{Fold change}=\frac{\text{Efficiency(target)}\hat{}(\Delta\text{C(t)targer(control}-\text{treatment))}}{\text{Efficiency(reference)}\hat{}(\Delta\text{C(t)reference(control}-\text{treatment))}}.$$

2.5.Natural killer cell cytolytic activity (NKCA)

K562 tumor cells were radioactively labeled with 100 uCi of [^{51}Cr] (New England Nuclear, Boston, MA). Radiolabeled K562 cells were washed and then incubated for 4 hour with NK92 cells. Following incubation, the supernatants were removed using a Skatron

harvesting press (Skatron Inc., Sterling, VA) and the associated radioactivity determined. Effector to target ratios for NKCA were 2, 1, 0.5 and 0.25:1 as described previously [50].

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.

2.6. Immunofluorescent Flow Cytometric Analysis of Intracellular and Surface Proteins

For detection of surface proteins, NK92 cells (1.0×10^5/assessment) were incubated with antibodies for 30 minutes on ice (agitated after 15 minutes). Cells were then washed twice with 0.1% BSA (Sigma Aldrich, St. Louis, MO) in PBS (Gibco, Grand Island, NY) and resuspended in 1% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO). Surface staining antibodies included anti-IL12Rβ2 (PerCP conjugated) (R&D Systems, Minneapolis, MN) and anti-IL-2Rα/CD25 (Alexa488 conjugated) (BD Biosciences, San Jose, CA). For intracellular protein analysis, cells were washed twice with 0.1%BSA following surface staining, then fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Jose, CA) for 20 min at 4°C. The cells were then washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA) and probed with antibodies specific for molecules of interest including anti-granzyme B (Alexa Fluor 647 conjugated) (BD Biosciences, San Jose, CA), anti-perforin (PE-conjugated) (BD Biosciences, San Jose, CA), and anti-glucocorticoid receptor (GR) (unconjugated) (Abcam, Cambridge, MA). Secondary anti-IgG (FITC conjugated) (Millipore, Temecula, CA) was added for 30 min at 4°C to unconjugated primary antibodies. For intracellular cytokine analysis of IFN-gamma (PE conjugated) (BD Biosciences, San Jose, CA) cells were incubated in leukocyte activation cocktail (BD Pharmigen, San Jose, CA) at 37°C for 4 hours prior to permeabilization and antibody staining. Samples were analyzed with a FACSCanto Fluorescence-Activated Cell Sorter in the Core Laboratory of the Cardinal Bernardin Loyola University Cancer Center equipped with a 15mW argon-ion laser and a red diode laser using FACSDiva software for data acquisition. After staining, cells were analyzed by flow cytometry. 10,000–30,000 events were recorded and analyzed with FlowJo v8.4.1. Flow cytometric analysis was confirmed by microscopy.

2.7. Western Blot

For Western blot analysis, nuclear and cytoplasmic compartments of NK92 cells were extracted from $1–3 \times 10^6$ 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 both lysed nuclei and cytoplasm were resuspended in Laemmlis SDS-Sample Buffer (4×) (Boston Bioproducts, Boston, MA). Samples were boiled for 10 minutes and proteins separated by electrophoresis with a 12%

agarose gel and transferred to a nitrocellulose membrane for blotting. Proteins were visualized with anti-NF-κB p65 (Abcam, Cambridge, MA), anti-cJun (Abcam, Cambridge, MA), anti-STAT4 (Santa Cruz, Santa Cruz, CA), anti-GR (Abcam, Cambridge, MA) and HRP conjugated goat anti-rabbit IgG secondary antibody (Millipore, Temecula, CA) and chemiluminescence reagent (ThermoScientific, Rockford, IL). Quality of nuclear and cytoplasmic compartment extraction was determined by Laminin B and GAPDH, respectively (Abcam, Cambridge, MA). Blot density was quantified using Image J software.

2.8. Chromatin Immunoprecipitation (ChIP) Assay

NK92 cells (5×10^6) 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. Cross-linked nuclei were extracted using the Fermentas ProteoJET Cytoplasmic and Nuclear Protein Extraction protocol (Fermentas, Burlington, ON) and lysed with 500 μL of high salt lysis buffer (Santa Cruz, Santa Cruz, CA). The samples were sonicated using a Branson Sonifier 250 on ice for a total of four, 15 second cycles per sample. Sonicated samples were diluted and aliquoted into 100μl volumes. Pre-cleared supernatants with ChIP-Grade Protein-G Magnetic Beads (Cell Signaling, Danvers, MA) were collected and immunoprecipitated overnight at 4°C with the following antibodies: anti-acetyl-H4K8 (Millipore, Temecula, CA), anti-acetyl-H3K9 (Cell Signaling, Danvers, MA), anti-acetyl H3K27 (Abcam, Cambridge, MA), anti-trimethyl H3K4 (Cell Signaling, Danvers, MA), anti-trimethyl H3K9 (Cell Signaling, Danvers, MA), anti-trimethyl H3K27 (Cell Signaling, Danvers, MA) and control rabbit Ig (Cell Signaling, Danvers, MA). ChIP-Grade Protein-G Magnetic Beads (Cell Signaling, Danvers, MA) were added and incubated an additional 4 hours at 4°C. The immune complexes were collected, washed, and eluted. Cross-linking was reversed by heating at 65°C overnight and 10 minutes at 95°C in elution buffer (Santa Cruz, Santa Cruz, CA). DNA was recovered using the MiniElute Reaction Cleanup Kit (Qiagen, Valencia, CA) and resuspended in 50 μl of dH_2O. Quantitative real-time PCR was performed using an Opticon 2 Real-Time Detector; PCR cycles were denaturation at 94°C (30 s) and annealing at 55–61°C (30 s), no extension phase was necessary. The DNA samples were amplified with primer pairs to the −22KB enhancer (F: 5’-GAA TTG GCT TGA CAC CTC TGT CCT-3’ and R: 5’-TTC CAT CTC TCG GCA AAG AGC AGT-3’) and proximal promoter of the *IFNG* locus (F: 5’-TCA TCG TCA AAG GAC CCA AGG AGT-3’ and R: 5’-ATG GTG ACA GAT AGG CAG GGA TGA-3’), the proximal promoters of *PRF1* (F: 5’-GGC ACA GTT CCA AGC ACT TCA CAA-3’ and R: 5’-AGC CTC ACT GTG CCT CAG TTT CTT-3’), *GZMB* (F: 5’-AGC CTG TTG CCT CTG TGA GAA AGT-3’ and R: 5’-TGG GAT TTG CTG GCA ACC TAG ACA-3’), *IL6* (F: 5’-CAG AAG AAC TCA GAT GAC TGG-3’ and R: 5’-GCT GGG CTC CTG GAG GGG-3’), and *TNF* (F: 5’-CGC TTC CTC CAG ATG AGC TC-3’ and R: 5’-TGC TCT CCT TGC TGA GGG A-3’). PCR data was analyzed using Opticon Monitor 3 Software (BioRad, Hercules, CA). Modification levels were calculated as percent input using the equation.

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

2.9.Chromatin Accessibility

The EpiQ Chromatin Analysis Kit (BioRad, Hercules, CA) was used to determine the accessibility of the *IFNG* enhancer, *IL6* proximal promoter, *GZMB* proximal promoter, *GAPDH* gene, and *RHO* gene. The methodology supplied in the kit was as directed for cells in suspension. Briefly, 250,000 NK92 cells were collected from treatments and resuspended in the supplied EpiQ chromatin buffer and either left undigested or nuclease digested. DNA was then isolated and quantification of accessibility, a measure of intact genomic regions, was performed by q-RT-PCR. The EpiQ Chromatin Analysis Kit Data Analysis Tool was used to determine percent accessibility. The control primers for the genomic regions *RHO* (F: 5'-AGG TCA CTT TAT AAG GGT CTG GGG G-3' and R: 5'-AGT TGA TGG GGA AGC CCA GCA CGA T-3') and *GAPDH* (F: 5'- ACC TCC CAT CGG GCC AAT CTC AGT C-3' and R: 5'-GGG TGA CTG TCG AAC AGG AGG AGG A-3') were supplied by Bio-Rad and used as directed as 0% and >95% accessibility respectively. Analysis of chromatin accessibility at the *IFNG* enhancer, *GZMB* promoter, and *IL6* promoter were performed using the primer sets listed in the ChIP methodology section.

2.10. Statistical methods

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

3. Results

3.1. Effect of chronic glucocorticoid (GC) treatment on proinflammatory cytokine production

The human NK cell line, NK92, was cultured with dexamethasone (10^{-10}M) for 5 days (termed chronic GC treatment) and the effect of GC determined by comparison to NK92 cells simply cultured in medium alone (untreated). When stimulated with IL-12, cells harvested after 4 and 5 days of GC treatment (Chronic GC + IL-12), exhibited an increased production of IFN gamma when compared to untreated cells stimulated with IL-12 (Untreated + IL-12) (Fig. 1A). GC treatment alone had no impact on basal IFN gamma production (Chronic GC vs. Untreated). The effect of chronic GC treatment was maximal at 5 days with 10^{-10}M GC. 10^{-9}M GC did enhance stimulated IFN gamma production after 5 days but the enhancement was less pronounced than with 10^{-10}M GC. GC (10^{-7}M and 10^{-8}M) inhibited stimulated IFN gamma production at 2, 4 and 5 days of treatment. Data are not shown. Hence the effect of GC at 10^{-10}M was further examined. Intracellular cytokine staining of NK cells confirmed these findings and demonstrated a 32.4±3.8% increase in intracellular IFN gamma mean fluorescence intensity (MFI) after 5 days of chronic GC treatment, when compared to untreated cells (Table 1). The shift in MFI was accompanied by a modest increase in the percentage of IFN gamma positive cells (Table I). This was not an IFN gamma specific phenomenon, as IL-2 stimulated IL-6 production increased from 32.2±1.8pg/mL to 61.6±2.4pg/mL after 5 days of GC treatment (Fig. 1B). mRNA isolated and quantified from NK cells after 5 days of GC treatment exhibited a 23-fold induction of IFN gamma mRNA after IL-12 stimulation, while untreated cells exhibited a 14-fold

induction (Fig. 1C). These results demonstrate chronic GC treatment to prime NK cells for enhanced proinflammatory cytokine production via enhanced transcription of cytokine genes after cellular stimulation.

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

Chronic GC treatment of NK cells produced a time dependent effect on NKCA, with an 18% decrease by day 4 and a 25% decrease by day 5 (Fig. 1D). Human NKCA is primarily mediated via granule dependent lysis of tumor cells; therefore, the mRNA level of two key NK cell granule constituents, perforin and granzyme B, was assessed. As early as day 2 of GC treatment, mRNA expression for both perforin (*PRF1*) and granzyme B (*GZMB*) was reduced when compared to untreated cells. By day 5 of GC treatment *PRF1* transcripts were reduced by 60% and *GZMB* transcripts by 40% compared, (Fig. 1E). Flow cytometry confirmed reductions in both perforin and granzyme B MFI levels after 5 days of chronic GC treatment (Fig. 1F and Table 1). These data demonstrate chronic GC treatment to reduce perforin and granzyme B levels with concomitant reductions in NKCA. Furthermore, the percentages of perforin or granzyme B positive cells were essentially unchanged after chronic GC treatment (Table 1). Taken together, these data demonstrate chronic GC treatment to decreases NK cell cytolytic activity and at the same time prime NK cells for enhanced proinflammatory cytokine production upon stimulation. Secondly, these data demonstrate this effect at the cellular level in that the percentages of cells positive for cytokines or lytic molecules were essentially unchanged. Rather a shift in the MFI at the individual cellular level was observed. As such, chronic GC treatment imparts an apparent dichotomous phenotype on NK cells, which results in reduced NKCA and an enhanced capacity for proinflammatory cytokine production.

3.3. Chronic GC treatment induced histone post translational modifications at proinflammatory gene regulatory regions

Ligand activated glucocorticoid receptor (GR) has been shown to interact with histone modifying enzymes and to influence histone tail post translational modifications at genomic regulatory regions [50; 52; 53]. Histone post translational modifications at genomic regulatory regions can not only affect active transcription rates but can also serve to prime genes for future transcription. Enhanced transcription after stimulation was observed after 5 days of chronic GC treatment (Fig. 1C). Hence, six histone post translational modifications at the promoter and −22KB enhancer region of the *IFNG* locus, and the *IL6* promoter were assessed in chronic GC treated and untreated cells. See Fig. 2. By chromatin immunoprecipitation, each of the three regulatory regions of untreated NK cells exhibited moderate H4K8 acetylation (Ac), H3K27Ac, and H3K4 trimethylation (me3); low but detectable H3K9Ac and H3K27me3; and undetectable/below background (IgG) H3K9me3 levels. In cells treated with GC for 5 days, the *IFNG* enhancer, which is critical for *IFNG* transcription [54; 55], exhibited a significant increase in H4K8Ac as well as H3K27Ac (Fig. 2A). This pattern of acetylation for H4K8 and H3K27 corresponded to the enhanced IFN gamma production and secretion following chronic GC treatment and IL-12 stimulation (Fig. 1A and C). No GC treatment effects were observed at the proximal promoter of *IFNG* (Fig. 2B) or the *TNF* promoter (data are not shown). Like the *IFNG* enhancer, the *IL6* proximal promoter exhibited a significant increase in H4K8Ac as well as H3K27Ac (Fig.

2C) after 5 day of GC treatment. This increase corresponded to the two fold increase in IL-6 production following chronic GC treatment and IL-2 stimulation (Fig. 1*B*). These data indicate that proinflammatory gene regulatory regions exhibit increased acetylation following chronic GC treatment and suggest histone post translational modifications to contribute to the enhanced proinflammatory cytokine production following cellular stimulation. For both the *IFNG* enhancer and the *IL6* promoter an increase in H3K4me3 at day 2 of GC treatment (grey bars) but not at day 5 of GC treatment was noted.

3.4. Chronic GC induced effects on chromatin accessibility

Acetylation increases the accessibility of gene regulatory regions and enhances transcription of genes upon cellular activation [56; 57; 58]. Chromatin from 5 day chronic GC treated and untreated cells was subjected to nuclease digestion. qRT-PCR was used to quantify the degree to which each regulatory region was resistant to digestion compared to *GAPDH*, a constitutively open and transcribed gene. Chronic GC treatment increased the accessibility of the *IL6* promoter by 18% and increased the accessibility of the *IFNG* enhancer by 37% (Fig. 2D). As no increase in transcription was observed prior to cytokine stimulation (Fig. 1A) it is likely that this increased accessibility is the result of the increased acetylation at proinflammatory cytokine gene regulatory regions (Fig. 2A and C) which primes NK cells for enhanced cytokine production when stimulated. Analysis of the *GZMB* promoter yielded no significant difference in accessibility (Fig. 2D). Although granzyme B transcripts were diminished, chronic GC treatment did not appear to alter chromatin accessibility, suggesting that *GZMB* is affected by GC in a manner different from cytokine regulatory regions.

3.5. Chronic GC treatment induced histone post translational modifications at PRF1 and GZMB regulatory regions

In contrast to the cytokine loci, regulatory regions for the granule constituents of untreated NK cells exhibited high levels of H4K8Ac, H3K9Ac, H3K27Ac, and H3K4me3 while H3K9me3 and H3K27me3 were at or below background (IgG) levels. These histone post translational modifications are indicative of genes undergoing active transcription (high H3K9Ac and H3K27Ac) with no heterochromatin marks (H3K9me3 and H3K27me3) [56; 57; 58]. However, with 5 days of chronic GC treatment (dark grey bars) the perforin (*PRF1*) promoter exhibited significantly reduced H3K9Ac and H3K27Ac, corresponding to reduced perforin transcripts (Fig. 3B and Fig. 1F). Likewise, the proximal promoter of *GZMB* exhibited significant reductions in H3K9Ac and H4K8Ac (Fig. 3B) after 5 days of GC treatment, corresponding to reduced transcripts for granzyme B (Fig. 1E). These data suggest chronic GC treatment to reduce NKCA at least in part through reduced acetylation of histones associated with the promoter regions of these two granule constituents. For the *GZMB* promoter a decrease in H3K4me3 at day 2 of GC treatment (grey bars) was noted.

3.6.Effect of chronic GC treatment on GR Cellular Levels and GR Sensitivity to GC

It is possible that continued GC exposure may desensitize NK cells to the effect of the hormone [59; 60] by reducing cellular levels of GR or by reducing the sensitivity of GR to GC. No difference in GR levels, as judged by MFI at either day 2 or day 5, was found between chronic GC treated and untreated cells. A representative histogram is shown Fig.

4A and quantification of GR MFI in Fig. 4B, with untreated cells set to 100%. To assess the functional activity of GR in NK cells treated for 5 days with GC, NK cells were exposed to a high concentration of GC (10^{-7}M) during 4 hours of stimulation with IL-12 (Switch to −7M). Fig. 4C demonstrates that 10^{-7}M GC equally reduced IFN gamma production in both chronic GC treated cells and in untreated cells. These data demonstrate no effect of chronic GC treatment on GR levels or sensitivity to GC.

3.7 Effect of chronic GC treatment on cytokine receptor levels and signal transduction pathways

It is possible that chronic GC treatment may affect levels of cytokine receptors or alternatively the capacity of NK cells to complete signal transduction. Chronic GC treatment had no impact on the levels of the IL-12 receptor beta chain or the IL-2 receptor alpha chain, implying that chronic GC did not alter surface receptor density of these two cytokine receptors (Fig. 5A and B). Downstream of GR signaling is the activation and subsequent nuclear localization of the canonical proinflammatory signaling transcription factors NFκBp65 and AP-1 (c-Jun) as well as STAT4. Western blot analysis and ImageJ quantification demonstrated 5 days of chronic GC treatment to have no impact on the total cellular levels of these three transcription factors (Fig. 5C). Likewise, 5 days of chronic GC treatment did not alter the total cellular level of NFκBp65 (Fig. 5D). Quantification of NFκBp65 and AP-1 (c-Jun) in cell nuclear fractions isolated from NK cells treated for 5 days with GC or not (Untreated) demonstrated no difference in nuclear localization after IL-12 stimulation. (Fig. 5E–F). These data demonstrate NK cells exposed to chronic GC treatment to have no apparent signal transduction defect nor any apparent deficit in cytokine surface receptor levels.

4. Discussion

This investigation evaluated the effects of chronic low concentration GC exposure on the human NK cell line, NK92. This exposure to GC, termed chronic GC treatment, reduced histone acetylation of the proximal promoter regions for two NK granule constituents, perforin and granzyme B. The reduction in histone acetylation corresponded with reduced mRNA and protein level for each and was associated with reduced NK cell cytolytic activity (NKCA). In contrast, chronic GC treatment increased histone acetylation at the −22 kb enhancer region of *IFNG* and the proximal promoter region of *IL6*, as well as chromatin accessibility for each of these gene regulatory regions, priming the regions for transcription upon cellular stimulation. Increases in histone acetylation were associated with quantitatively increased mRNA and protein levels for these cytokines. These epigenetic and immunologic effects were demonstrated at the individual cellular level, in that chronic GC treatment affected levels of perforin, granzyme B, and IFN-gamma within individual cells, with little or no change in the percentage of cells positive for each of these NK cell effector proteins.

Conceptually, histone tail proteins possess highly basic amino-terminal tail domains located outside of the core nucleosome particle, which are accessible to multiple post-translational covalent modifications, especially acetylation [61]. Lysine acetylation neutralizes histone

proteins, whereas deacetylation restores the positive charge of lysines. Following acetylation, nucleosome mobility on the DNA is enhanced, increasing accessibility of the promoter for transcription machinery [62]. Thus, acetylation increases and deacetylation decreases transcription factor access to regulatory regions. With 5 days of GC treatment, a quantitative reduction in the acetylation of H3K9 at the *PRF1* and *GZMB* proximal promoters was noted. H3K9Ac is typically found at active promoters and enhancers and is highly correlated with transcription [63]. In the case of the *PRF1* promoter a reduction in H3K27Ac and for the GZMB promoter H4K8Ac were also observed. These histone post translational modifications are typically found at active gene regulatory regions [56]. Reductions in histone acetylation at these gene regulatory regions would reduce gene transcription. As such, these reductions in histone acetylation likely contribute to the reduced production of transcripts for both of these effector proteins. In contrast, GC treatment increased acetylation of H4K8 and H3K27 for both the *IFNG* enhancer and the *IL6* promoter. Increased histone acetylation at these gene regulatory regions would be expected to increase production of transcripts for both of these proteins upon cellular stimulation. For untreated cells, H3K4me3 with no H3K9me3 or H3K27me3 was noted. H3K4me3 typically marks transcription start sites and is associated with transcription potential at gene regulatory regions [64; 65]. H3K9me3 marks constitutive heterochromatin and H3K27me3 marks silenced genes [63; 66; 67; 68] indicating these gene regulatory regions to be located within euchromatin, either actively transcribed (*PRF1* and *GZMB*) or with the potential for transcription (*IFNG* and *IL6*).

By use of this *in vitro* culture system it was possible to evaluate the effect of GC on NK cell histone post translational modifications over time. After 2 days of chronic GC treatment no significant change in the acetylation status of either the *PRF1* or *GZMB* promoter region was noted. Only a slight numerical reduction in H3K9Ac was detected for both promoter regions at day 2. Significant reductions in H3K9Ac and H3K27AC were only identified by day 5 of chronic GC treatment. The reduced acetylation status of these residues corresponded with maximal reductions in perforin and granzyme B levels as well as maximal reductions in NK cell cytolytic activity. It is worth noting that after 2 and 5 days of chronic GC treatment, transcripts for *PRF1* and *GZMB* were reduced, with detected reductions in perforin at days 4 and 5 and for granzyme B only at day 5. These data suggest that the transient reduction in transcript levels at day 2 were not sufficient to effect NKCA. The effect of GC on NKCA was only manifest after 5 days of continuous culture with GC when maximal reductions in acetylation at both promoters was observed. It is worth noting that a significant reduction in H3K4me3 was observed at day 2 of GC treatment for the *GZMB* promoter. H3K4me3 is associated with an open chromatin structure and transcriptional activation [69; 70]. It is possible that a reduction in this histone post translational modification contributed to reduced transcript levels on that day. The unexpected observation was the acetylation status of the enhancer region of *IFNG* and the promoter region of *IL6*. Both were increased with 5 days of chronic GC treatment. These epigenetic modifications coincided with increased transcription and protein levels of these proinflammatory cytokines when NK cells were stimulated, suggesting that increased acetylation status primed these promoters regions for increased transcription upon cellular stimulation. Interestingly, H3K4me3 was increased with GC treatment for both the enhancer

region of *IFNG* and the promoter region of *IL6*, suggesting that these regulatory regions were open and primed for transcription by day 2. It is possible that this histone post translational modification may pioneer the changes in acetylation noted by day 5. The effect of GC on *PRF1* and *GZMB* regulatory regions directly affected function, while the effect on proinflammatory cytokine gene regions was indirect resulting in a primed or poised condition, apparent only after cellular stimulation. These data demonstrate that GC can dichotomously regulate NK cell effector function epigenetically through two apparently independent mechanisms. These mechanisms do not appear to relate to a direct influence of chronic GC on; the cellular levels of GR, cytokine receptor density, GR sensitivity or signal transduction pathways. Chronic GC did not alter steady state GR or cytokine receptor protein levels. Although ligand activated GR can directly interact with proinflammatory transcription factors like AP-1 (Fos-Jun heterodimers) and NF-κB (p65-p50heterodimers) [71; 72; 73], chronic GC treatment did not alter their steady state levels. Nor did chronic GCs treatment affect lymphocyte responsiveness via the JAK-STAT signaling pathway and or via NF-kB or AP-1. Taken together, the effect of chronic GC treatment on NK cell function is through epigenetic modification of chromatin accessibility, both suppression of NKC A and priming of proinflammatory cytokine loci.

These observations may be of particular relevance to understanding stressful conditions in which GC levels are influenced by the experiences of chronic and or immediate stress. For example in humans, prior adverse experiences, which dysregulate GC have been shown to “prime” inflammatory responses, promoting excessive cytokine production [74; 75; 76; 77; 78; 79]. Likewise, in experimental animals stress has been shown to potentiate neuro-inflammation [20; 21; 22; 80; 81] increasing proinflammatory cytokine production in response to LPS challenge [82; 83]. In those experimental studies, blockade of the GC receptor with the GC receptor antagonist RU486 abolished the stress-induced inflammatory response, while exogenous GC administration, mimicked the effect of chronic stress. It is important to note that chronic stress did not by itself tonically increase proinflammatory cytokine levels, rather the effect of prior stress shifted the animal’s response toward a preferential proinflammatory phenotype [20: 21; 22]. It has been suggested that the proinflammatory effects of GC are limited to the brain [83]. However, GC have been shown to potentiate the peripheral (liver) proinflammatory response to a challenge of LPS, if GCs are administered prior to challenge. The observations reported herein may also provide insight into various human conditions in which an apparent dichotomous phenotype is demonstrable. For example, psychological stress has been associated with reduced peripheral blood NKCA and with increased serum or produced levels of proinflammatory cytokines [4; 19; 85; 86; 87]. In those cases psychological stress was typically associated with hypothalamic-pituitary-adrenal (HPA) axis activation and GC dysregulation. Of particular note is the condition of individuals with major depression, which is a disorder that is accompanied by not just immune dysregulation but also HPA axis activation [43; 88; 89; 90; 91; 92; 93]. Several systematic reviews and meta-analyses have found that major depression is reliably associated with a reduction in NKCA with an increase in circulating levels of proinflammatory cytokines [94; 95; 96]. However, no direct relationship was found between NKCA and proinflammatory cytokines [93]. The observations of this study, support the possibility that both immune suppression and immune enhancement can exist within a

single cell population and that it may be essential to stimulate the cell population to uncover such a dichotomous phenotype.

There are limitations to this investigation. The work has been accomplished in a single human NK cell line, although this work does follow our previous investigative approach with this particular cell line [50]. Also, we have explored only a finite number of proinflammatory cytokines and granule constituents, but these are hallmark molecules for each. Further, we have investigated only 6 different histone post translational modifications, but the evaluation of those has identified unique differences relevant to transcription status. Finally, analysis of the observed effects was accomplished only in the presence of GC. The effect of GC removal on the observed effects was not evaluated.

In conclusion, a low concentration of GC reduced NKCA as well as the histone acetylation of the promoter regions of *PRF1* and *GZMB*, which corresponded with reduced levels of perforin and granzyme B. In contrast, low GC concentration increased enhancer/promoter histone acetylation and accessibility for *IFNG* and *IL6*. These GC induced epigenetic effects resulted in a “preparative” or “priming” effect on the enhancer/promoter in that increased mRNA and protein levels were only detected upon stimulation (cellular activation) of the GC treated cells. Taken together, treatment with a low concentration GC decreased NK cell cytolytic activity and at the same time primed NK cells for enhanced proinflammatory cytokine production. This apparent dichotomous NK phenotype corresponded with histone post translational modifications at the promoter/enhancer gene regions of these immune effector molecules.

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Highlights

- Glucocorticoids reduce NK cell cytotoxicity while at the same time prime NK cells for proinflammatory cytokine production.
- Reduced NK cell lytic activity is associated with reduced histone promoter acetylation for perforin and granzyme B.
- Increased proinflammatory cytokine production is associated with increased histone acetylation of regulatory gene regions.

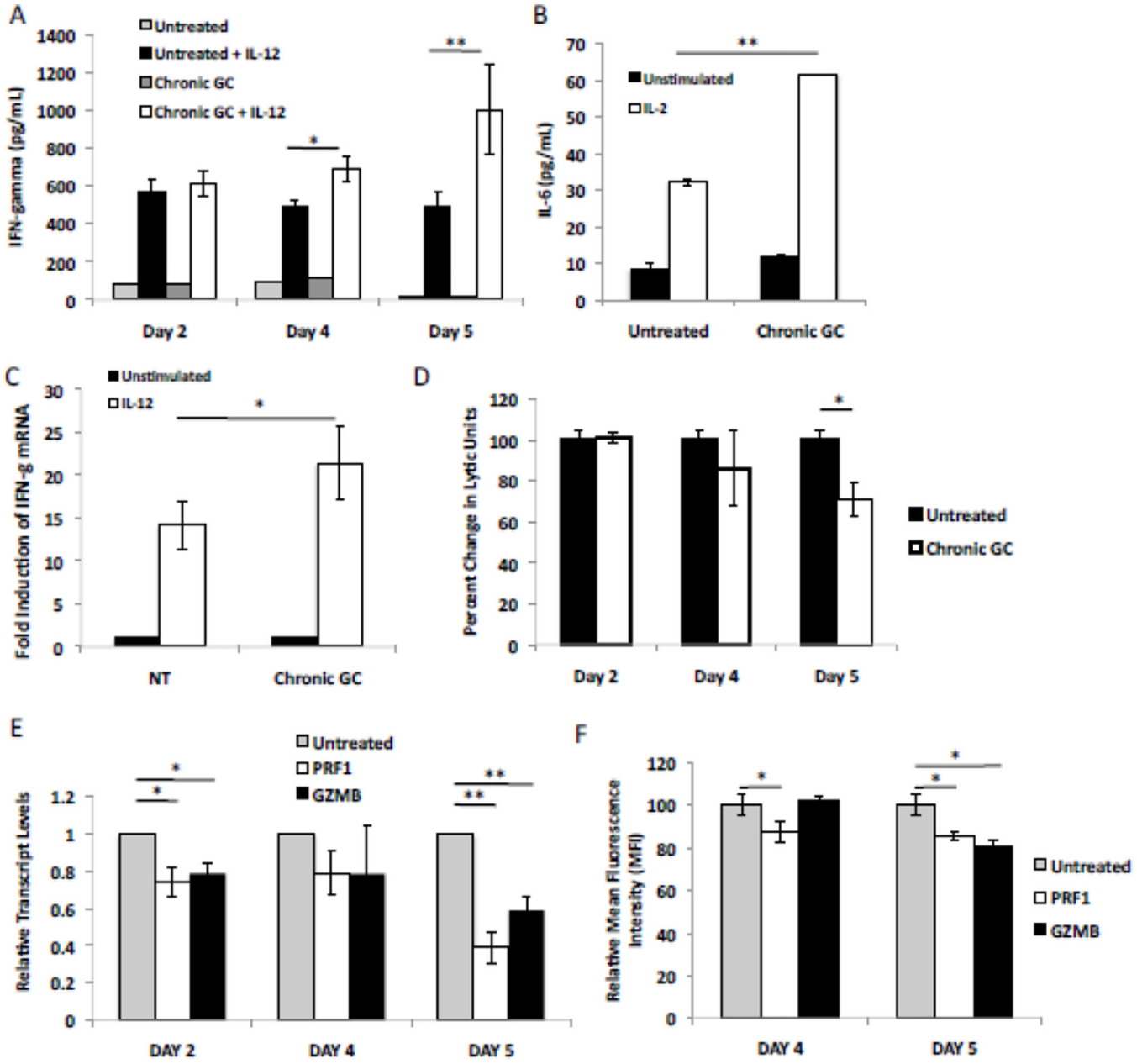

Fig. 1.

Effect of chronic glucocorticoid (GC) treatment on NK cell proinflammatory cytokine production and natural killer cell cytotoxic activity (NKCA). (**A**) IFN gamma production (as measured by ELISA) by NK92 cells, either untreated or treated with GC for various time periods, and then either stimulated or not (unstimulated) with IL-12 for 4 hours, n=3. (**B**) IL-6 production as measured by ELISA by NK92 cells harvested from 5 day cultures (either untreated or treated with GC) and either unstimulated or stimulated with IL-2(5,000 U), for 4 hours, n=3. (**C**) IFN gamma transcript levels for IL-12 (40 pg) stimulated and unstimulated cultures with and without chronic (5 day) GC exposure. IFN-gamma transcript levels from unstimulated, untreated cells were set to 1. (**D**) NKCA for target K562 cells as percent change in lytic units ± SEM of NK92 harvested at indicated days of treatment, n=3.

(**E**) NK92 cells, either untreated or treated with GC for various time periods were harvested and relative transcript levels determined for two granule constituents, perforin (*PRF1*) and granzyme B (*GZMB*). Results were standardized to transcript levels in untreated NK92 cells (grey bars) set to 1. (**F**) Flow cytometric analysis of granule constituent protein levels after 4 and 5 day chronic GC treatment. Graphs demonstrate mean fluorescent intensity (MFI) for perforin and granzyme B. Untreated cells were set to 100%. *; $p<0.05$, **; $p<0.01$.

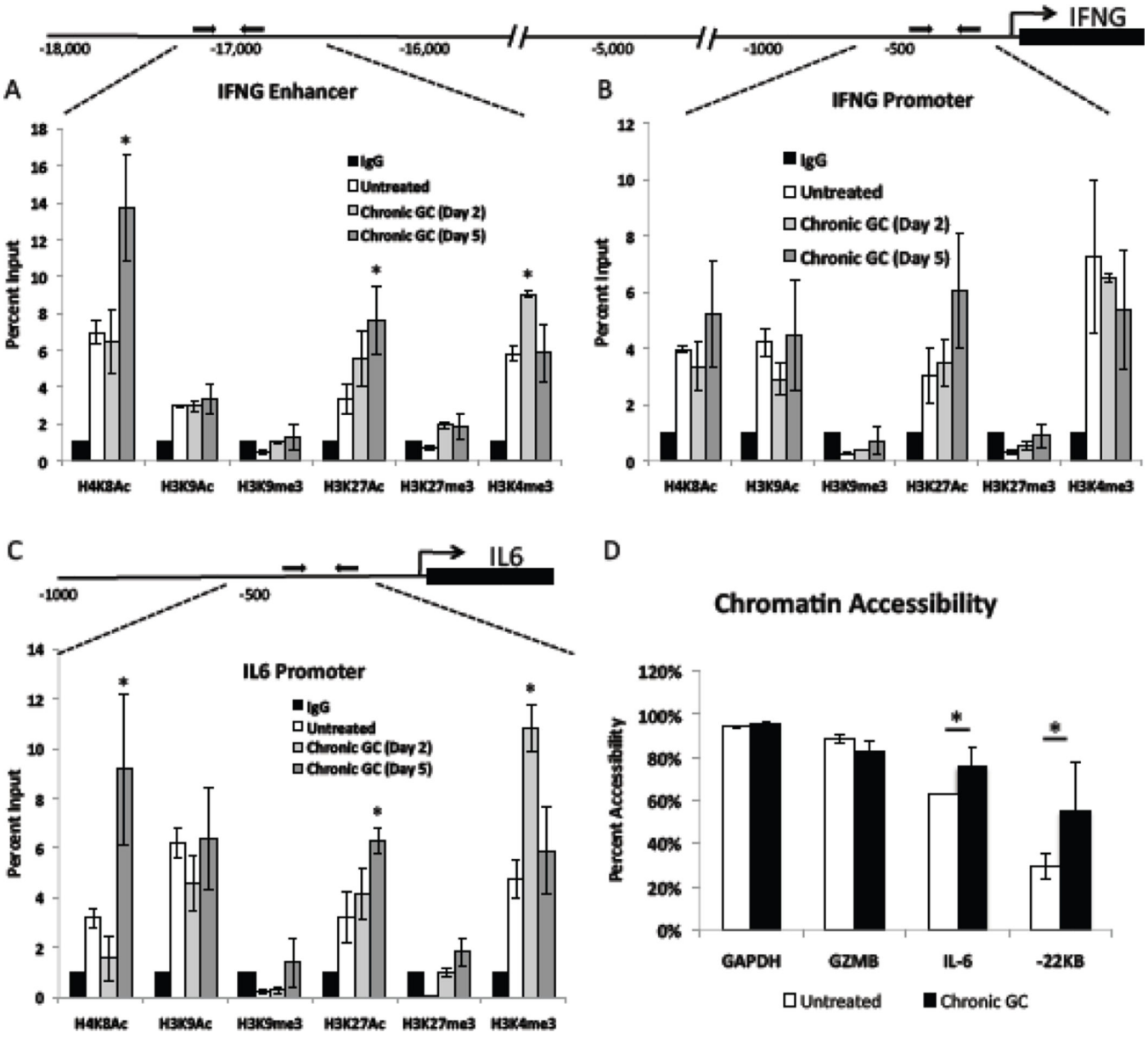

Fig. 2.

Histone post translational modifications at NK proinflammatory gene regulatory regions. Schematic diagrams and percent input calculations of posttranslational histone modifications at the (**A**) *IFNG* enhancer, (**B**) *IFNG* promoter, and (**C**) the *IL6* promoter after 2 days (grey bars) and 5 days (dark grey bars) of chronic GC treatment, compared to untreated cells (white bars) and to the IgG (black bars) negative control. Untreated cell values are the average for each modification at day 2 and day 5. No difference in these levels over time was observed. (**D**) Percent change in sensitivity of indicated genomic regions to nuclease digestion as determined by the Bio-Rad EpiQ chromatin accessibility kit. Accessibility is calculated as the ability of nuclease to digest indicated regions setting GAPDH accessibility as 100% in untreated (white bars) and 5 day chronic GC treated cells

(black bars). Data represent the average of three independent experiments performed in duplicate n=6 ± SEM *; $p<0.05$.

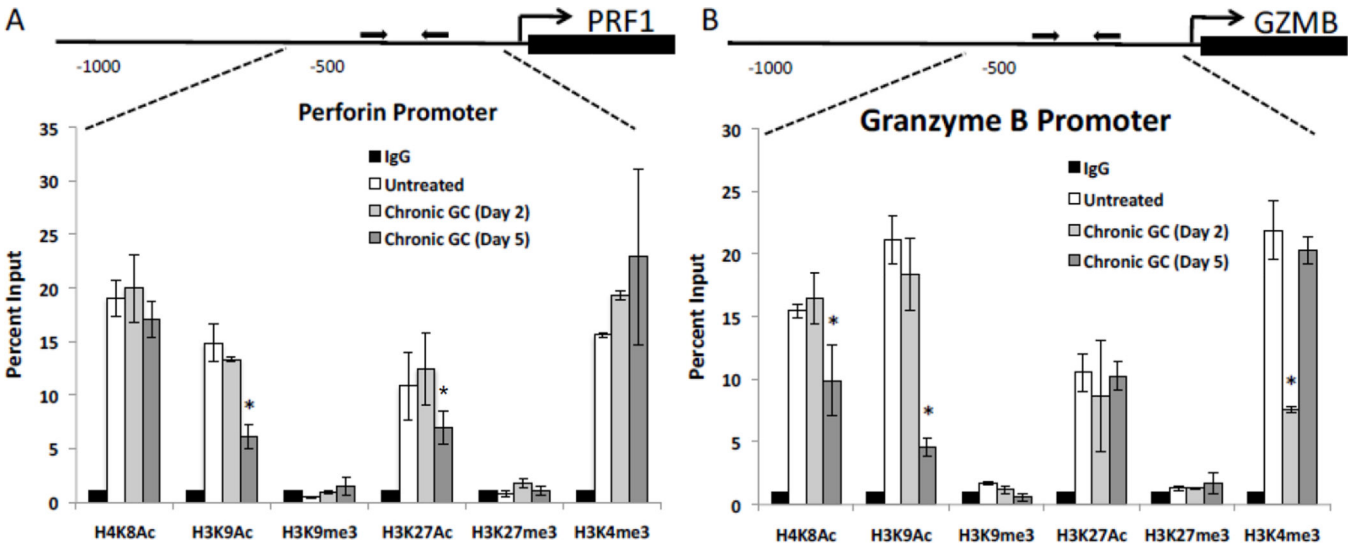

Fig. 3.

Histone post translational modifications at NK cells granule constituent regulatory regions. Schematic diagrams and percent input calculations of posttranslational histone modifications at the (**A**) proximal promoter of the *PRF1* gene and the (**B**) proximal promoter of the *GZMB* gene after 2 days (grey bars) and 5 days (dark grey bars) of chronic GC treatment, compared to untreated cells (white bars) and to the IgG (black bars) negative control. Untreated cell values are the average for each modification at day 2 and day 5. No difference in these levels over time was observed. Data represents the average of three independent experiments performed in duplicate n=6 ± SEM *; $p<0.05$.

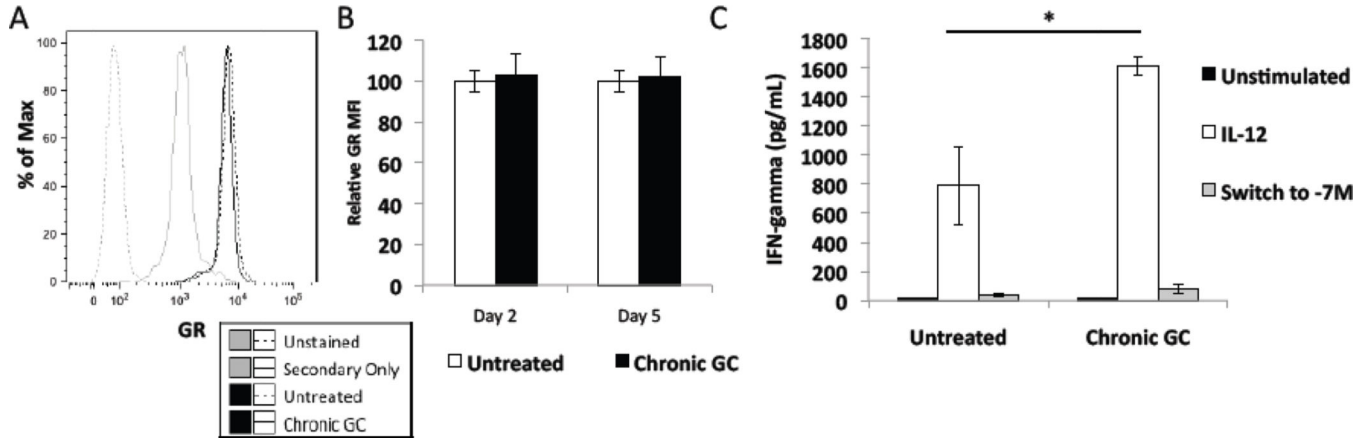

Fig. 4.

Effect of chronic GC treatment on the glucocorticoid receptor (GR). (**A**) Representative histogram of GR protein levels in untreated and chronic GC treated (5 days) NK92 cells assessed by flow cytometric analysis. (**B**) Quantification of MFI from flow cytometric analysis of GR mean fluorescent intensity levels (MFI) of cells harvested after 2 days and 5 days of GC treatment. MFI of untreated cells was set to 100%. (**C**) IFN-gamma production by untreated (black bars) and chronic GC (5 days) (white bars) treated NK92 cells that then received an additional 4 hour treatment with a high concentration of GC (10-^{7}M) (switch to −7M) and IL-12 (40 pg). Data represent the average of three independent experiments n=3 ± SEM *; $p<0.05$.

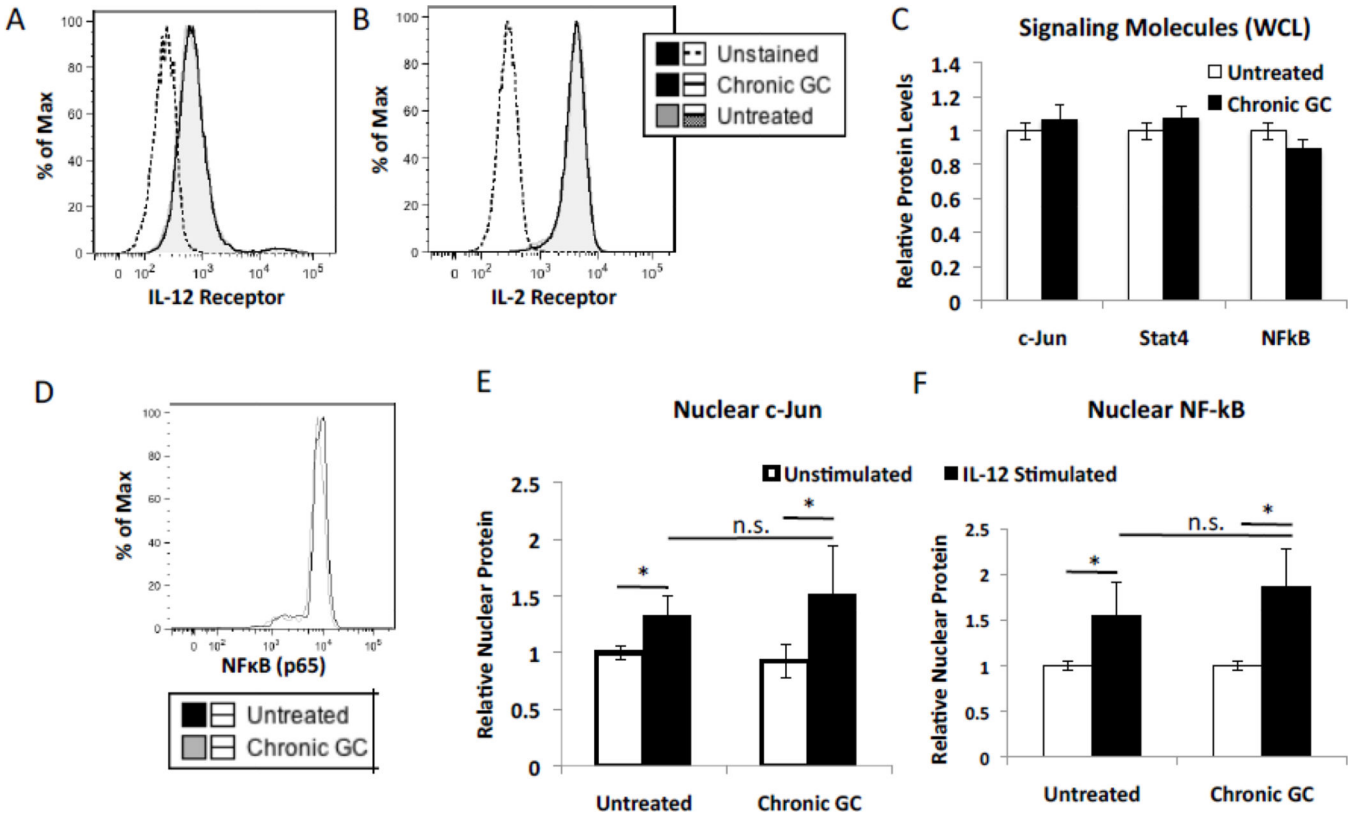

Fig. 5.

Effect of chronic GC (5 day) treatment on NK cell receptor expression and signaling pathways. Flow cytometric analysis of (**A**) IL-12 and (**B**) IL-2 receptor surface expression by NK92 cells treated with GC for 5 days or not (untreated). (**C**) ImageJ quantification of whole cell lysates probed by western blot analysis for c-Jun, Stat4, and NF-kB (p65) levels in chronic GC treated and untreated cells. (**D**) Flow cytometric analysis of NF-kB (p65) in 5 day chronic GC treated and untreated cells. ImageJ quantification of nuclear (**E**) c-Jun and (**F**) NF-kB (p65) in chronic GC treated and untreated cells prior to and after IL-12 stimulation (40 pg). In all cases, relative protein levels in GC treated cells were compared to untreated cells set to 1. n=3 *; $p<0.05$.

Table 1

Effect of chronic glucocorticoid (GC) treatment on proinflammatory cytokine production and granule constituent levels in NK92 cells

Mean Fluorescent Intensity (MFI) and/or Percentages of Cytokine and Intracellular Protein Positive Cells				
Cellular Protein	**Untreated**	**Chronic GC Treatment (5 day)**	***P value***	**(%) Change**
IFN-gamma (MFI)	1452 ± 71	1922 ± 91	0.0005	32.4 ± 3.8
IFN-gamma Positive (%)	85.0 ± 1.0	90.0 ± *1*.7	0.001	5.7 ± 0.37
Perforin (MFI)	7421 ± 192	6371 ± 138	0.017	−14.1 ± 1.86
Perforin Positive (%)	95.3 ± 0.29	94.3 ± 1.36	0.309	−0.98 ± 1.42
Granzyme B (MFI)	11009 ± 150	10115 ± 188	0.017	−8.13 ± 1.71
Granzyme B Positive (%)	98.2 ± 0.40	97.8 ± 0.31	0.318	−0.34 ± 0.31

Values are mean fluorescent intensity (MFI) of surface and intracellular proteins ± S.E.M. after 4 hour stimulation with leukocyte activation cocktail stimulation, N= at least three independent experiments. Isotype control MFI was always < 150. % Change= (MFI Untreated- MFI Chronic GC treatment)/(MFI Untreated) × 100. P values, No Treatment vs. GC Treatment.