ATF2 Impairs Glucocorticoid Receptor-mediated Transactivation in Human CD8+ T Cells

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### Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ATF2</td>
<td>activating transcription factor 2</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
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<td>GCR</td>
<td>glucocorticoid receptor</td>
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<td>GCs</td>
<td>glucocorticoids</td>
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<tr>
<td>H4 K5</td>
<td>histone H4 lysine 5 residue</td>
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<td>HAT</td>
<td>histone acetyltransferase</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>MKP-1</td>
<td>mitogen induced MAPK phosphatase 1</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>P300</td>
<td>adenovirus E1A-associated 300kDa protein</td>
</tr>
<tr>
<td>SRC-1</td>
<td>steroid receptor coactivator-1</td>
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<td>TIP60</td>
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ABSTRACT

Chronic inflammatory diseases often have residual CD8⁺ T cell infiltration despite treatment with systemic corticosteroids suggesting divergent steroid responses between CD4⁺ vs. CD8⁺ cells.

To examine steroid sensitivity, dexamethasone (DEX)-induced histone H4 lysine (K)5 acetylation and glucocorticoid receptor (GCR)α translocation were evaluated. DEX treatment for 6h significantly induced H4 K5 acetylation in normal CD4⁺ cells (p=0.001), but not in CD8⁺ cells. DEX responses were functionally impaired in CD8⁺ as compared to CD4⁺ cells, using MKP-1 (1h) (p=0.02) and IL-10 mRNA (24h) (p=0.004) induction as a read-out of steroid-induced transactivation. Normal DEX-induced GCRα nuclear translocation and no significant difference in GCRα and GCRβ mRNA expression were observed in both T cell types. Also, no significant difference in SRC-1, p300 and TIP60 expression was found. However, ATF2 expression was significantly lower in CD8⁺ compared to CD4⁺ cells (p=0.009). Importantly, inhibition of ATF2 expression by siRNA in CD4⁺ cells resulted in inhibition of DEX-induced transactivation in CD4⁺ cells.

The data indicates refractory steroid-induced transactivation but similar steroid-induced transrepression of CD8⁺ cells as compared to CD4⁺ cells due to decreased levels of the histone acetyltransferase ATF2.
INTRODUCTION

Currently glucocorticoids (GC)s are the most effective anti-inflammatory therapy used for treatment of chronic inflammatory and immune diseases.\(^1,2\) However the sensitivity to GCs varies considerably among immune cells.\(^1,3,4\) For instance, clinical data demonstrates residual CD\(^8^+\) T cell infiltration despite treatment with systemic GCs with more severe disease outcomes. These cells could therefore be one of the key mediators for resistance to steroid therapy. It was found that in patients with multiple myeloma a decrease in the CD\(^4^+\)/CD\(^8^+\) ratio due to an increased number of HLA-DR expressing\(^5\) and cancer germline specific CD\(^8^+\) cells\(^6\) is usually a good indicator of poor steroid response. In GC resistant cases of systemic lupus erythematosus, CD\(^8^+\) T cells have been shown to be refractory to steroid-mediated apoptosis\(^7\) and this is monitored as an indicator for the therapeutic efficacy of steroids. Relapses of multiple sclerosis are treated commonly with high-dose intravenous methylprednisolone.\(^8\) Several independent studies have reported that steroid treatment can significantly decrease the numbers of CD\(^4^+\) T cells in these patients;\(^8\) however, the same studies observed no change or even an increase in the number of CD\(^8^+\) T cells after treatment in poorly controlled patients.\(^9\) In asthma patients a decline in lung function as an asthma outcome has been shown to correlate with the number of lung infiltrating CD\(^8^+\) cells.\(^10\) In patients with chronic obstructive pulmonary disease (COPD) it had been shown that CD\(^8^+\), CD68\(^+\) cells and neutrophils are refractory to treatment with inhaled steroids, highlighting a need for understanding differential cell response to GCs.\(^11,12\)

GCs exert their biological effect through a specific receptor, glucocorticoid receptor \(\alpha\) (GCR\(\alpha\)), which is expressed in virtually all cells. GCR\(\alpha\) is a DNA-binding protein, located in the cell cytoplasm. Its nuclear translocation is induced upon ligand binding. GCs suppress production of multiple inflammatory proteins via transrepression and transactivation.\(^13\) GCR\(\alpha\) directly interacts with pro-inflammatory transcription factors preventing transcription of inflammatory genes (transrepression).\(^1,13,14\) Activated GCR\(\alpha\) also directly binds to its recognition sites in the promoters of certain genes to activate their transcription (transactivation), resulting in production of anti-inflammatory proteins, such as mitogen-activated protein kinase phosphatase.
(MKP-1), which inhibits MAP kinase signaling pathways. Recently new insights have been gained into the molecular mechanisms how GCs suppress inflammation through transactivation and transrepression, as well the importance of histone modification in steroid responsiveness.

Several molecular mechanisms had been reported to be involved in the GC resistance, including increased expression of GCRβ. GCRβ is the homologous isoform of GCRα in human cells, which is generated from alternative splicing of the human GCR gene. GCRβ differs from GCRα in its carboxyl terminus, where the last 50 amino acids of GCRα are replaced by a non-homologous, 15 amino acid sequence. As a result of this difference, GCRβ may compete with GCRα for binding to GRE sites or compete for the transcriptional coactivator molecules, inhibiting steroid responses. The expression level of GCRβ and GCRα in different cell types is the factor that determines variations in cellular responses to steroids. To gain information about responses of different cell types to steroids, we analyzed whether the responses of primary human CD8+ cells are refractory to steroids as compared to CD4+ cells and the potential molecular mechanism(s) for this.
MATERIALS AND METHODS

Reagents

Anti-human CD3 antibody (Orthoclone OKT®3 sterile solution, Ortho Biotech Products, L.P., Raritan, NJ) and dexamethasone (DEX) (Sigma Chemicals Co., St. Louis, MO) were used for cell stimulation. Allophycocyanin (APC) labeled CD4 (clone RPA-T4), CD8 (clone RPA-T8) antibodies were purchased from BD Pharmingen (San Diego, CA). Human CD4+ T cell isolation kit II, human CD8+ T cell isolation kit II (Miltenyi Biotec, Auburn, CA) were used for the isolation of CD4+ and CD8+ T cell populations, respectively. Primers and probes for mitogen induced MAPK phosphatase-1 (MKP-1), IL-10, ATF2, GADPH were purchased from Applied Biosystems (Foster City, CA). GCRα and GCRβ primers were custom ordered from Applied Biosystems based on the sequences published by DeRijk et al.29

Subjects

This study was approved by the Institutional Review Board at National Jewish Medical and Research Center, Denver, CO. Eight normal healthy adult donors with no history of atopic and respiratory diseases were enrolled in the current study. Informed consent was signed during the time of blood draw.

Isolation of human PBMC and magnetic cell sorting

PBMC were isolated by Ficoll-Hypaque® density gradient centrifugation from heparinized venous blood of healthy donors as previously described.30 Cells were subsequently seeded to the slides to stain for acetylated histone H4 K5 or sorted into CD4+ and CD8+ T cells by negative depletion using magnetic cell sorting based on the manufacture’s instructions (Miltenyi Biotec). The purity of the enriched cells was evaluated by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) and was always over 90% positive.
Real-time PCR assay for GCR, MKP-1 and IL-10 mRNA

Total RNA from purified T cells was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA), reverse-transcribed into cDNA using reverse transcription regents (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions as described.31 To measure steroid-induced transactivation, DEX-induced MKP-1 and IL-10 mRNA were evaluated in CD4\(^+\) and CD8\(^+\) cells. For DEX-induced MKP-1 experiments, the cells were incubated with or without 10\(^{-7}\) M DEX for 1h before RNA extraction; for DEX-induced IL-10 experiments, the cells were incubated with or without 10\(^{-7}\) M DEX for 24h before RNA extraction. The quantitative real-time PCR was performed as previously described.31 Briefly, the reactions were carried out using the dual-labeled fluorogenic probe method. ABI prism 7000 sequence detector (Applied Biosystems) was used to run real-time PCR and collect fluorescence data. Relative gene expression levels were calculated and normalized to the corresponding levels of the housekeeping gene (GADPH). Standard curves for all targets were generated using the fluorescent data from two-fold serial dilutions of 1000ng total RNA of the target sample. Standard curves for GCR\(\alpha\) and GCR\(\beta\) were generated from ten-fold serial dilutions of the GCR\(\alpha\) and GCR\(\beta\) plasmids as described by us earlier.26

GGR\(\alpha\) nuclear translocation

GCR\(\alpha\) intracellular translocation in response to 10\(^{-7}\) M DEX (Sigma Chemical Co., St. Louis, MO) treatment was analyzed by immunofluorescent staining. In these experiments, freshly isolated PBMC were seeded at 1\(\times\)10\(^6\) cells/ml on poly-D-lysine coated cover slips. Cells were then treated with 10\(^{-7}\) M DEX or cultured in media alone for 1h, fixed in 4% paraformaldehyde in PBS and stained with anti-CD4 APC or anti-CD8 APC antibodies for 30 min on ice. Following incubation the cells were washed with PBS, permeabilized for 15 min RT in permeabilization solution [PBS containing 0.1% (v/v) Tween 20, 0.1% (w/v) bovine serum albumin (Sigma) and 0.01% (w/v) saponin (Sigma)] and blocked with a commercial blocking solution (Superblock, Scytek, Logan, UT) for 15 min at room temperature. The cells were then
incubated with an affinity purified polyclonal antibody to GCRα (Affinity Bioreagents, Golden, CO) diluted in permeabilization solution (1:250) overnight at 4°C, washed, then incubated with donkey anti-rabbit IgG, F(ab’)2-cy3 conjugated secondary antibody (Jackson Laboratories, West Grove, PA) (1:200) and the nucleus counterstained with 300nm 4’,6’-diamidino-2-phenylindole (DAPI) (Sigma) for 1h at room temperature, washed and mounted on slides. Purified nonimmune rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as an isotype control. The slides were analyzed by fluorescent microscopy (Leica, Germany) with an imaging analysis software Slidebook™ (Intelligent Imaging Innovations, Denver, CO), the data was presented as a ratio of mean fluorescence intensity (MFI) for nuclear and cytoplasmic cy3 (GCRα) staining as described by us earlier in CD4+ and CD8+ cells. Fifty of CD4+ and CD8+ cells were analyzed per each slide.

The TransAM™ GR transcription assay kit (Active Motive, Carlsbad, CA) was used to quantify GCRα cellular translocation of the bulk cell population. Freshly isolated CD4+ and CD8+ cells were cultured with 10^{-7} M DEX or media alone for 1h. Then nuclear and cytoplasmic cellular extracts were prepared from cells with a NE-PER® Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL). 5 µg of nuclear extracts from untreated and DEX treated cells were added to the plate coated with GRE consensus sequence (5’-GGTACAnnnTGTTCT-3’), incubated for 1h at room temperature with mild agitation and washed three times. GCR present in nuclear extracts bound specifically to the oligonucleotide and was detected with an antibody directed against GCR, recognized by secondary anti-rabbit HRP conjugated antibody following addition of the developing solution. Absorbance was read on a spectrophotometer at 450 nm with a reference wavelength of 655 nm. Nuclear extract from DEX treated HeLa cells was used as a positive control. Competitor GRE consensus sequence oligonucleotide (40 pmol/well) was added to the wells prior to the addition of nuclear extracts to prevent GCR binding to the probe immobilized to the plate to validate the specificity of the GCR-GRE interaction. GCR activation was expressed as OD450 nm in all nuclear extracts.
Suppression of anti-CD3 induced CD4+ and CD8+ T cells proliferation and cytokine production by DEX

Purified T cells (1×10^5/well) were stimulated with 0.5 μg/ml of soluble anti-CD3 in the absence or presence of 10^-9 and 10^-7 M DEX. Irradiated PBMC (1×10^4/well) were used as antigen-presenting cells for the assay. The cultures were incubated for 72h at 37°C in humidified, 5% CO₂ incubator. The cell proliferation was assessed based on [³H]-thymidine incorporation as previously described.³³

To examine DEX-induced suppression of the cytokine production, cells were treated as above in the 96-well plates for 24h. The supernatants were collected, frozen and stored at -80°C until use. Commercial ELISA kits were used to measure TNFα, IFN-γ and IL-13 in cell culture supernatants (R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer’s instructions. Absorbance readings were transformed to cytokine concentrations using standard curves. IC₅₀, defined as the concentration of DEX that inhibits the proliferation or cytokine production by anti-CD3-stimulated lymphocytes to 50% of the level seen in the absence of DEX, was used as the parameter to measure the steroid sensitivity of the cells.

Assessment of histone H4 lysine (K)5 acetylation by immunostaining

Human PBMC (1×10^6 cells/ml) were cultured in 4-well poly-D-lysine coated Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) in the presence or absence of 10^-7 M DEX for 6h. Cells were washed with HBSS and fixed in 4% paraformaldehyde in PBS for 5 min followed by blocking in a commercial blocking solution (Superblock; Scytek, Logan, UT) for 15 min at room temperature. Cells were first incubated with either anti-CD4 APC or anti-CD8 APC antibodies for 1h at 4°C, and permeabilized for another 15 min in permeabilization solution [PBS containing 0.1% (v/v) Tween 20, 0.1% (w/v) BSA (Sigma), and 0.01% (w/v) saponin (Sigma)], then incubated with anti-acetylated H4 K5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in permeabilization solution (1:500) overnight at 4°C. Purified non immune rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) was used as an isotype control. After
washing, the cells were incubated with a donkey anti-rabbit IgG, F(ab')2-Cy3 conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200), and the nucleus was counterstained with 300 nm 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma) for 45 min at room temperature and washed in PBS/0.1% Tween 20 for 15 min. Stained cells were observed by fluorescence microscopy (Leica) using a ×63 objective. 100 cells were analyzed per each slide after coding the slides to blind the slide reader. The MFI of cy3 staining (acetylated K5) in CD4+ and CD8+ cells was assessed by the analysis software within the computer-generated masks for nuclear (DAPI staining) regions of the cells. The data was presented as cy3 (acetylated K5) MFI before and after DEX treatment in CD4+ and CD8+ T cells.

**Western blotting**

Purified CD4+ and CD8+ T cells were treated with DEX (10⁻⁹M, 10⁻⁷M) before lysis in ice-cold RIPA buffer containing protease inhibitor cocktail (Sigma). 30µg of total protein were separated by SDS-PAGE and transferred to the nitrocellulose membrane. MKP-1 was assayed using anti-MKP-1 antibody (Santa Cruz Biotechnology).

Nuclear extracts were prepared from freshly isolated CD4+ and CD8+ T cells with a NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). 10µg of nuclear protein per condition were run on 4-15% gradient gel (Bio-Rad Laboratories, Hercules, CA) and transferred to the nitrocellulose membrane. The membranes were blotted with anti-SRC-1, ATF2 and TIP60 (Santa Cruz Biotechnology) antibodies. For p300 detection, 60µg of whole cell extracts were run on 7.5% gel (Bio-Rad Laboratories), transferred to the membrane for 4h at 20V in the cold room. The membrane was then incubated with 2 µg/ml anti-p300 antibody (Upstate, Lake Placid, NY). To control the quality of nuclear proteins preparation the membranes were stripped and re-probed with anti-C23 antibody (Santa Cruz Biotechnology) used as nuclear control protein. Developed X-ray films were scanned and densitometry of the bands was quantified using NIH Image software (v. 1.63) (this software is available on the Internet at http://rsb.info.nih.gov/nih-image).
Silencing of ATF2 expression by specific siRNA

Small interfering RNAs (siRNAs) were used to inhibit ATF2 gene expression. ATF2 siRNA, non-specific siRNA were purchased from Santa Cruz Biotechnology. Commercial Nucleofector human T cells kit (Amaxa, Gaithenberg, MD) and special transfection program for human T cells on the Nucleofector Device (Amaxa) were used. In brief, \(5 \times 10^6\) purified \(\text{CD}4^+\) T cells were suspended in 100 \(\mu\)l of transfection solution and transfected with 1 \(\mu\)g of ATF2 siRNA, or nonsilencing control siRNA using the V014 program (approach previously described by Goleva et al.\(^{19}\)). Transfected cells were immediately diluted with pre-warmed T cells growth media (Amaxa) and cultured in 24-well plates or on poly-D-lysine coated coverslips. To confirm inhibition of ATF2 mRNA and protein expression by ATF2 siRNA, transfected cells were serially assayed by real-time PCR and microscopy 24h to 72h after transfection for ATF2 expression with methods described above. To determine the effect of siRNA delivery on cell viability, the trypan blue dye exclusion test was used. The cell viability was usually 70-80% at 48h and 50-60% at 72h after siRNA transfection.

Statistical analysis

Data were expressed as mean±SEM. The paired \(t\)-test was used to compare functional responses of \(\text{CD}4^+\) and \(\text{CD}8^+\) cells obtained from the same donors (hence, paired). The test was also used to compare pre and post DEX responses within \(\text{CD}4^+\) and \(\text{CD}8^+\) cells types. Before testing, paired difference distributions were examined for outliers, which can indicate violation to the normality assumption of the parametric \(t\)-test. No outliers were apparent. A \(p\)-value of less than 0.05 was considered statistically significant. All reported \(p\)-values were based on two-sided tests.
RESULTS

Differential gene activation by GCs in human CD4+ vs. CD8+ T cells

To characterize GC-mediated transactivation in human CD4+, as compared to CD8+ T cells, we investigated the effect of DEX on MKP-1 gene induction by real-time PCR. MKP-1 is an important GC-induced anti-inflammatory gene, which reflects early GCR transactivation response.26,35 There was no significant difference in MKP-1 mRNA levels in resting CD4+ and CD8+: 3.05±0.99 ng MKP-1/ng GADPH in CD4+ vs. 4.48±1.12 ng MKP-1/ng GADPH in CD8+ (n=8). In human T cells, DEX rapidly induced MKP-1 mRNA gene expression. However, we found significantly different levels of MKP-1 induction by DEX in CD4+ and CD8+ T cells (Fig. 1A). CD4+ T cells showed significantly greater induction of MKP-1 expression after short term DEX exposure than CD8+ T cells (3.13±0.42 fold in CD4+ T cell vs. 1.83±0.14 in CD8+ T cells, n=8, p=0.02) (Fig. 1A).

To provide more definitive evidence regarding the divergent GCR transactivation activity in CD4+ vs. CD8+ T cells, we measured the effects of DEX on another GC-induced anti-inflammatory gene, IL-10, known to be induced by GCR transactivation. The basal levels of IL-10 mRNA in CD4+ and CD8+ were not significantly different: 0.43±0.11 vs. 0.71±0.29 ng IL-10/ng GADPH (n=8). It was found that DEX treatment for 24h significantly increased IL-10 gene expression in CD4+ T cells, whereas nearly no effect on IL-10 gene expression in CD8+ T cells was seen (IL-10 fold induction by DEX was 1.95±0.35 in CD4+ T cells, and 0.95±0.28 in CD8+ T cells, n=8, p = 0.004) (Fig. 1B).

Along with mRNA, significantly lower MKP-1 protein induction by DEX was found in CD8+ cells as compared to CD4+ cells. The cells were treated with 10−7M and 10−9M DEX for 9h or cultured in media alone. The DEX-mediated increase in MKP-1 protein level was dose dependent both in CD4+ and CD8+ T cells; however, much stronger induction of MKP-1 was observed in CD4+ than in CD8+ T cells was noted (Fig. 1C).

DEX induces similar GCRα nuclear translocation in CD4+ and CD8+ T cells
To determine whether the differences in GCR transactivation properties of CD4+ vs. CD8+ T cells were caused by the difference in GCRα nuclear translocation in response to steroids, we measured the GCRα cellular translocation in response to DEX in normal CD4+ vs. CD8+ T cells. GCRα nuclear translocation was assessed in response to 10^-7 M DEX by immunostaining. In the absence of DEX, GCRα was localized uniformly in T cells; DEX induced GCRα nuclear translocation within 1h. Increasing the time of DEX treatment for greater than 1h had no further effect on GCRα nuclear translocation, therefore we used this time point to assay additional subjects. As shown in Fig. 2A-C, DEX induced similar levels of GCRα nuclear translocation in both CD4+ and CD8+ T cells. Cy3 MFI for the nuclear region of CD4+ were 447.1±52.9 and 585.7±62.0 before and after DEX treatment (p=0.008, n=4); and CD8+ were as follows: 476.7±64.0 vs. 615.2±72.0 (p=0.03, n=4).

An alternative technique was applied to evaluate GCR cellular translocation in bulk CD4+ and CD8+ T cells. GCR was measured in nuclear extracts from these cells based on its interaction with GRE consensus motive immobilized to the plate (TransAM GR transcription factor assay). No difference in the amount of nuclear GCR was found in freshly isolated CD4+ and CD8+ T cells. Addition of DEX to the cells resulted in a significant increase in the amount of nuclear GCR both in CD4+ and CD8+ T cells (Fig. 2D). Both cell types showed similar amounts of nuclear GCR after DEX treatment.

Because GC anti-inflammatory response is mediated through GCRα, and alternatively spliced isoform - GCRβ, can interfere with GCRα by forming heterodimers with GCRα and compete for steroid receptor coactivator and GRE, displaying a dominant negative activity, we analyzed the expression of GCRα and GCRβ isoforms in CD4+ vs. CD8+ T cells. A quantitative real-time PCR assay was used to compare GCRα and GCRβ mRNA amounts between purified CD4+ and CD8+ T cells. Primers and probes were designed to be complementary to the sequence within the region of exon 9α for GCRα, or exon 9β for GCRβ (as previously described). Our results demonstrated that there is no significant difference in GCRα mRNA and GCRβ mRNA expression (Table 1) between human CD4+ and CD8+ T cells.
The molar ratios of GCRα mRNA to GCRβ in CD4+ and CD8+ were 2183±506 vs. 1834±424, respectively (p=0.53, n=8). In addition, microscopy results also showed that GCRα and GCRβ protein expression in CD4+ T cells were similar to CD8+ T cells (data not shown).

**Similar gene suppression by DEX in CD4+ vs. CD8+ T cells**

The anti-inflammatory effects of GCs rely both on the direct activation of genes, e.g. the induction of MKP-1 and IL-10 (transactivation) and their indirect effects on inflammatory gene suppression via interaction with other transcription factors (transrepression).\(^1\) The results above showed a significant difference in steroid-induced transactivation responses in CD4+ vs. CD8+ T cells: therefore it was of importance to compare steroid-induced transrepression responses in these two T cell populations.

Purified CD4+ or CD8+ T cells were stimulated by anti-CD3 in the presence or absence of DEX for 72h. Irradiated PBMC were used as antigen-presenting cells for the assay. The effect of DEX on T-cell proliferation induced by anti-CD3 was assessed on the basis of tritiated thymidine incorporation. As shown in Fig. 3, DEX significantly inhibited CD4+ and CD8+ T cell proliferation with DEX IC\(_{50}\) of 5.73±1.34 nM for CD4+ cells and 7.60±0.87 nM for CD8+ cells. At the same time TNFα and IFNγ were measured in cell culture supernatants from 24h stimulated anti-CD3 stimulated cells. Production of TNFα and IFNγ by anti-CD3 stimulated CD4+ and CD8+ cells were comparable. A similar degree of DEX induced suppression of cytokine production was observed in CD4+ and CD8+ T cells (DEX IC\(_{50}\) for TNFα production was 6.33±2.18 nM for CD4+ cells and 6.37±2.45 nM for CD8+ cells; DEX IC\(_{50}\) for IFNγ production was 0.59±0.04 nM for CD4+ cells and 0.51±0.07 nM for CD8+ cells).

We attempted to measure Th2 cytokine IL-13 in 24h cell culture supernatants from CD4+ and CD8+ cells stimulated with anti-CD3 in presence of irradiated APC. However, IL-13 was below the detection limit of the assay. This is not surprising since normal not allergic donors were used in this study.
Differential effect of DEX on histone acetylation in CD4+ vs. CD8+ T cells

Histone acetylation status is one of the key factors in regulation of inflammatory gene transcription.36 Because histone H4 K5 acetylation is a marker of GC-mediated transactivation,17 we examined the ability of GCs to induce H4 acetylation at residue K5. Using immunostaining, we found (Fig. 4) that DEX treatment for 6h significantly induced H4 K5 acetylation in normal CD4+ T cells (MFI 397.0±20.4, 518.0±31.0 before and after DEX, respectively; p=0.001, n=8). There was a marginal change in H4 K5 acetylation detected in CD8+ T cells (MFI 392.1±18.3 vs. 421.8±22.7 p=0.06, n=8).

Expression of histone acetyltransferases that acetylate histone H4 K5 in CD4+ vs. CD8+ T cells

Histone acetyltransferases (HAT) regulate gene transcription by histone acetylation. Acetylation of the ε-group on lysines residue reduces the charge of histones and subsequently releases the tightly wound DNA, allowing the recruitment of additional large protein complexes.36,37 We hypothesized that the difference in DEX-induced acetylation and, hence, transactivation in CD8+ cells vs. CD4+ cells could be a consequence of deficient HAT expression by CD8+ cells.

To test this hypothesis, nuclear protein extracts were prepared from CD4+ and CD8+ cells isolated by negative selection. SRC-1, ATF2, TIP60 and p300 have been reported to have HAT activity and are known to acetylate K5 of histone H4.38,39 Using Western blot analysis, we measured expression of these proteins in CD4+ and CD8+ cells. No difference in SRC-1 and TIP60 expression between these two cell populations was found (Fig. 5A, 5C). We were unable to consistently detect p300 in T cell nuclear extracts from all donors, most likely due to insufficient cell amount used (6×10^6 CD4+ or CD8+ was the maximal cell number we could recover). While using whole cell extracts from CD4+ and CD8+ cells we did not detect a difference in p300 expression between these two cell types (Fig. 5B). The immunostaining and real-time PCR also revealed that there was no difference in p300 expression in CD4+ vs. CD8+
cells (data not shown). The amounts of ATF2 varied within 8 donors studied. Importantly, a significant decrease in ATF2 expression in CD8+ cells was found as compared to CD4+ cells (Fig. 5D, 5E). The densitometry ratio of ATF2 to C23 was 0.53±0.11 in CD8+ cells as compared to 0.75±0.11 in CD4+ cells (n=8, p=0.009).

The effect of ATF2 gene silencing on DEX-induced gene transactivation in human CD4+ T cells

To evaluate whether different levels of ATF2 expression can modulate steroid induced transactivation, we silenced the ATF2 gene expression in primary human CD4+ T cells and examined the effects of DEX on MKP-1 and IL-10 gene induction. Purified CD4+ T cells from normal donors were transfected by electroporation with ATF2 siRNA or nonsilencing control siRNA. Real-time PCR (Fig. 6A) and microscopy results (Fig. 6B) indicated that introduction of ATF2 siRNA 48h after transfection specifically inhibited ATF2 expression in the CD4+ T cells. The estimated silencing efficiency was 60.41± 7.22% (n=4). Therefore we selected this time point to compare MKP-1 induction followed by incubation with DEX for 1h, and IL-10 induction for 24h. Fig. 6C shows that silencing of ATF2 resulted in a significant decrease of DEX induced MKP-1 production (MKP-1 mRNA fold induction was 3.42±0.42, n=4, p=0.006 compared to the nonsilencing siRNA with values of 5.58±0.70). As well, significantly reduced IL-10 gene induction was observed in ATF2 silenced CD4+ T cells compared to nonsilencing RNA group (1.05±0.06 vs. 2.44±0.38 in nonsilencing RNA group, n=4, p=0.03) (Fig. 6D).

This data supports the concept that ATF2, via acetylation of the histone H4 K5 residue, controls GC-mediated gene transactivation in T lymphocytes and accounts for differences in steroid responsiveness of CD8+ vs. CD4+ cells.
DISCUSSION

The current study assessed the steroid responsiveness of human peripheral blood CD8+ T cells as compared to CD4+ T cells. Functional assays were performed to evaluate steroid-induced transactivation and transrepression since the anti-inflammatory and immunosuppressive effects of GCs rely on these two molecular mechanisms, respectively. Steroid-induced transactivation assays, including IL-10 and MKP-1 gene induction, demonstrated significantly reduced GCR-mediated transactivation in CD8+ T cells as compared to CD4+ cells. To assess DEX-induced transrepression, purified CD4+ or CD8+ T cells were stimulated in the presence and absence of DEX. Our results showed inhibitory effect of DEX on cytokine secretion and cell proliferation in both T cell populations suggesting that DEX has similar transrepression activity in these two cell types. No difference in the Emax of DEX was seen on proliferation or cytokine release between CD4+ and CD8+ cells. In contrast, when we measured the transactivation effects of DEX on resting T cells, significantly different responses to GCs between CD4+ and CD8+ were found. The difference in DEX induced transactivation between CD4+ and CD8+ cells was also detected with 10^{-6}M DEX (the highest physiological dose) (data not shown), suggesting that both DEX effect on the sensitivity of cells (EC50) and maximal response seen (Emax) were significantly altered in CD8+ cells as compared to CD4+ cells. These data suggested that CD8+ T cells are refractory to steroids because of a failure of steroids to switch on anti-inflammatory gene expression, rather than a failure to switch off inflammatory genes. Our data supports the idea that steroid responses can be regulated not only by GCR transrepression, but also by GCR transactivation.

Since reduced GCRα cellular translocation in response to steroids and divergent expression of GCRα vs. dominant negative isoform, GCRβ, by cells can modify cellular response to steroids, we evaluated these parameters of steroid response in our system. There was no difference in DEX-induced GCRα nuclear translocation of CD4+ vs. CD8+ T cells. As well, similar levels of GCRα and GCRβ mRNA were found in both cell types.
Histone acetylation has been found to differentially regulate gene transcription, and acetylation of histone H4 itself serves as an important mechanism for signaling-mediated chromatin remodeling. It has been demonstrated that activated GCR forms complexes with CREB-binding protein and its homologues p300, which have intrinsic histone acetyltransferase activity, and induces histone acetylation of lysine 5 and 16. The effect of GCs on K5 has been established to be a target for steroid-induced transactivation.\textsuperscript{17,38} In our studies, we found reduced DEX-induced transactivation in CD8\textsuperscript{+} T cells compared to CD4\textsuperscript{+} cells, and this attenuated effect was related to H4 histone acetylation on K5 in response to DEX. This study was designed to test the hypothesis that differential expression of HATs could be associated with decreased GC responses in CD8\textsuperscript{+} as compared to CD4\textsuperscript{+} T cells. Four enzymes – SRC-1, p300, ATF2, TIP60 – have been reported to regulate H4 K5 acetylation.\textsuperscript{38,39} Using Western blotting difference in SRC-1, p300 and TIP60 expression between these two T cell populations was detected. But significantly lower level of ATF2 protein in CD8\textsuperscript{+} than CD4\textsuperscript{+} T cells was found.

ATF2 - activating transcription factor 2 (CREB2), is a member of the leucine zipper family of DNA binding proteins. This protein binds to the cAMP-responsive element (CRE), an octameric palindrome. The protein forms a homodimer or heterodimer with c-Jun and stimulates CRE-dependent transcription. This protein is also a HAT that specifically acetylates histones H2B and H4 \textit{in vitro}; thus it may represent a class of sequence-specific factors that activate transcription by direct effects on chromatin components.\textsuperscript{40} To demonstrate that ATF2 expression has an important regulatory role in cellular steroid responses, we used siRNA transfection technology to silence ATF2 mRNA and examined the consequence of ATF2 elimination on GCR-mediated gene transcription. Indeed, we found that specific ATF2 siRNA, but not control siRNA, could significantly inhibit ATF2 protein and mRNA expression in human CD4\textsuperscript{+} T cells after transfection. In ATF2 silenced, but not control siRNA silenced CD4\textsuperscript{+} T cells, MKP-1 and IL-10 mRNA induction by steroids was significantly decreased. These data, for the first time, provides direct evidence supporting that ATF2 expression level is directly related to cellular steroid responses.
Our present studies demonstrate that peripheral blood CD8+ T cells are less sensitive to DEX as compared to CD4+ T cells accounting for the clinical observation that CD8+ T cells are often remaining in persistently inflamed tissues of patients despite systemic steroid therapy.5-11 Moreover, the current study investigated the molecular mechanism of relative steroid insensitivity of CD8+ vs. CD4+ T cells. We demonstrate for the first time that CD8+ T cells have a lower level of HAT ATF2 expression than CD4+ T cells. The decreased ATF2 in CD8+ appears to be able to functionally reduce the response of this cell type to steroids because RNA silencing of ATF2 inhibited the steroid responsive CD4+ T cells as well. These data suggest that molecular manipulation of ATF2 expression and activity may offer a novel approach to modify cellular steroid responsiveness.
ACKNOWLEDGMENTS

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The authors have no conflict of interest.
REFERENCES


Table 1: GCRα and GCRβ mRNA expression between human CD4+ and CD8+ T cells

<table>
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<tr>
<th>Subjects</th>
<th>GCRα mRNA expression</th>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>1</td>
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Mean±SEM  27.33±0.21  27.39±0.12  0.014±0.002  0.013±0.001  36.83±0.34  36.77±0.21  0.016±0.004  0.014±0.002

*According to the standard curves measured from GCRα and GCRβ plasmids, respectively.
FIGURE LEGENDS:

Figure 1. Divergent effects of DEX on anti-inflammatory genes induction in human CD4+ and CD8+ cells. DEX-induced activation of MKP-1 (A) and IL-10 (B) gene expression by purified human CD4+ and CD8+ cells. MKP-1 (A) and IL-10 (B) mRNA induction by DEX as compared to media treated cells were analyzed by real-time PCR. Purified human CD4+ and CD8+ T cells were collected at 1h after DEX treatment to analyze MKP-1 induction and 24h after DEX treatment to analyze IL-10 induction. (C) Differential induction of MKP-1 protein expression by DEX in CD4+ vs. CD8+ cells. The cells had been treated by DEX or cultured in medium only for 9h. A representative Western blot out of three independent experiments performed is shown.

Figure 2. GCRα cellular translocation in human CD4+ and CD8+ lymphocytes in response to 10^-7 M DEX treatment in vitro. Freshly isolated PBMC were stimulated for 1h with 10^-7 M DEX or remained untreated. Representative images of GCRα cellular translocation in CD4+ (A) and CD8+ (B) T cells in response to DEX treatment are shown (original magnification 630×; blue, DAPI – nuclear staining; red, cy3 – GCRα; green, APC – CD4+ (A) or CD8+ (B) T cells surface staining) (the cells from four different donors were evaluated by this assay). Note cytoplasmic localization of the GCRα before DEX treatment and increase in nuclear localization of the GCRα after DEX treatment both in CD4+ and CD8+ T cells. (C) GCRα (Cy3) MFI for the nuclear region of CD4+ and CD8+ before and after DEX treatment. (D) Addition of DEX resulted in significant increase in the amount of nuclear GCR both in purified CD4+ and CD8+ cells. GCR was measured in nuclear extracts from CD4+ vs. CD8+ T cells ±DEX (1h) based on its interaction with GRE consensus motive immobilized to the plate (TransAM™ GR transcription factor assay) (Data were expressed as mean±SEM, n=6).
Figure 3. DEX IC₅₀ required for inhibition of anti-CD3 stimulated proliferation and cytokine secretion by purified human CD4⁺ and CD8⁺ T cells from normal donors. No significant difference was observed between CD4⁺ vs. CD8⁺ T cells. Data were expressed as mean±SEM, n=3).

Figure 4. DEX-induced histone H4 lysine (K)⁵ acetylation in human CD4⁺ and CD8⁺ T cells. Representative images of acetylated H4 K5 in CD4⁺ and CD8⁺ cells in response to 6h of 10⁻⁷ M DEX treatment in vitro are shown (original magnification 630×; blue, DAPI–nuclear staining; red, cy3–acetylated histone H4 K5; green, APC–surface staining with anti-CD4 (A) or anti-CD8 APC antibodies (C)). The mean fluorescence intensity (MFI) of cy3 staining (acetylated histone H4 K5) in CD4⁺ (B) and CD8⁺ (D) cells was assessed by analysis software within the computer-generated masks for the cell nuclei. 50-100 cells were analyzed for each donor studied (n=8).

Figure 5. The expression of histone acetyltransferases SRC-1, p300, TIP60 and ATF2 in normal CD4⁺ and CD8⁺ lymphocytes. Nuclear proteins or whole cell proteins were extracted from purified CD4⁺ and CD8⁺ T cells. The expression of SRC-1 (A), p300 (B), TIP60 (C) and ATF2 (D) was evaluated by Western blot. C23 was used as a loading control for nuclear proteins. Nuclear extracts from Jurkat T cells and HeLa cells were used as a positive control for anti-p300 antibody. Vertical lines have been inserted to indicate where a gel lane was cut. These gels came from two different experiments. (E) The ratio of AFT2 to C23 was calculated as a ratio of density for AFT2 and C23 bands measured with a NIH Image v.1.63 software.

Figure 6. Inhibition of ATF2 gene expression using specific siRNA in human CD4⁺ decreases the transactivation activity of steroids. Introduction of ATF2 siRNA into CD4⁺ T cells resulted in specific inhibition of ATF2 expression as shown by real-time PCR (A) and immunostaining (B) (original magnification 630×; blue, DAPI – nuclear staining; red, cy3–
ATF2). The pictures are representatives of four independent experiments. Silencing of ATF2 resulted in significant decrease of DEX-induced MKP-1 (C) and IL-10 (D) production by human CD4\(^+\) T cells (n=4) as compared to non-specific siRNA control group.
Figure 1

A. MKP-1 fold induction by DEX

B. IL-10 fold induction by DEX

C. Table with data for MKP-1 and C23 levels after treatment with DEX in CD4⁺, Hela, and CD8⁺ cells.
Figure 2
Figure 3
Figure 4

A. Acetylated K5 histone H4 (Cy3) CD4 (APC)

B. MFI of H4 K5

C. Acetylated K5 histone H4 (Cy3) CD8 (APC)

D. MFI of H4 K5
Figure 6

A. 

ng ATF2/mg GADPH

0 250 500 750 1000

ATF2 siRNA Control siRNA

P=0.01

B. 

ATF2 siRNA 48 h Control siRNA 48 h

C. 

MKP-1 fold induction

2 4 6 8

ATF2 siRNA Control siRNA

P=0.006

D. 

IL-10 fold induction

0 1 2 3 4

ATF2 siRNA Control siRNA

P=0.03
ATF2 impairs glucocorticoid receptor-mediated transactivation in human CD8+ T cells

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