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Expression of the Glucocorticoid Receptor from the 1A Promoter Correlates with T Lymphocyte Sensitivity to Glucocorticoid-Induced Cell Death

Jared F. Purton,*† Julie A. Monk,‡ Douglas R. Liddicoat,*† Konstantinos Kyparissoudis, Samy Sakkal,‡ Samantha J. Richardson,† Dale I. Godfrey,2* and Timothy J. Cole2,3†

Glucocorticoid (GC) hormones cause pronounced T cell apoptosis, particularly in immature thymic T cells. This is possibly due to tissue-specific regulation of the glucocorticoid receptor (GR) gene. In mice the GR gene is transcribed from five separate promoters designated: 1A, 1B, 1C, 1D, and 1E. Nearly all cells express GR from promoters 1B–1E, but the activity of the 1A promoter has only been reported in the whole thymus or lymphocyte cell lines. To directly assess the role of GR promoter use in sensitivity to glucocorticoid-induced cell death, we have compared the activity of the 1A promoter with GC sensitivity in different mouse lymphocyte populations. We report that GR 1A promoter activity is restricted to thymocyte and peripheral lymphocyte populations and the cortex of the brain. The relative level of expression of the 1A promoter to the 1B–1E promoters within a lymphocyte population was found to directly correlate with susceptibility to GC-induced cell death, with the extremely GC-sensitive CD4+/CD8− thymocytes having the highest levels of GR 1A promoter activity, and the relatively GC-resistant αβTCR+/CD4low/CD8low thymocytes and peripheral T cells having the lowest levels. DNA sequencing of the mouse GR 1A promoter revealed a putative glucocorticoid-response element. Furthermore, GR 1A promoter use and GR protein levels were increased by GC treatment in thymocytes, but not in splenocytes. These data suggest that tissue-specific differences in GR promoter use determine T cell sensitivity to glucocorticoid-induced cell death.


Glucocorticoids (GC) are widely known for their role in the stress response, where high circulating concentrations of hormone (>100 nM) promote actions on the cardiovascular, renal, nervous, and immune systems to re-establish homeostasis. The profound immunosuppressive and anti-inflammatory effects of GCs have led to this hormone being the most commonly used pharmacological agent for the treatment of leukemia, autoimmune diseases, and inflammatory diseases such as arthritis. High stress-induced levels of GCs cause atrophy of the thymus and other lymphoid tissues, yet all other tissues remain intact. This strong cytolytic effect of GCs on thymocytes has made it one of the classical models of apoptotic death, with only the most mature thymocytes being resistant to GC-induced cell death (GICD).

Despite this, sensitivity to GCs does not appear to be important for the normal development of these cells (4–6). Interestingly, thymocyte sensitivity to GCs does not appear to correlate with steady state glucocorticoid receptor (GR) levels, because highly sensitive CD4+/CD8− thymocytes express lower levels of GR than more mature CD24low/αβTCRhigh thymocytes that are relatively resistant to GCs (7).

A direct relationship between GR protein levels and GC effects has been demonstrated previously (5, 8). It is therefore interesting that most cell types, mainly of nonlymphoid origin, appear to survive GC treatment by decreasing transcription of GR mRNA to reduce GR protein levels (9, 10), and that sequences in the GR promoter seem to regulate this process (11). This down-regulation is tissue specific, because GR mRNA and protein levels were increased in the GC-sensitive human CEM-7 and mouse S49 T lymphocyte cell lines upon GC treatment (12, 13), and increased GR expression was suggested to be essential for subsequent GICD in T lymphoblasts (14).

The down-regulation of GR protein in GC-resistant cells after GC treatment occurs by multiple mechanisms (10). The primary one appears to be a decreased rate of GR gene transcription, although there is also evidence for decreased GR protein half-life (15, 16). Decreased GR half-life was caused by hormone binding to the GR and was dependent upon receptor phosphorylation (16, 17). Importantly, the reduction in the GR gene transcription rate after GC treatment was also dependent on receptor activation and phosphorylation, suggesting that the GR regulates its own expression (17).

The GR can alter target gene transcription by binding as a homodimer to specific GC response elements (GREs) in target gene promoter regions (18). The GR gene is comprised of nine transcribed exons, the first of which encodes the majority of the 5′ untranslated region of GR mRNA (19). An analysis conducted on...
DNA sequences 5' of exon 2 in mice has revealed at least five distinct promoter regions, designated 1A, 1B, 1C, 1D, and 1E, which each gave rise to a unique untranslated exon 1 (20, 21). Exons 1B–1E are all located immediately upstream of exon 2 within a CpG island and appear to be housekeeping promoters, because they are all expressed in every tissue examined to date (20, 21). Exon 1A, however, is located at least 32 kb upstream from exon 2, and expression from this promoter in mice has previously only been detected in tissues of high GR content, such as whole thymus and the WEHI-7 and S49 T cell lines (20, 21). This promoter organization appears to be conserved, because similar arrangements of housekeeping promoters and an upstream thymus-specific promoter for the GR gene have also been reported in humans and rats (22–25).

Very little is known about how promoter usage contributes to the transcriptional activity of the GR gene and the mechanisms determining GC sensitivity in particular cell types. This study is the first to correlate the activity of the GR 1A promoter in different lymphocyte populations, including thymocytes, with their sensitivity to GCID. GC treatment of T cells or stimulation with Abs directed to CD3 and CD28 in vitro was found to enhance GR 1A promoter activity. These results suggest that tissue-specific regulation of the GR gene by differential promoter usage is responsible for the well-documented sensitivity of different lymphocyte populations and T cells to GCID.

Materials and Methods

Mice and reagents

C57BL/6 mice between 4 and 6 wk of age were obtained from Monash University central animal house (Clayton, Australia). Dexamethasone (DEX) was purchased from Sigma-Aldrich (Castle Hill, Australia), dissolved in ethanol, and adsorbed into corn oil by heating, to a final stock concentration of 10 mg/ml. Mice were weighed and injected i.p. with either DEX (1–50 mg/kg body weight) or corn oil alone as a vehicle control. Mice were killed 48 h later, and organs were removed for analysis. The survival index (SI) of a given lymphocyte population was calculated as: SI = (cell recovery from a DEX-treated mouse)/(mean cell recovery of the vehicle-treated mouse) in cold PBS containing 2% FCS, counted, and stained for DNA content.

Cell culture reagents

Thymocytes and splenocytes were prepared from C57BL/6 stock mice, counted, and cultured in RPMI 1640 medium (Invitrogen Life Technologies, Auckland, New Zealand), containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia), 2 mM GlutaMax, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Culture medium for fetal thymi also included 1 mM sodium pyruvate and 15 mM HEPES buffer (Invitrogen Life Technologies). Thymus stromal cells were prepared for RNA preparation by culturing fetal thymuses in the presence of 2-deoxyuridine (0.36 mg/ml) for 5 days. Thymocytes and splenocytes were cultured with 10−6 M DEX (Sigma-Aldrich) diluted from a 10−2 M solution stored in ethanol, or with a 1/10,000 dilution of ethanol alone as a vehicle control.

Flow cytometry

Cell suspensions were prepared from thymus, spleen, and liver of DEX- or vehicle-treated mice in cold PBS containing 2% FCS, counted, and stained with combinations of the following Abs for 20 min at 4°C, before analysis with a two-laser, four-color FACS Calibur (BD Biosciences, San Diego, CA): anti-CD4-allophycocyanin (clone RM4-5), anti-CD8-PerCP (clone 53-6.7), anti-CD24-FITC (clone M1/69), anti-αβTCR-allophycocyanin (clone H57-597), anti-NK1.1-PE (clone PK136), anti-B220-PE (clone RA3-6B2), anti-CD43-FITC (clone B11), anti-CD44-CyChrome (clone IM7), anti-CD69-PE (clone H1.2S3), and anti-CD25-PE (clone PC61) All Abs were purchased from BD Pharmingen (San Diego, CA). The NK cell-specific CD1d tetramer loaded with α-galactosylceramide was provided by S. Sidobre and M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Annexin V-FITC (BD Pharmingen) staining was performed according to the manufacturer’s instructions. Unconjugated rat anti-mouse CD16 (2.4G2 clone) was used in all flow cytometry experiments to block nonspecific FcR-mediated binding. All cell sorting was performed using a FACStar Plus (BD Biosciences), and different cell populations were enriched to >98% purity.

B cell depletions and T cell stimulation assay

B cell depletions were performed as previously described (26). Briefly, lymph node suspensions were prepared, counted, and incubated in 5-ml volumes of PBS at a concentration of 1 × 106 cells/ml on petri dishes coated previously with polyclonal anti-IgG and anti-IgM (Caltag Laboratories, Burlingame, CA) at a concentration of 15 μg/ml at room temperature for 1 h. Purified T cells were gently washed off and counted for stimulation assays. Anti-CD3 and anti-CD28 stimulation was conducted according to a previously described method (27). Briefly, anti-CD3 (clone KT3) and anti-CD28 (clone OX40L) were diluted together to 10 μg/ml each in PBS; 500 μl/well was used to coat six-well plates for overnight incubation at 4°C. Wells were washed, and 1 × 106 purified T cells were added in 3 ml of culture medium for 20 h before being harvested and frozen for isolation of total RNA. A small number of stimulated and unstimulated cells were stained using Abs specific for αβTCR and CD69 and analyzed by flow cytometry.

Complement-mediated lysis

Thymocyte suspensions containing 0.02 μg/ml DNase I (Roche, Castle Hill, Australia) were stained with an IgM Ab that recognized CD24 (clone J11D; grown in-house) for 10 min at 4°C, then incubated with 2 ml of rabbit complement (GTI, Waukesha, WI) for 30 min at 37°C. Viable cells were isolated on a Histopaque 1.083 gradient (Sigma-Aldrich); counted; stained with Abs specific for CD44 (clone M1/69), αβTCR, and the CD1d tetramer; sorted to >98% purity; and used for preparation of total RNA.

Cloning and sequencing of the mouse GR gene exon 1A

The GR gene exon 1A was isolated from a 2.5-kb EcoRI-XhoI fragment of a Phage MG21 as described previously (20). DNA sequences were determined by the dideoxynucleotide chain termination method using deoxyadenosine 5′-dideoxynucleotides (Applied Biosystems) and T7 DNA polymerase as described previously (20). The GenBank accession number for the sequence is AY429467. The mouse GR 1A promoter was compared with that of the human GR 1A sequence using the BESTFIT program from ANGIS (Australian Genomic Information Center, Sydney, Australia).

RNase protection assay

A mouse exon 1A exon 2 cDNA fragment was generated by RT-PCR using total RNA from normal adult mouse thymus and the following primers: forward, 5′-CATCTGCAAGCTTCTCAGCCGAG-3′; and reverse, 5′-CCAGATTCTAGGAAATCCCTCGTCTGCT-3′. The PCR fragment was subcloned into the pBluescript (Stratagene, La Jolla, CA). Total RNA was prepared from lymphocyte populations using TRIzol reagent (Invitrogen Life Technologies, Auckland, New Zealand). Total RNA (0.5–1 μg) was hybridized overnight at 58°C with a uniformly 32P-labeled antisense RNA probe made using a riboprobe kit (Pharmacia, Sydney, Australia), and was analyzed as previously described (28). The GR1A/2 antisense RNA probe was transcribed from a 272-nt CDNA fragment (29) containing 67 nt of the mouse GR exon 1A and 205 nt of exon 2, cloned in pBluescript (Stratagene). Briefly, 32P-RNA and excess unbound probe in the hybridization reaction were digested by RNase T1 and RNase A, leaving intact only those portions of the probe that had hybridized to specific GR mRNA sequences. Samples were separated by electrophoresis on an 8% polyacrylamide sequencing gel (Bio-Rad, Hercules, CA) for 1 h at 1600 V, exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA), and analyzed by a Typhoon 8600 scanner (Molecular Dynamics). Different bands were quantitated using ImageQuant version 5.1 software (Molecular Dynamics) after correction against the background. This quantitation was used to calculate the percentage of GR mRNA made from the 1A promoter for each RNA sample: GR 1A promoter usage (%) = 1A band/(1A band + housekeeping promoter bands) × 100.

Western blotting

Tissue and cell samples were homogenized in RIPA buffer (1× PBS (pH 7.4), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml PMsf, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin), incubated on ice for 30 min, and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was decanted and centrifuged, and the final cleared supernatant was used for Western blot analysis. The protein concentrations of the samples were determined using the Bradford assay (30). Samples (100–150 μg) and molecular weight standards (Amersham Biosciences, Sydney, Australia) were separated by SDS-PAGE.
(4.5% acrylamide stacking gel, pH 6.8, then a 10% acrylamide resolving gel, pH 8.9) for 3 h at 250 V. After electrophoresis, proteins were transferred onto nitrocellulose membranes (ADVANTEC, Pleasanton, CA) for 16 h at 30 V. Membranes were incubated in 5% skim milk powder in PBS containing 0.1% Tween 20 (PBST) for 1 h, then with the primary Ab (GR[R20]:sc1004; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000 in 5% skim milk powder in PBST for 1 h. After rinsing with PBST (three times, 10 min each time), membranes were incubated with the secondary Ab (HRP-linked donkey anti-rabbit Ig; Amersham Biosciences) diluted 1/50,000 in PBST for 1 h. Membranes were then rinsed (three times, 10 min each time, with PBST; twice, 5 min each time, with PBS), and GR was detected using ECL (Amersham Biosciences). To standardize for protein loading, membranes were also probed with an anti-actin-specific Ab (A2066; Sigma-Aldrich, St. Louis, MO). Signal was detected using a DV435 BV CCD camera (Andor, Belfast, Northern Ireland), and bands were quantified using Gel Pro version 4.5 analysis software (Media Cybernetics, Silver Spring, MD).

Statistical analysis
Statistical analysis was performed using PRISM statistical analysis software (GraphPad, San Diego, CA). The Mann-Whitney rank-sum U test was performed unless otherwise indicated. For correlation analysis, both the Spearman rank nonparametric correlation test and the Pearson parametric correlation test were used, with similar results.

Results
Immature thymocytes are extremely GC sensitive, and only the most mature survive treatment with DEX
It has been reported previously that only the most mature thymocytes are GC resistant (2). Enriched populations of mature thymocytes have traditionally been prepared by injecting mice with 100 mg/kg cortisone acetate 48 h before harvest (31). However, only totally GC-resistant cells remain after such a high dose of GC, which allows no determination of the relative GC sensitivity of various thymocyte populations compared with each other. In this study we have tested the sensitivity of thymocyte subpopulations to GC-induced cell death at a range of DEX doses. Thymocytes were shown to be very sensitive to GCs; small doses of DEX caused large decreases in cell recovery (Fig. 1 A). A dose of only 5 mg/kg DEX produced a major (60%) reduction in cell number, and a moderate dose of 20 mg/kg DEX caused a 90% reduction. At this dose, the highly sensitive, CD4^+CD8^+ (double-positive (DP)) thymocytes were nearly totally depleted (Fig. 1, B,i and C,i). The proportions of each of the different CD4- and CD8- (Fig. 1B,i) and the CD24- and αβTCR- (Fig. 1B,ii) defined thymocyte populations were all significantly altered by DEX treatment. The enrichment of the CD4^-CD8^- and CD4^-CD8^+ (single positive (SP)) thymocytes suggested that these cells were relatively GC resistant, although analysis of the cell numbers recovered of these populations showed that all CD4- and CD8-defined populations were significantly reduced by 20 mg/kg DEX (Fig. 1C,i). When SP thymocytes were subdivided based on their expression of αβTCR and

![Figure 1](https://www.jimmunol.org/)

**FIGURE 1.** Thymocyte populations have different levels of sensitivity to DEX treatment. Mice were injected i.p. with DEX (1–50 mg/kg), and organs were harvested 48 h later. Thymocyte suspensions were made; counted; stained with combinations of Abs specific for CD4, CD8, CD24, αβTCR, NK1.1, CD44, CD69, or γδTCR; and analyzed by flow cytometry. A. Thymus cell numbers recovered from DEX-treated mice. B. Representative dotplots show frequencies of CD4^- and CD8^- defined (i) or CD24^- and αβTCR-defined (ii) thymocyte populations of mice treated with or without 20 mg/kg DEX. C. Cell numbers of thymocyte populations recovered after treatment with various doses of DEX, defined by CD4 and CD8 (i) or CD24 and αβTCR (ii), based on the gates shown in B. D. SI of various thymocyte populations after treatment with 20 mg of DEX/kg body weight. Data are pooled from six experiments comprising 11 vehicle-treated (0 mg/kg DEX) mice and seven mice treated with 20 mg/kg DEX. At least three mice were analyzed at every dose.
CD24, only the \( \alpha \beta \)TCR\textsuperscript{high}CD24\textsuperscript{low} or \( \alpha \beta \)TCR\textsuperscript{high}CD24\textsuperscript{int/low} subsets were not significantly reduced by a dose of 20 mg/kg, whereas \( \alpha \beta \)TCR\textsuperscript{high}CD24\textsuperscript{high} were clearly depleted (Fig. 1C.ii). The ratio of cell recovery from various thymocyte populations from 20 mg/kg DEX-treated and control thymuses was used to obtain a quantitative value, termed the SI, indicating their sensitivity to GICD (Fig. 1D). Thus, the high GC sensitivity of DP thymocytes was reflected by a low SI value. Total \( \alpha \beta \)TCR\textsuperscript{high}CD4 or CD8 SP thymocytes had higher SI values, but were still GC sensitive relative to the GC-resistant \( \alpha \beta \)TCR\textsuperscript{int}CD24\textsuperscript{low} and \( \alpha \beta \)TCR\textsuperscript{int}CD24\textsuperscript{int/low} thymocytes. It was noteworthy that thymic NKT cells, defined by the expression of NK1.1 and \( \alpha \beta \)TCR, were highly GC sensitive despite the fact that they expressed an \( \alpha \beta \)TCR\textsuperscript{int}CD24\textsuperscript{int/low} phenotype (Fig. 1D). The relative resistance of \( \alpha \beta \)TCR\textsuperscript{high}CD24\textsuperscript{int/low} thymocytes to GICD was confirmed by a lack of annexin V staining of these cells after 6 h of DEX treatment, in contrast to GC-sensitive DP thymocytes, which showed a clear increase in annexin V binding in response to DEX (Fig. 2).

Splenocytes were relatively GC resistant, because the percent reduction of cell numbers in the spleen was significantly less than that in the thymus for all doses tested (Fig. 3). Treatment with 5 or 10 mg/kg DEX, for example, caused less than half the loss of cell number (25 and 30%, respectively) that was observed in the thymus (60 and 72%). The proportion of major splenocyte subsets, including CD4 T cells, CD8 T cells, and B cells (Fig. 3B.i and data not shown), in DEX-treated spleens, at all doses remained unchanged regardless of their expression of activation or maturation markers, such as CD43, CD44, or CD69. The exceptions were NK cells, NKT cells, CD4\textsuperscript{+}CD25\textsuperscript{+} T cells, and Mac-1\textsuperscript{+}GR-1\textsuperscript{+} cells, which all showed significant increases in proportion after DEX treatment, and CD11c\textsuperscript{+}CD8\textsuperscript{+} dendritic cells, which were significantly decreased (Fig. 3B.ii and data not shown). Despite the variations in proportions of some subsets, all splenocyte populations displayed significant decreases in cell number after DEX treatment (Fig. 3C and data not shown), which was reflected in their reduced SI values (Fig. 3D). Treatment with 20 mg/kg DEX also caused a significant decrease in cell number (Fig. 3D). Therefore, the SI of various splenocyte populations after treatment with DEX was significantly lower than that of the thymus.
reduction in T and B cell numbers in liver, whereas NK and NKT cell numbers did not change in this organ (data not shown).

**DNA sequencing of the mouse GR 1A promoter reveals a putative GRE**

A region contained in a 2.5-kb EcoRI-XhoI DNA fragment of a λ phage (MG21) corresponded to the 5’ end of a novel GR cDNA and was designated exon 1A (20). This exon 1A region of the mouse GR gene was determined by pulse-field gel electrophoresis to be ~30 kb upstream of the previously described proximal promoters (1B–1E) and exon 2 of the mouse GR gene, and the DNA sequence of exon 1A was found to be identical with that reported recently (20, 21). The mouse sequence is displayed in uppercase, and the human sequence is shown in lowercase. The sequence published in this study encompasses the nucleotide sequence encoding mouse and human GR gene exon 1A.

The mouse sequence is displayed in uppercase, and the human sequence is shown in lowercase. The sequence published in this study encompasses the nucleotide sequence encoding mouse and human GR gene exon 1A.

**FIGURE 4.** Analysis of the mouse GR gene exon 1A and upstream promoter DNA sequence demonstrates high homology to the human GR gene exon 1A sequence and reveals a putative GRE. A best-fit analysis of the nucleotide sequence encoding mouse and human GR gene exon 1A. The mouse sequence is displayed in uppercase, and the human sequence is shown in lowercase. The sequence published in this study encompasses the site of transcription initiation to that of a putative GRE half-site. The start site of transcription that was also somewhat conserved in the human sequence is designated position +1 and is shown in bold for both species. Both a box and a label indicate the positions of a putative GRE site (eight of 12 match for the consensus; GGTACANNNTGTTCT) are underlined, and a putative TATA box, in the mouse sequence.

**FIGURE 5.** Expression of the GR gene from the 1A promoter in mice is restricted to T cells and the brain cortex. RNase treatment of a 32P-labeled oligonucleotide probe hybridized to various RNA samples produced a 272-bp protected fragment from GR mRNA that had been transcribed from the GR 1A promoter or a 205-bp fragment that corresponds to GR transcripts that have been transcribed from the remaining promoters (designated 1B–1E). A. RNase protection assay performed on RNA prepared from various whole organs or cell lines. B. RNase protection assay performed on different tissues removed from wild-type and RAG-1−/− mice.

To investigate the relative activity of the upstream GR 1A promoter compared with that of the proximal GR promoters (1B–1E), RNase protection analysis was performed on total RNA isolated from various mouse tissues and cell lines. A 272-bp cDNA fragment composed of exon 1A (last 67 nt) and exon 2 (first 205 nt) GR cDNA sequences was generated by RT-PCR (data not shown) and used to generate a 32P-labeled antisense RNA probe. Consequently GR transcripts initiated from the 1A promoter would protect the full length of the antisense RNA probe, forming a 272-bp protected RNA fragment. However, GR transcripts that have been initiated by the proximal housekeeping promoters (1B, 1C, 1D, or 1E) (20, 21) would only protect the exon 2 section of the probe, producing a 202-bp band after gel analysis. The sum of both protected RNA fragments would be equal to the total GR mRNA, allowing the amount of exon 1A-containing GR mRNA to be calculated as a percentage of the total GR mRNA transcripts. The housekeeping promoters 1B–1E were detected as being the major, if not the only, GR gene promoters used in most tissues and cell lines analyzed (Fig. 5A). Expression from the GR 1A promoter was found to be restricted to the thymus, spleen, the T cell lines WEHI-7 and S49, and brain (Fig. 5A). Further examination revealed that GR 1A promoter use in the brain of wild-type mice was restricted primarily to the cortex, with the cerebellum, for example, displaying no detectable expression (Fig. 5B and data not shown). RNase protection analysis performed with total RNA obtained from tissues of RAG1−/− mice, which lack all but the most immature lymphocyte precursors, confirmed that lymphocytes were not contributing to the GR 1A signal observed in the cortex of the brain (Fig. 5B). Furthermore, in the absence of lymphocyte development, GR1A promoter use was virtually undetectable in the thymus and spleen of RAG1−/− mice (Fig. 5B).
Thymocytes that produce a higher proportion of their GR mRNA from the 1A promoter are more sensitive to GICD

To confirm that thymocytes were the source of the GR gene 1A promoter activity that had been detected in the whole thymus (Fig. 5A), RNase protection assays were performed on isolated thymocytes and thymic stromal cells (Fig. 6A). GR 1A promoter activity was observed in adult and embryonic fetal mouse thymocytes, but not within thymic stromal cells. To examine the levels of expression of GR mRNA from the 1A promoter among different thymocyte populations, RNase protection analysis was performed on total RNA prepared from populations of CD3<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>, CD4<sup>−</sup>CD8<sup>−</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> thymocytes isolated by flow cytometric sorting. To obtain sufficient numbers of the relatively rare, GC-resistant, TCR high CD24 int/low thymocytes for RNA preparation, which comprise only 5% of the thymus, CD24<sup>high</sup> cells were removed from thymocyte cell suspensions by complement-mediated lysis before sorting. CD1d tetramer<sup>+</sup> NKT cells were separated from the TCR high CD24 int/low thymocytes by flow cytometric sorting. Sufficient numbers of the highly GC-resistant αβ<sub>TCR</sub><sup>high</sup>CD24<sub>int/low</sub> thymocytes could not be purified for RNA isolation, because these cells comprise <1% of the intact organ. Every subset of sorted thymocytes examined by RNase protection analysis expressed the 1A promoter to some degree, from the immature CD3<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup> triple-negative cells to the most mature αβ<sub>TCR</sub><sup>low</sup>CD24<sub>int/low</sub> thymocytes (Fig. 6B and data not shown). The level of GR mRNA transcribed from the 1A promoter was calculated as a percentage of whole GR mRNA for each sorted thymocyte population (Fig. 6C). The activity of the GR 1A promoter was found to be significantly lower in the αβ<sub>TCR</sub><sup>low</sup>CD24<sub>int/low</sub> thymocytes compared with all other thymocyte populations tested, with the highest levels being found in DP thymocytes and NKT cells. The Spearman rank correlation test found a strong negative correlation (r = −0.943) when the GR 1A promoter activity for each thymocyte population was plotted against its SI value (Fig. 6D). This correlation strongly suggests that the level of GR mRNA expressed from the GR 1A promoter...
before GC treatment determines a thymocyte population’s sensitivity to GICD.

**Splenic lymphocytes display lower levels of GR 1A promoter activity compared with thymocytes**

Given that peripheral lymphocytes were found to be more resistant to GICD relative to thymocytes, RNA was purified from B220<sup>+</sup> or αβTCR<sup>-</sup> lymphocytes sorted from peripheral lymph nodes or whole splenic lymphocytes, and RNase protection assays were performed to investigate their relative use of GR promoters. The GR was transcribed from the 1A promoter at a low level in all purified peripheral lymphocyte populations and the brain cortex (Fig. 7), comparable to that observed on the GC-resistant αβTCR<sup>+</sup> CD24<sup>int/low</sup> thymocytes (Fig. 6C). Additionally, to investigate whether the activation status of a T cell altered its promoter usage, total RNA was prepared from purified T cells that were cultured in the presence or the absence of CD3/CD28 stimulation. The percentage of GR mRNAs transcribed from the 1A promoter was significantly higher in T cells that had been stimulated during culture compared with those that were not stimulated, although this level was not significantly higher than that observed in noncultured, freshly isolated, peripheral lymphocytes (Fig. 7).

**DEX treatment increases GR levels and GR 1A promoter use in thymocytes, but not in splenocytes**

To examine the effects of GC treatment on GR levels, Western blot analysis was performed on extracts of lymphocytes removed from DEX- or vehicle-injected mice. Exposure to DEX for 6 h in vivo caused a 2-fold increase in GR levels in DEX-treated thymocytes (Fig. 8, A and C), which at this early time point displayed only a small, but reproducible, level of apoptosis (data not shown). However, a 2-fold reduction of GR levels was detected in isolated splenocytes (Fig. 8, B and C). To investigate the transcriptional response of the GR gene promoters to GC treatment, total RNA from lymphocytes from the thymus and spleen was analyzed by RNase protection assay for GR 1A promoter activity. Six hours after DEX treatment in vivo, an increase in GR 1A promoter use was seen for thymocytes, whereas no change was observed for splenocytes from the same mice. This result was even more striking, showing almost a 2-fold increase in GR 1A promoter usage, when thymocytes were cultured for 4 h in the presence of DEX, whereas again no change was detected for splenocytes under the same culture conditions (Fig. 8E). These data suggest that the increased sensitivity of thymocytes to GICD may be a consequence of GC-mediated up-regulation of GR via the GR 1A promoter in these cells, but not in peripheral lymphocytes.

**Discussion**

This study is the first to show that a correlation exists between the GC sensitivity of lymphocyte populations and the level of expression of the GR gene 1A promoter before GC treatment. Our data control for protein loading. Signals were quantified using Gel Pro version 4.5 analysis software as described in Materials and Methods. B, Western blot analysis of splenocyte protein samples probed for GR and actin immunoreactivity as described in A. C, The ratio of GR to actin protein levels are depicted as the mean ± SE for three mice per group. D, The percentage of GR 1A promoter usage for cell samples from the same mice used in C is represented as the mean ± SE. E, Thymocyte and splenocyte cell suspensions from untreated mice were cultured for 4 h in the presence of 10<sup>-6</sup> M DEX or vehicle and analyzed by RNase protection for percent GR 1A promoter usage. Error bars depict the mean of four separate samples ± SE.<sup>*</sup>, p ≤ 0.05 (by Student’s t test).
demonstrate that GR expression is increased in DEX-treated thy-
mocytes, but is decreased in DEX-treated splenocytes, and that this
was associated with increased usage of the GR 1A promoter in
thy lymphocytes, but not splenocytes, in response to DEX in vivo and
in vitro. This suggests that the higher transcription from the GR 1A
promoter results in up-regulation of GR protein levels in GC-sen-
sitive cells after hormone treatment. Although this differential reg-
ulation of GR protein levels has previously been observed in cul-
tured cell lines (14, 33), this is the first study to demonstrate this
phenomenon in vivo and to show its likely role in determining the
differential response of thymic vs peripheral lymphocytes to GC.

The expression of the GR gene 1A promoter in the cortex of the
brain may have a similar purpose to that in thymocytes, in making
cells more sensitive to GC levels. This would ensure that cells of
the cerebral cortex can respond to rising stress-induced levels of
GC. Recent studies in rats after chronic corticosterone administra-
tion have shown that the pyramidal neurons of the prefrontal cor-
tex undergo significant dendritic reorganization that probably re-
lects functional changes important for the regulation of stress-
related neuronal responses (34).

The expression of GR 1A-specific mRNA within murine T cells
is also conserved in humans and rats (23, 24). DNase footprint
analysis of the human GR gene 1A promoter revealed a putative
GRE within the 1A exon (24). When this region was cloned with
a luciferase reporter construct into a GR-expressing Jurkat cell
line, GC treatment significantly enhanced luciferase activity, sug-
gestting that the GRE was functional (24). This study also cited
evidence that the GC-resistant IM-9 cell line reduced GR 1A pro-
moter use upon treatment with 10⁻⁶ M DEX, but found that the
same treatment caused GC-sensitive CEM-7 cells to increase their
GR 1A promoter use (24). A key finding of a recent study that
quantitated transcription from the various GR promoters in these
cell lines after GC treatment was that the 1A promoter was the
most highly regulated (35). Thus, the regulation of GR mRNA
from the GR 1A promoter appears to be a major determinant of
susceptibility to GICD. Further evidence that tissue-specific pro-
moters are a conserved mechanism used to regulate steroid recep-
tor gene expression comes from the closely related mineralocorti-
coid and estrogen receptors, which are also expressed from
multiple promoters in tissue-specific patterns (29). The increased
GR 1A promoter use by thymocytes after DEX treatment suggests
that the putative GRE present within exon 1A may be important in
maintaining the expression of the GR gene from the 1A promoter.
The mechanisms by which the GR 1A promoter influences GR
protein levels in GC-resistant and -sensitive cells are currently un-
clear and require further investigation.

A derivative of the mouse lymphoma cell line S49, which had
undergone several rounds of selection for GC sensitivity, was ob-
served to express extremely elevated levels of GR mRNA from the
GR 1A promoter, compared with the GR housekeeping promoters
1B–1E (21). Evidence was cited that expression of GR mRNA from the
1A promoter was also found to correlate with a putative
membrane-bound form of the GR (21). Membrane-specific pro-
gesterone receptors were recently cloned from the sea trout, yet
were found to be genetically distinct from the nuclear progesterone
receptor (36). We are unable to comment directly on the cellular
location of the GR expressed and synthesized from the 1A pro-
moter, other than the fact that thymocytes treated with or without
GC in vivo displayed no expression of the 150-kDa species of the
GR, which is considered to be the membrane-bound GR. However,
the influence of membrane GC-responsive receptors on the regu-
lation of cytoplasmic GR expression cannot be ruled out.

In addition to the most mature thymocytes, the only peripheral
lymphocytes whose numbers were not diminished by GC treat-
ment were liver NKT cells. Although this has been reported pre-
viously (37), we find that in contrast, thymic NKT cells were
highly GC sensitive, establishing a clear functional distinction be-
tween these two NKT cell populations.

Our results suggest that differential promoter use may be a de-
terminant of peripheral lymphocyte GC sensitivity during immune
responses. This may represent a mechanism to ensure that highly
activated T cells remain responsive to GCs produced by the stress
response, which may be required as the immune response subsides.
Of particular interest is the fact that TCR signaling has been re-
ported to prevent apoptosis of T cell hybridomas, thymocyte cell
lines, and splenocytes caused by GR signaling (38–40), yet we
have found that these two antagonistic stimuli both enhance GR 1A
promoter use in T cells. Two possible explanations for this discrep-
ancy are 1) the use of CD28 costimulation in the current study and not
in previous studies may alter the antagonism between TCR and GR
signaling; and 2) the readout of GR 1A promoter use may mask ef-
fects on total GR gene transcription. Intriguingly, TCR signaling has
been reported to enhance GRE-mediated transcription (38), suggest-
ing a possible role for the putative GRE in the GR 1A promoter
activity observed after T cell stimulation.

Thus, we propose that a major determinant leading to GICD is
a high level of GR 1A promoter activity and an ability after GC
treatment to further increase GR protein levels. Ramdas et al. (14)
found that a 2-fold increase in GR levels after GC treatment was
required for GICD of a leukemic T cell line, which is comparable
to the increased GR levels we observed in thymocytes after GC
treatment in vivo.

Several possible mechanisms might explain the variable sensi-
tivity of lymphocyte subsets to GC, including differential:
autoregulation, and a predisposition to GICD. This has important
implications for the potential to selectively inhibit the undesirable
immune-depleting effects of GC in clinical practice.

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References

 influence stress responses? Integrating permissive, suppressive, stimulatory, and

cells in the mouse thymus: cell population changes during cortisone-induced


