The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction

Ana C. Liberman,* Damian Refojo,* Jimena Druker,* Marta Toscano,† Theo Rein,‡ Florian Holsboer,‡ and Eduardo Arzt*,[1]

*Laboratorio de Fisiología y Biología Molecular, Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires and IFIBYNE-CONICET, Buenos Aires, Argentina; ‡Max Planck Institute of Psychiatry, 80804 Munich, Germany; and †Division of Immunogenetics, Hospital de Clínicas Jose de San Martin, Faculty of Medicine, University of Buenos Aires, Avenida Cordoba, City of Buenos Aires, Argentina

ABSTRACT **Glucocorticoids (GCs) immunosuppression acts via regulation of several transcription factors (TF), including activating protein (AP)-1, NF-κB, and NFAT. GCs inhibit Th1 cytokines and promote a shift toward Th2 differentiation. Th1 phenotype depends on TF T-bet. In this study, we examined GC regulation of T-bet. We found that GCs inhibit T-bet transcriptional activity. We show that glucocorticoid receptor (GR) physically interacts with T-bet both in transfected cell lines and in primary splenocyte cultures with endogenous GR and T-bet. This interaction also blocks GR-dependent transcription. We show both *in vitro* and *in vivo* at endogenous binding sites that the mechanism underlying T-bet inhibition further involves reduction of T-bet binding to DNA. Using specific mutations of GR, we demonstrate that the first zinc finger region of GR is required for T-bet inhibition. GCs additionally inhibit T-bet both at mRNA and protein expression levels, revealing another layer of GR action on T-bet. Finally, we examined the functional consequences of GR/T-bet interaction on IFN-gamma, showing that GCs inhibit transcriptional activity of T-bet on its promoter. In view of the crucial role of T-bet in T cell differentiation and inflammation, we propose that GR inhibitory interaction with T-bet may be an important mechanism underlying the immunosuppressive properties of GCs.—Liberman, A. C., Refojo, D., Druker, J., Toscano, M., Rein, T., Holsboer, F., Arzt, E. The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction. *FASEB J.* 21, 1177–1188 (2007)**

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The immunosuppressive and anti-inflammatory action of glucocorticoids (GCs) involves the inhibition of both cytokine gene expression and their pleiotropic action on target cells (1–7). GCs are widely used as clinical tools to suppress inflammatory, autoimmune and allergic diseases (8). These molecules exert their biological effects through binding to the GC receptor (GR), which is a ligand activated transcription factor (TF) regulating either positively or negatively the expression of target genes. The positive gene regulation usually involves the binding of GR homodimers to the GC response elements (GREs) found in the promoter sequences of GC target genes (9). A second and more elusive mode of action is independent of the direct interaction of GR with DNA. It is exerted through the interference or crosstalk with the activity of other transcriptional regulators, such as Rel A (NF-κB), AP-1, or members of the STAT family by mechanisms based on protein–protein interactions, a mechanism also termed transrepression (10–21). As AP-1, NF-κB, and STATs regulate genes involved in inflammation and immune activation, GR antagonism with these TFs is believed to underlie the anti-inflammatory and immune-suppressive actions of GCs (10–22).

The GR DNA-binding domain (DBD) is important for both transactivation and transrepression (23–25). DNA binding and subsequent activation of gene expression require the dimerization of GR and binding to a palindromic GRE (9). Mutagenesis of the GR DBD showed that disruption of the D-loop in the second zinc finger abolishes the ability of GR to dimerize, thus inhibiting GC-mediated activation. However, mutations in this region did not affect transrepression of AP-1. On the other hand, mutations in the first zinc finger of the GR DBD disrupted transrepression of AP-1 but did not inhibit GR transactivation through the GRE (24), suggesting that activation and transrepression are separate phenomena. Interestingly, by introducing different mutations into the GR DBD, it has been demonstrated that transrepression of NF-κB and AP-1 is mediated by

[1] Correspondence: Laboratorio de Fisiología y Biología Molecular, FCEN, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina. E-mail: earzt@fbmc.fcen.uba.ar
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different interaction surfaces within the GR DBD (23–25). S425G is a DBD mutant shown to block transrepression of NF-κB (25) and A458T is a D-loop dimerization interface mutation that blocks GR dimerization and activation of transcription (25). Both mutants are important tools for studying the molecular mechanisms involved in GC anti-inflammatory and immunosuppressive actions.

T-bet is a T helper1 (Th1)-specific transcription factor that controls the expression of the potent inflammatory cytokine IFN-gamma (IFN-γ) (26–28), hallmark of Th1 cell-mediated immunity (29–31). T-bet expression correlates with IFN-γ expression in Th1 and NK cells (27,32). Ectopic expression of T-bet transactivates the IFN-γ gene and induces both endogenous IFN-γ production, up-regulation of IL-12 receptor β2 chain (IL-12Rβ2), and chromatin remodeling of individual IFN-γ alleles (26, 33, 34). Overexpression of T-bet by retroviral gene transduction into primary T cells, developing Th2 cells or terminally committed, fully polarized Th2 cells is able to generate IFN-γ-producing Th1 cells, simultaneously producing the inhibition of the Th2 cytokines IL-4 and IL-5 (26). Mice lacking T-bet fail to develop Th1 cells and display a dramatic reduction of IFN-γ production by $CD4^+$ T cells (27). Moreover, T-bet-deficient mice show normal lymphoid development but profound defects in mounting Th1 immune responses (35) and a corresponding increase in Th2 cytokines. Thus, T-bet is a key TF that controls lineage commitment of Th cells by simultaneously activating Th1 genetic programs and by repressing the development of the opposing Th2 subset (26).

Recent evidence indicates that GCs mediate selective suppression of Th1 cellular immunity (36–41). Since T-bet is a key TF responsible for Th1 differentiation and for IFN-γ regulation, and considering that GCs inhibit IFN-γ production (8, 42, 43), we decided to explore whether T-bet may be a target for the immunosuppressive actions of GCs. In this study, we demonstrate that GCs inhibit the activity of T-bet by a mechanism involving transrepression, which might represent an important mechanism to understand the immunosuppressive properties of GCs.

MATERIALS AND METHODS

Splenocytes and cell line cultures

Spleens were removed aseptically from naive BALBc mice and dispensed through a metal mesh into PBS solution in order to obtain single-cell suspensions, as described previously (44). Splenocytes were resuspended at a density of 2.5×10^6 cells per ml in a RPMI 1640 culture medium supplemented with 10% charcoal-stripped steroid-free FBS (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 10^{-5} M mercaptoethanol and penicillin (100 U/ml), and plated (2 ml/well) in six-well plates. Cultures were incubated at 37°C in a humid atmosphere of 5% CO_2 for 24 or 48 h. Purification of T cells was achieved by a conventional technique involving cell adhesion to plastic as described (44). Briefly, splenocytes were plated in 6-well plates at a density of 4×10^6 cells/ml in RPMI 1640 supplemented with serum and antibiotics. After 120 min, nonadherent cells were aspirated and extensively washed with PBS, and fresh medium was added. This procedure was repeated again after 120 min. Monocyte contamination was less than 1%. Purity of the cell population was assayed by immunofluorescence using specific monoclonal antibodies (Serotech Laboratories Limited, Toronto, Canada): CD2, CD4, CD8, CD14, CD19, and CD45, which define antigens on T cells/NK cells, Th lymphocytes, T cytotoxic/suppressor cells, monocytes/macrophages, B cells, and leukocytes (leukocyte common antigen), respectively. Splenocytes were stimulated at the beginning of the culture with the T cell mitogen concanavalin A (Con A) (Con A, 2.5 μg/ml). The effect of GCs on splenocytes cultures was studied during the last 1, 2, 4, 8, and 24 h of incubation for Western blot assays and 2, 6, 12, 24, or 48 h for Northern blot assays. Mouse IFN-γ was measured according to the manufacturer's instructions by ELISA (Pierce Biotechnology, Rockford, IL, USA).

Differentiated splenocyte cultures were obtained by stimulation of purified T cells with Con A (2.5 μg/ml) during 24 h and then followed by stimulation under Th1-differentiating conditions using IL-12 (5 ng/ml), and anti-IL-4 (10 μg/ml) antibody during 9 days. Also, IL-2 (10 U/ml) was also added for clonal expansion of the differentiated cells. Cells were harvested on days 0, 1, 2, 3, 5, 6, 7, 8, and 9 poststimulation. The optimal T-bet/GR-expressing condition was obtained at day 5. Therefore, 5-day-enriched cell cultures were washed twice with PBS, and fresh medium was added. Then, cells were stimulated using ionomycin and 0.1 μM phorbol 12-myristate 13-acetate (PMA) in the presence of 100 nM dexamethasone (Dex) during 30 min or 1 h of incubation and coimmunoprecipitation of endogenous T-bet and GR was performed.

The murine cell line EL-4, extensively used for studies with Th TF (45–50), was grown in the same medium. EL-4 cells were treated, where indicated, with GC and 1 μM ionomycin, and 0.1 μM PMA (all from Sigma Chemical, St. Louis, MO, USA), to generate T-bet-activating conditions. The transfection experiments were also repeated in human Jurkat T cells, cultured in the same conditions.

The synthetic GC used were Dex and hydrocortisone (Sigma Chemical, St. Louis, MO, USA) 0.1–1000 nM, which cover a physiological-pharmacological range of GC dose. Some experiments were performed in the presence of the specific GR antagonist RU38486 (Sigma Chemical) (1 μM), added 30 min before the addition of GCs, to prove the specificity of GR action.

Plasmids and transfection assays

The plasmid constructs were kindly provided and previously described as follows: the murine IFN-γ promoter (which contains the 5′ flanking region, promoter and 42 bp of 5′-untranslated sequence from IFN-γ) coupled to the chloramphenicol acetyl transferase gene, was provided by Dr. Howard S. Fox (51) and was subcloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA) using *Sma*I and *Hind*III enzymes (IFN-γ-Luc); murine pJG4.5mT-bet, obtained from Dr. Laurie H. Glimcher (26), was subcloned with *Not*I into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA) (pcDNA3-T-bet); the double-stranded oligonucleotides, encoding the T-bet binding site (26), were annealed, blunt-ended and filled in using klenow enzyme (Invitrogen), and subcloned with *Sma*I into the pTATA-GL3-basic luciferase reporter vector (Promega, Madison, WI, USA); the human glucocorticoid receptor expression vector subcloned in a cytomegalovirus (CMV) promoter CMV-hGR, the CMV-β-galactosidase, and the TK-GRE2-Luc promoter were supplied by Dr. Dietmar Spengler

(52); the wild-type human glucocorticoid receptor phGR-SB receptor and its GR DNA-binding domain (DBD)-derived mutants A458T and S425G were provided by Dr. Andrew C. B. Cato (24); the construct containing the multimerized NF-κB-binding sites linked to a minimal promoter upstream of the luciferase gene (κB-Luc promoter) was obtained from Dr. Michael Bell (53); the mouse mammary tumor promoter cloned upstream of the luciferase gene (MMTV-Luc), which contains multiple GC-responsive elements (GREs), located between positions –202 and –79 upstream of the RNA start site, was supplied by Dr. R. Evans (54).

Transfection of EL-4 cells (10^6 cells/ml) was performed by electroporation using a GenePulser (Bio-Rad Laboratories, Hercules, CA, USA) at 280 V/975 microfarads with a total of 30 μg of DNA. Cells were washed with PBS, and extracts were prepared with reporter lysis buffer (Promega). After treatments, cells were harvested, and luciferase activity was measured, as described previously (55) using the luciferase measure kit (Promega) with a Junior luminometer (Berlthod, Bad Wildbad, Germany). CMVhGR expression vector was cotransfected in EL-4 and Jurkat cells in order to make them responsive to GCs. In all cases, cells were cotransfected with the CMV-β-galactosidase plasmid expression vector, used as a control for transfection efficiency to standardize the results (56). Some transfection experiments were performed in the presence of the specific GR antagonist RU38486 (1 μM), to prove the specificity of GR action. Some transfections were also done without the GR expression vector to confirm the involvement of the GR.

Western blot and coimmunoprecipitation assays

After the indicated stimulation time, primary splenocyte cultures were washed once with PBS (pH 7.0), and cell lysates were prepared in standard cracking buffer and boiled for 2 min. Equal levels of protein were analyzed by 10% acrylamide-bisacrylamide gel and transferred to nitrocellulose membranes (Sigma Chemical). The membranes were incubated with the mouse monoclonal anti-T-bet (100 ng/ml) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated specific secondary antibody (dilution: 1:3000) (Bio-Rad Laboratories), and detection was with the ECL kit, according to the manufacturer's instructions (Pierce Biotechnology). Anti-GAPDH (dilution: 1:10000) (Abcam, Cambridge, UK) antibody was used routinely as a loading control.

Cellular extracts of EL-4 cells, stimulated under activating conditions (PMA and ionomycin) as indicated above and in the presence of Dex during 30 min or 1h, transfected with CMVhGR or the mutant S425G receptor and pcDNA3Tbet expression vectors, were obtained and immunoprecipitated as described previously (55). Briefly, cells were collected using a lysis buffer: Tris-HCl, 50 mM, pH 7.4; EGTA, 1 mM; Nonidet P-40, 1%; NaCl, 150 mM; orthovanadate, 2 mM; phenylmethylsulfonyl fluoride, 1 mM; protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), immunoprecipitated with mouse monoclonal anti-T-bet or rabbit polyclonal anti-GR (1 μg/ml) (Santa Cruz Biotechnology), resolved on a 10% acrylamide-bisacrylamide gel, and transferred to nitrocellulose membranes.

The membranes were incubated with the indicated antibodies (GR or T-bet) and detected as described above. An identical procedure was followed to perform the coimmunoprecipitation assays for endogenously expressed T-bet and GR in splenocyte cultures on day 5 of differentiation.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (56). Briefly, EL-4 cells transfected with T-bet and GR, were stimulated under activating conditions (PMA and ionomycin), as indicated above in the presence of Dex during 1 or 6 h, then collected and washed with PBS. Nuclear proteins were extracted as follows. Cells were lysed in 100 μl of buffer A (HEPES 10 mM pH 7.9, KCl 10 mM, EDTA 100 μM, Nonidet P-40 0.1%, protease inhibitor cocktail (Roche Diagnostics). After centrifugation, the nuclear pellet was lysed with 20 μl of buffer B (HEPES 20 mM, pH 7.9, NaCl 0.42 M, EDTA 1 mM, EGTA 1 mM). Double-stranded oligonucleotides encoding the T-bet binding site (forward: 5′-GTGAATTTCACACCTAGGTGTGAAATT-3′, reverse: 5′-AATTTCACACCTAGGTGTGAAATT-3′) was end-labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase (Invitrogen). Equal amounts (20 μg) of each sample were incubated in a total volume of 20 μl of buffer containing 500 ng of poly (dI-dC)·(dI-dC). A 400-fold excess of unlabeled T-bet binding site was included in the reaction where indicated. After incubation on ice for 10 min, end-labeled oligonucleotides (20,000 cpm) were added and the incubation was continued for 20 min at room temperature. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel. The gels were dried under vacuum and autoradiographed at −70°C. Supershift experiments were performed using 1 μg of the anti-T-bet specific antibody (Santa Cruz Biotechnology).

Chromatin immunoprecipitation assays

For chromatin immunoprecipitation (ChIP) analysis, stimulated under activating conditions (PMA and ionomycin), as indicated above in the presence or absence of Dex during 1 or 6 h, transiently transfected EL-4 cells were fixed with 1% formaldehyde for 10 min at 37°C, washed with PBS, and lysed first with a cytoplasmatic lysis buffer (HEPES, 50 mM, pH 8; EDTA, 1 mM; EGTA, 0.5 mM; NaCl, 140 mM; glycerin, 10%; Nonidet P-40 0.5%, Triton X 100 0.25%) and then with a ChIP lysis buffer (Tris-HCl 10 mM pH 8, EDTA 1 mM, EGTA 0.5 mM, NaCl 200 mM) to lyse the nucleus. The chromatin was sheared to 250–1,000 bp of length by sonication with three pulses of 10 s at 30% amplitude (Fisher Scientific Sonic Dismembrator, Troy, NY, USA). Mouse monoclonal anti-T-bet antibody (Santa Cruz Biotechnology) was added (1 μg per immunoprecipitation) and incubated overnight. Protein A-sepharose beads (Sigma Chemical, St. Louis, MO, USA) were added for 4 h. The immunoprecipitated fraction was washed 5 times with ChIP lysis buffer and once with PBS. The antibody complexes were eluted, and the cross-links were reversed by adding ChIP digestion buffer (Tris-HCl, 50 mM pH 8, EDTA 1 mM, NaCl 100 mM, SDS 0.5%, Proteinase K 100 μg/ml) to each sample and heating 3 h at 60°C and then 6 h at 65°C. The DNA was phenol/chloroform extracted and precipitated with 20 μg of glycogen as a carrier. The DNA was resuspended in 20 μl of RNase-free water and was used for polymerase chain reaction (PCR) analysis. For each experiment, PCRs were performed with different numbers of cycles or with dilution series of input DNA to determine the linear range of the amplification. All of the results shown fall within this range. PCR primers used were T-bet binding site within the IFN-γ promoter: (forward): 5′-CAATTCAAAACT-TCTCTGAG-3′ (reverse): 5′-CCGTTCTATGTTCTCTATTA-3′, β-actin: (forward): 5′-TGACGGGGTCACCCACACTGTGCCCA-TCTA-3′ (reverse): 5′-CTAGAAGCATTTGCGGTGGACGATG-GAGGG-3′. Mock control conditions were performed without antibodies, and a nonspecific immunoglobulin G_1 (IgG_1) antibody was added as a control. Semiquantification was performed in the exponential phase of PCR amplification by densitometric analysis using NIH-image software, and data were normalized to the DNA input used for each individual immunoprecipitation.

Northern blot and real-time PCR analyses

Northern blot of endogenously expressed T-bet of splenocyte cultures was performed as described previously (56). Briefly, 20 μg RNA samples, isolated by the guanidine isothiocyanate phenol-chloroform extraction method, were denatured with glyoxal, electrophoresed on a 1.2% agarose gel, and transferred overnight to a Hybond-N nylon membrane (GE Healthcare, Fairfield, CT, USA). Filters were baked for 2 h at 80°C and stained with methylene blue to check for RNA integrity. They were prehybridized for 1 h at 42°C (50% formamide, 5 × SSPE, 5 × Denhardt′s solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA) and hybridized at 42°C for 12 h with T-bet. Blots were washed at increasing salt and temperature stringency with a final wash of 30 min at 42°C in 0.1 × saline-sodium citrate (SSC), containing 0.1% SDS. The same procedure was used with the β-actin probe, but hybridization and final wash were performed at 60°C. Dried filters were exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY, USA) at −70°C with intensifying screens for 20 days. A 1.1 Kb (*Bam*HI digest) T-bet cDNA fragment (26) and a 1 Kb *Pst*I fragment of β-actin cDNA (56) as a loading control, were labeled with a random-priming kit using [α-^{32}P] dCTP (specific activity, 2–4×10^8 cpm/μg) (GE Healthcare, Fairfield, CT, USA). The mRNA levels were quantified by using phosphoimager analysis (Fuji Photo Film Co., Aichi, Japan). The blots were reprobed after eluting the first probe with 5 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.1 × Denhardt′s solution, at 65°C for 2 h. For real-time PCR, cDNA was used at a concentration of 10 ng input RNA per μg of reaction volume. Primers are as follows: T-bet: (forward): 5′-GCCAGGGAACCGCTTATATG-3′; (reverse): 5′-GACGATCATCTGGGTCACATTGT-3′. β-actin: (forward): 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (reverse): 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′. Real-time quantitative PCR with Platinum SYBR Green I (Invitrogen), was then performed in 50-μl reactions in quadruplicate with 1:20 fraction of each cDNA sample and the corresponding primers, using an DNA Engine OPTICON ™ apparatus (Bio-Rad Laboratories). Samples are quantified using relative standard curves for each amplification reaction, and results were normalized to the internal control β-actin.

Statistics

Statistics were performed by ANOVA in combination with the Scheffé′s test. Data are shown as mean ± SEM.

RESULTS

Con A-induced T-bet mRNA and protein expression is inhibited by GCs

Endogenous T-bet was stimulated in splenocyte cultures obtained from naive mice with Con A (2.5 μg/ml) for 24 h in the presence or absence of 10 nM Dex during these 24 h, and mRNA was extracted for Northern blot analysis. Densitometric quantification shows inhibition of Con A-induced T-bet mRNA expression by Dex (**Fig. 1*A***). Real-time PCR quantification of T-bet mRNA relative to β-actin expression confirms the inhibition of T-bet expression (Fig. 1*B*). Similar results were obtained at 2, 6, 12, and 48 h (not shown). Also, similar results were obtained with 100 nM Dex and 10 and 100 nM hydrocortisone at all times tested (not shown). The inhibition of T-bet mRNA expression by Dex was blocked by the transcription inhibitor actinomycin D, confirming that the novo gene expression is required for the action of GCs and that the inhibition was not due to decreased mRNA stability by GCs (data not shown).

To determine whether Dex-mediated inhibition of T-bet mRNA correlated with a decrease in T-bet protein levels, we performed Western blot assays in total and purified splenocyte cultures stimulated with Con A for 24 h in the presence of Dex during the last 1, 2, 4, 8, and 24 h of incubation. We observed an inhibition of Con A-induced T-bet protein synthesis (Fig. 1*C* and *D*). The specific GR antagonist RU38486 was able to revert Dex inhibition of T-bet protein synthesis, proving the specificity of GR action (Fig. 1*E*). These results show that GCs inhibit T-bet endogenous expression.

T-bet transcriptional activity is inhibited by GCs

We further investigated whether GCs are also able to regulate the transcriptional activity of T-bet. Transfection of GR/T-bet-negative-EL-4 T cells (45–50) with T-bet response elements that we cloned upstream of the luciferase gene (T-bet-RE-Luc) and 9 μg of GR expression vector (CMV-hGR) in the absence or presence of 9 μg of T-bet expression vector (pcDNA3-T-bet) resulted in an almost five-fold increase of the luciferase activity (**Fig. 2*A***). Cotransfection of increasing amounts of GR-expressing plasmid in the presence of Dex (100 nM) led to a potent, dose-dependent inhibition of this induction (Fig. 2*A*). The reversion by the specific antagonist RU38486 proved the involvement of GR in the inhibition mediated by Dex on T-bet activity (Fig. 2*A*). In addition, no effect of GCs was observed when GR was not present in the transfection experiment (Fig. 2*A*). Similar results were obtained in Jurkat T cells (not shown).

As the inhibition of T-bet activity could be the result of reduced T-bet protein expression by Dex inhibition of the CMV promoter driving T-bet expression vector, we analyzed T-bet protein levels by Western blot analysis under the same conditions as in the transfection experiments. We observed no inhibition of T-bet protein levels by Dex treatment (data not shown), proving that GCs directly inhibit the transcriptional activity of T-bet.

Mutual transcriptional interference between GR and T-bet

Since GR repressed the transcriptional activity of T-bet, we were interested to know whether the inhibition is reciprocal. EL-4 cells transfected with GR expression vector either alone or in combination with different amounts of T-bet expression vector resulted in a pronounced activation of MMTV-Luc activity (Fig. 2*B*). Cotransfection of T-bet together with GR in the presence of 100 nM Dex resulted in the repression of GR-mediated transcription in a dose-dependent man-

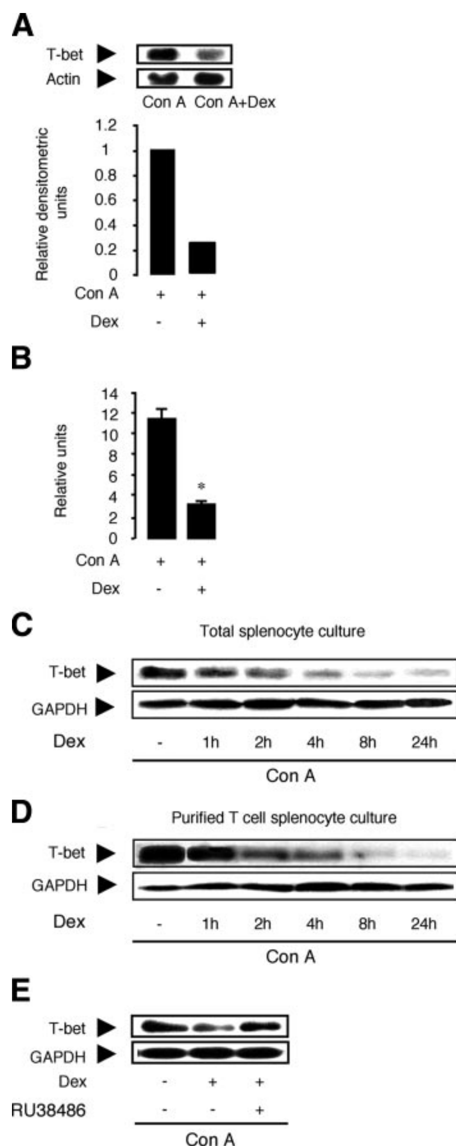

Figure 1. Glucocorticoids inhibit T-bet mRNA expression and protein levels. *A, B)* Primary splenocyte cultures were activated with Con A (2.5 μg/ml) during 24 h in the presence or absence of the glucocorticoid dexamethasone (Dex, 10 nM) during 24 h. Then, mRNA from splenocytes cultures was prepared for T-bet and GATA-3 mRNA analysis by Northern blot analysis (20 μg per lane, as detailed in Materials and Methods). *A)* Single bands corresponding to T-bet were obtained. The intensity of T-bet and β-actin signals was quantified by densitometry and expressed as relative intensity of T-bet with respect to β-actin mRNA levels. One out of three independent experiments with similar results is shown. *B)* Real-time PCR analysis in exactly the same conditions as the Northern blot analysis was performed using Platinum SYBR Green 1 in 50-μl reactions in quadruplicate of each cDNA. Samples were quantified using relative standard curves for each amplification reaction, and results were normalized to the internal control β-actin. Results, as relative units, are expressed as mean ± SEM (n=3, *B:* $^*P < 0.001$ *vs.* Con A). One out of three independent experiments with similar results is shown. *C, D)* Total and purified splenocyte cultures were activated with Con A for 24 h (2.5 μg/ml) in the presence or absence of the glucocorticoid dexamethasone (Dex, 100 nM) during the last 1, 2, 4, 8, and 24 h of incubation. *E)* The specific glucocorticoid receptor antagonist, RU38486 (1 μM), was added 30 min before dexamethasone (100 nM) treatment of the cells (2 h of Dex treatment and reversion by RU38486 is shown). Then, cell lysates were prepared for T-bet analysis by Western blot analysis. Single bands corresponding to T-bet were obtained. GADPH signal was used as a loading control. One out of three independent experiments with similar results is shown.

ner, and no effect of GCs was observed, when GR was not present in the transfection experiment (Fig. 2*B*).

Since the MMTV reporter contains many regulatory sites, we aimed to compare the above repressive action in another GRE reporter, the TK-GRE2-Luc containing only two palindromic GR-binding sites coupled to the TK promoter, with GR and T-bet expression vectors. Cotransfection of increasing amounts of T-bet together with constant amounts of GR in the presence of Dex inhibited the transcriptional activity of GR in a dose-dependent manner (Fig. 2*C*). No effect of GCs was observed when GR was not present in the transfection experiment. Thus, this minimal GRE reporter has an equal response to T-bet repression than the multiple GRE-containing MMTV plasmid.

Altogether, these experiments demonstrate that T-bet and GR reciprocally repress their transcriptional activity.

GR inhibition of T-bet transcriptional activity is mediated by a transrepression mechanism

The mutual inhibition between GR and T-bet suggested that the underpinning mechanism could be transrepression involving protein-protein interaction. Control experiments carried out in EL-4 cells show that, as described previously (24, 25), S425G transrepression mutant was able to transactivate TK-GRE2-Luc but not transrepress κB-Luc activity when compared to wild-type GR. On the contrary, A458T transactivation mutant was not able to transactivate TK-GRE2-Luc but was able to transrepress κB-Luc activity (**Fig. 3*A*** and ***B***).

The ability of these GR mutants to repress T-bet-RE-Luc reporter construct is shown in Fig. 3*C*. We found that the A458T mutation had no effect on the ability of GR to repress T-bet activity, whereas the S425G mutation greatly reduced the ability of GR to repress T-bet, therefore suggesting that transrepression might be the mechanism by which GR is inhibiting T-bet activity.

Physical association of GR and T-bet in transfection experiments

Our data strongly indicated that mutual inhibition could be achieved by a direct interaction between GR and T-bet proteins. To confirm this possibility, we performed immunoprecipitation experiments in EL-4 cells cotransfected with GR and T-bet expression vectors. As shown in **Fig. 4*A*** and ***B***, GR did coimmunoprecipitate with T-bet either when the anti-T-bet or the anti-GR antibodies were used in the experiments, re-

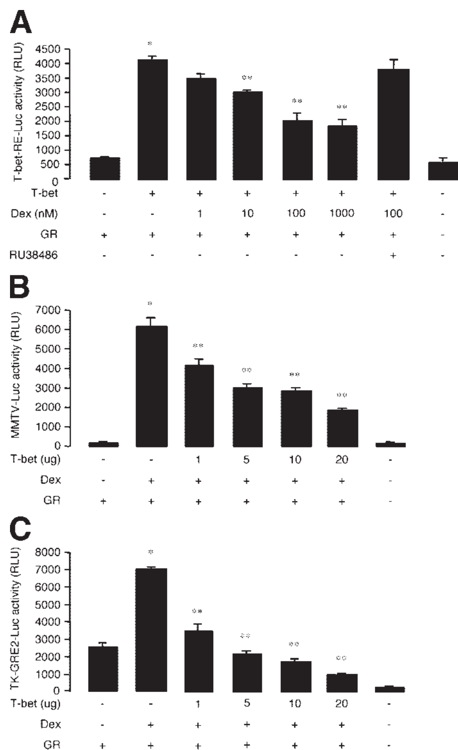

Figure 2. Glucocorticoid receptor (GR) and T-bet transcriptional activity inhibition. *A)* EL-4 cells were transiently transfected with 9 μg of a reporter plasmid, which contains T-bet response elements upstream of the luciferase gene (T-bet-RE-Luc) in the presence (+) or absence (–) of 9 μg of pcDNA3-T-bet expression vector (T-bet), and 9 μg of the CMVhGR GR expression vector. After 2 h in culture medium, cells were stimulated for 20 h with (+) or without (–) dexamethasone (Dex, 1, 10, 100, and 1000 nM) or the GR-specific antagonist RU38486 (1 μM). Results are given as relative luminometric units (RLU), normalized to β-galactosidase activity, are expressed as mean ± SEM ($n=4$, *A*: $^{*}P<0.001$ *vs.* basal without Dex, $^{**}P<0.001$ *vs.* T-bet without Dex). One out of four independent experiments with similar results is shown. *B)* EL-4 cells were cotransfected with 9 μg of the mouse mammary tumor promoter cloned upstream of the luciferase gene reporter plasmid (MMTV-Luc) plus 9 μg of the CMVhGR glucocorticoid receptor expression vector (GR) in the presence (+) or absence (–) of 1, 5, 10, and 20 μg of pcDNA3-T-bet expression vector (T-bet). After 16 h in culture medium, cells were stimulated for 8 h with (+) or without (–) dexamethasone (Dex, 100 nM). Results are given as RLU, normalized to β-galactosidase activity, are expressed as mean ± SEM ($n=4$, *B*: $^{*}P<0.001$ *vs.* basal without Dex, $^{**}P<0.05$ *vs.* Dex without T-bet). One out of four independent experiments with similar results is shown. *C)* EL-4 cells were cotransfected with 9 μg of a reporter plasmid construct containing two palindromic GR-binding sites coupled to the TK promoter reporter plasmid (TK-GRE2-Luc) plus 9 μg of the glucocorticoid receptor expression vector CMVhGR (GR) in the presence (+) or absence (–) of 1, 5, 10, and 20 μg of pcDNA3-T-bet expression vector (T-bet). After 16 h in culture medium, cells were stimulated for 8 h with (+) or without (–) dexamethasone (Dex, 100 nM). Results are given as RLU, normalized to β-galactosidase activity, are expressed as mean ± SEM ($n=4$, *C*: $^{*}P<0.001$ *vs.* basal without Dex, $^{**}P<0.05$ *vs.* Dex without T-bet). One out of four independent experiments with similar results is shown.

spectively. Controls using the same immunoprecipitating antibody indicated correct immunoprecipitation and blotting of GR and T-bet (Fig. 4*C* and *D*). Whole extract lysates were used as controls to demonstrate the presence of GR and T-bet proteins.

To further explore whether this mutual repression was a result of GR transrepressive properties we attempted to immunoprecipitate transfected GR transrepression mutant S425G and T-bet under the conditions mentioned above. Figure 4*E* and *F*, show that S425G did not coimmunoprecipitate with T-bet either when the anti-T-bet or the anti-GR antibodies were used. Controls using the same immunoprecipitating antibody for GR and T-bet (Fig. 4*G* and *H*) were included, and whole extract lysates were again used as controls. These findings corroborated the assumption that the mutual transrepression between T-bet and GR involves protein-protein interaction.

Physical association of endogenous GR and T-bet

Since ectopic overexpression of proteins by transfection may lead to artificial associations, we sought to study the physical interaction between endogenous GR and T-bet proteins. Optimal conditions for induction of T-bet were established first: Purified splenocyte cultures, obtained by activation with T cell mitogen Con A were stimulated under Th1-inducing conditions with IL-12 and anti-IL-4 antibody. Cells were harvested each day during a period of 9 days and analyzed for GR and T-bet expression by Western blot analysis assay (**Fig. 5*A***). Optimal T-bet/GR-expressing conditions for interaction analysis were found at day 5 post -stimulation; therefore, coimmunoprecipitation of endogenous GR and T-bet was assayed on day 5 (Fig. 5*B*–E). As shown in Fig. 5*B* and *D*, GR did coimmunoprecipitate with T-bet either when the anti-T-bet or the anti-GR antibodies were used in the experiments. Controls using the same immunoprecipitating antibodies indicate correct immunoprecipitation and blotting of T-bet and GR (Fig. 5*C* and *E*). Whole extract lysates were used as controls to confirm the presence of endogenous GR and T-bet proteins.

GR blocks DNA binding of T-bet in the presence of GCs

To further characterize the mechanism of GC-mediated repression of T-bet activity, we analyzed the effect of activated GR on T-bet DNA binding *in vitro* by EMSA assays using T-bet-binding sites as probes in EL-4 cells cotransfected with GR and T-bet expression plasmids (**Fig. 6*A***). We found that Dex treatment caused a marked reduction in the ability of T-bet to bind DNA (lane 2). As a competition control reaction, an excess of unlabeled T-bet oligonucleotide was included (lane 3). The identity of this complex was T-bet, as determined by a supershift experiment using an anti-T-bet antibody (lane 4). Thus, T-bet binding to the DNA is reduced by GR.

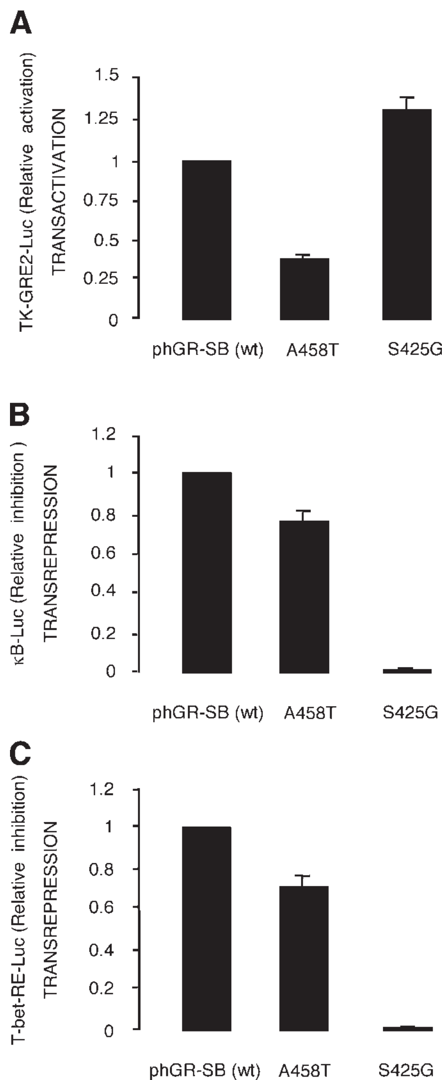

Figure 3. GR inhibition of T-bet transcriptional activity by different GR expression vectors. *A)* EL-4 cells were transiently transfected with 9 μg of a reporter plasmid containing two palindromic GR-binding sites coupled to the TK promoter reporter plasmid (TK-GRE2-Luc) plus 9 μg of phGR-SB (wt) wild-type glucocorticoid receptor expression vector or with the glucocorticoid receptor mutants A458T or S425G. After 16 h in culture medium, cells were stimulated for 8 h with (+) or without (–) dexamethasone (Dex, 10 nM). Results, as relative activation [to basal conditions, phGR-SB (wt) = 1] of TK-GRE2-Luc, normalized to β-galactosidase activity, are expressed as mean ± SEM. One out of four independent experiments with similar results is shown. *B)* EL-4 cells were cotransfected with 9 μg of κB-Luc reporter plasmid upstream of the luciferase gene plus 9 μg of phGR-SB (wt) wild-type glucocorticoid receptor expression vector or with the glucocorticoid receptor mutants A458T or S425G. After 16 h in culture medium, cells were stimulated for 8 h in the presence of IL-1β (10 ng/ml), which induces κB-Luc activity, in the presence or absence of dexamethasone (Dex, 100 nM). Results, as relative inhibition [to basal conditions, phGR-SB (wt) = 1] of κB-Luc, normalized to β-galactosidase activity, are expressed as mean ± SEM. One out of four independent experiments with similar results is shown. *C)* EL-4 cells were transiently transfected with 9 μg of a reporter plasmid, which contains T-bet response elements upstream of the luciferase gene (T-bet-RE-Luc) in the presence or absence of 9 μg of pcDNA3-T-bet expression vector (T-bet) and 9 μg of wild-type glucocorticoid receptor expression vector (ph-GR-SB) or with the glucocorticoid receptor mutants A458T or S425G. After 16 h in culture medium, cells were stimulated for 8 h with or without dexamethasone (Dex, 100 nM). Results, as relative inhibition [to basal conditions, phGR-SB (wt) = 1] of TK-GRE2-Luc, normalized to β-galactosidase activity, are expressed as mean ± SEM. One out of four independent experiments with similar results is shown.

To verify that the inhibitory action of GR on binding of T-bet to its cognate binding sites is also operative in the chromosomal context *in vivo*, we performed ChIP assays. EL-4 cells were cotransfected with GR and T-bet expression vectors under the same conditions, as described above for the EMSA assays. Chromatin immunoprecipitation from Dex-treated cells with an antibody against T-bet revealed a potent inhibition of T-bet-binding activity (Fig. 6*B*, lanes 1–3, Fig. 6*C*). The T-bet-DNA binding site was not immunoprecipitated with an isotype control IgG_1 antibody (lane 5). Thus, these results extend our *in vitro* observations with the EMSA assay by demonstrating the ability of GR to interfere with the interaction of T-bet with its endogenous, chromosomal binding sites.

Functional consequences of GCs inhibition of T-bet for IFN-γ transcription

We aimed at testing the functional relevance of our discovery of the GR-T-bet crosstalk at a promoter central to the immune system. We first confirmed that, GCs inhibit IFN-γ production in splenocyte cultures in a dose-dependent manner (**Fig. 7*A***), as previously shown (8,42,43). To investigate the possible involvement of GR/T-bet mutual interaction in the regulation of IFN-γ promoter activity, EL-4 cells were transfected with a −3447-IFN-γ promoter cloned upstream of the luciferase gene and with GR expression vector. Overexpression of T-bet, resulted in a almost seven-fold increase of the luciferase activity, and cotransfection of GR in the presence of Dex led to a potent inhibition of this induction (Fig. 7*B*). The specific antagonist, RU38486 proved the involvement of the GR and additional controls showed that no effect was observed in the presence of activated GR alone or when GR was not present in the transfection experiment (Fig. 7*B*). Under the same conditions as in the transfection experiments, Western blots were carried out to analyze GR and T-bet protein levels. No inhibition of GR and T-bet protein expression from the plasmids by Dex treatment was observed (data not shown), proving that under these conditions, direct interaction of GR and T-bet is the relevant mechanism in the regulation of the transcriptional activity in the IFN-γ promoter and not the reduction of T-bet expression (as shown in Fig. 1). Figure 7*C* shows that the inhibition by activated GR of T-bet induced IFN-γ promoter activity is dose dependent.

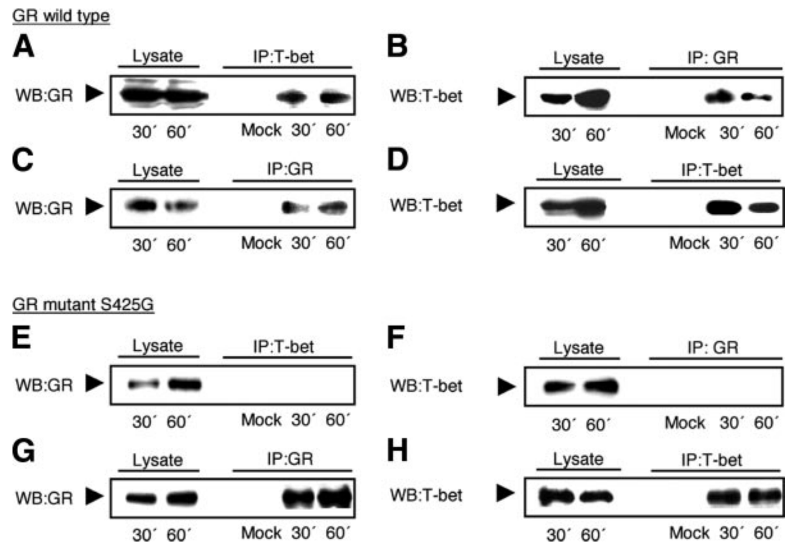

Figure 4. Coimmunoprecipitation of transfected glucocorticoid receptor or glucocorticoid receptor mutant S425G and T-bet. EL-4 cells were transfected with 20 μg of the CMVhGR glucocorticoid receptor expression vector (GR) and 20 μg of pcDNA3-T-bet expression vector (T-bet) (*A–D*) or 20 μg of GR mutant S425G and 20 μg of pcDNA3-T-bet expression vector (T-bet) (*E–H*). After 16 h in culture medium, EL-4 cells were treated for 30 or 60 min with dexamethasone (Dex, 100 nM) under activated conditions using 1 μM ionomycin and 0.1 μM PMA. Cell lysates were immunoprecipitated (IP) with protein A-G sepharose in combination with the following antibodies (1 μg/ml)—in *A, D, E,* and *H*: anti-T-bet antibody; in *B, C, F and G*: antiglucocorticoid receptor antibody (GR). The immunoprecipitated fractions and the whole lysates were analyzed by Western blot (WB) (bands of 65 for T-bet kDa and 94 kDa for GR), as described in Materials and Methods, by using the following antibodies (100 ng/ml)—*A, C, E,* and *G:* anti-GR antibody; in *B, D, F* and *H:* anti-T-bet antibody; and incubation with HRP-conjugated secondary antibodies (dilution: 1:3000). One out of four independent experiments with similar results is shown.

DISCUSSION

The ability of GCs to inhibit cellular immunity, although broadly described, is still poorly understood at the mechanistic level. In this study, we report that GR blocks the gene expression activating function of T-bet, a key TF involved in Th1-mediated cellular immunity. Moreover, GR physically interacts with T-bet and blocks its ability to bind to DNA and to activate gene transcription. We also show that the first N-terminal zinc finger region of GR is required for this transrepression mechanism. Thus, our data contribute to the understanding of the molecular mechanism underlying the immunomodulatory actions of GCs.

In general, GCs mediate their effects by binding to the intracellular GR, which triggers its nuclear translocation. Once in the nucleus, the activated GR regulates transcription of downstream genes in a positive or negative way by binding to specific GRE or negative GRE (nGRE), respectively, on target regulatory DNA sequences. GCs are also able to repress gene activation indirectly by a transrepression mechanism involving protein-protein interaction, for example, with family members of transcription factors such as RelA or AP-1 (10–14, 16–20). Our coimmunoprecipitation experiments show that transrepression by protein-protein interaction is the mechanism by which GCs also inhibit T-bet activity. The physical interaction between activated GR and T-bet results in the inhibition of the transactivation potential of both, as shown by transfection experiments using as reporters of GR and T-bet activity the TK-GRE2-Luc and T-bet-RE-Luc reporters, respectively. GC actions were reverted by the synthetic antagonist RU38486, which acts as a competitor for binding to GR, showing GR specificity of our observations. Furthermore, EMSA experiments reveal that GCs inhibit T-bet binding to the DNA, which, at least in part, may be a consequence of the molecular interaction between T-bet and GR. Importantly, ChIP experiments demonstrate that this inhibitory mechanism functions at endogenous sites in the chromatin context.

The nature of the interaction between GR and T-bet apparently is similar to that observed between GR and AP-1 or NF-κB; in all cases, there is a mutual repression. The zinc finger DBD of GR is essential for transrepression of AP-1 and p65 activity (23–25). We found that the zinc finger of the DBD is also important for the physical interaction between GR and T-bet. The mutant S425G has a serine to glycine substitution at position 425, which removes a hydroxyl group that is supposed to alter hydrogen bondings between the zinc finger domain and other proteins, thus suggesting that this binding is important for transrepression mediated by protein-protein interaction not only of NF-κB, but also of T-bet. Interestingly, this substitution not only alters the function of, but also the interaction with T-bet. As alluded to above, in addition to the protein-protein interaction hypothesis, but there is another mechanism proposed for GC-mediated repression of promoters mediated by GR binding to nGRE (57). Recently, an nGRE element was found at position −990 of the *fasL* promoter, which overlaps with an NF-κB binding site. GR was able to down-regulate *fasL* promoter by competing with NF-κB for binding to the common response element. No consensus nGRE sequences were found in the IFN-γ promoter and also not in the T-bet-RE-Luc reporter plasmid, rendering this possibility as an explanation for the inhibitory action highly unlikely. Nevertheless, the lack of well-conserved response elements hamper the identification of nGRE, and only a few reports have been published on nGREs (58). Therefore, the mechanism described here does not completely rule out the possibility that a combination of DNA-binding to cryptic nGREs and protein-protein interactions might account for the overall repression of

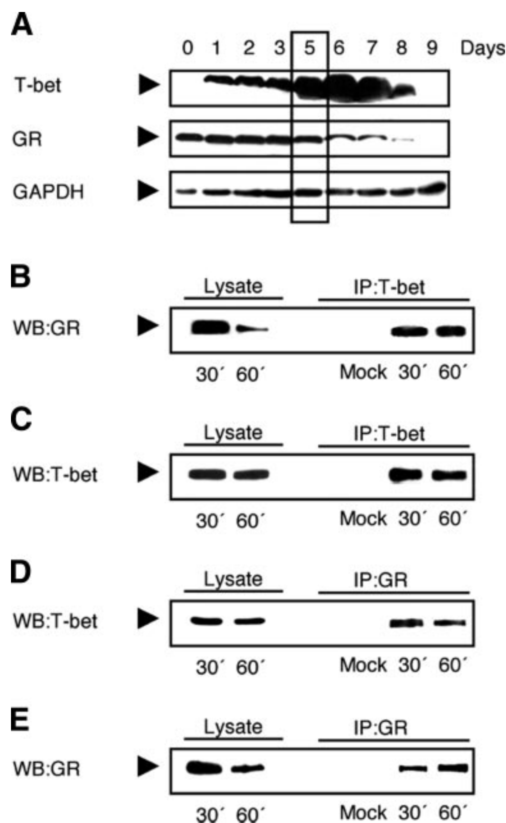

Figure 5. Coimmunoprecipitation of endogenous glucocorticoid receptor and T-bet. *A)* Purified T cells were activated with Con A (2.5 μg/ml) during 24 h and then under Th1-differentiating conditions using IL-12 (5 ng/ml), and anti-IL-4 antibody (10 μg/ml) during 9 days. IL-2 (10 U/ml), was also added for clonal expansion of the differentiated cells. Cells were harvested on days 0, 1, 2, 3, 5, 6, 7, 8, and 9 poststimulation. Cell lysates were prepared for T-bet and GR analysis by Western blot analysis. Single bands corresponding to T-bet and GR were obtained (65 kDa for T-bet and 94 kDa for GR). GADPH signal was used as a loading control. The optimal T-bet/GR-expressing conditions were achieved at day 5 poststimulation. One out of three independent experiments with similar results is shown. *B–E)* Five-day enriched cell cultures were washed twice with PBS, and fresh medium was added. Then, cells were treated for 30 or 60 min with dexamethasone (Dex, 100 nM) under activated conditions using 1 μM ionomycin and 0.1 μM PMA. Cell lysates were immunoprecipitated (IP) with protein A-G sepharose in combination with the following antibodies (1 μg/ml)—in *B* and *C*: anti-T-bet antibody; in *D and E*: antiglucocorticoid receptor antibody (GR). The immunoprecipitated fractions and the whole lysates were analyzed by Western blot (WB), as described in Materials and Methods, by using the following antibodies (100 ng/ml): in *B* and *E*: anti-GR antibody; in *C* and *D*: anti-T-bet antibody; and incubation with HRP-conjugated secondary antibodies (dilution: 1:3000). One out of four independent experiments with similar results is shown.

IFN-γ gene regulation by GCs. It would also be interesting to establish the involvement of GR beta (GRβ) isoform, which has also immunoregulatory actions (59).

IFN-γ is a central immunoregulatory cytokine responsible for several immunological effects, making its regulation important for protective cellular immunity (29–31). As mentioned above, studies of the IFN-γ gene have shown that T-bet elements play an important role in the induction of transcription and production of this cytokine (26–28, 32–35, 60, 61). Cotransfection of EL-4 cells, which do not produce IFN-γ with T-bet

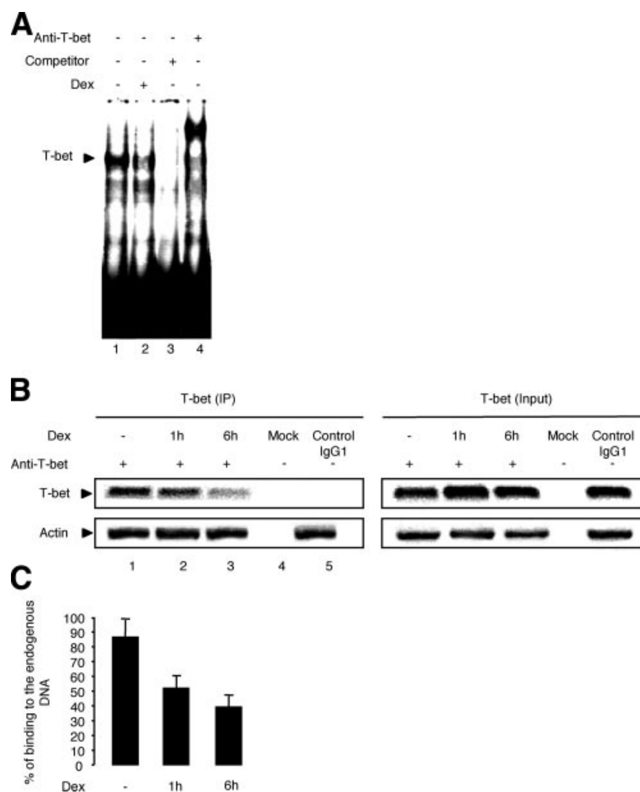

Figure 6. Glucocorticoids inhibit T-bet binding to the DNA and T-bet interaction with endogenous DNA sequences. *A)* EL-4 cells were transfected with 20 μg of the CMVhGR GR expression vector and 20 μg of pcDNA3-T-bet expression vector. After 16 h in culture medium, cells were treated for 1 (not shown) and 6 h with dexamethasone (Dex, 100 nM) under activated conditions using 1 μM ionomycin, and 0.1 μM PMA. EMSA and competition reactions were performed using labeled T-bet response elements oligomers and 20 μg of nuclear extracts from EL-4 cells. For competition reactions, an excess of unlabeled T-bet oligonucleotides was included in the reaction mixtures. The assay was performed using 500 ng of poly (dI-dC)·(dI-dC) in the reaction buffer. Nuclear extracts were preincubated with 1 μg of Anti-T-bet antibody for supershift experiments. One out of three independent experiments with similar results is shown. *B)* EL-4 cells were transfected with 20 μg of the CMVhGR GR expression vector and 20 μg of pcDNA3-T-bet expression vector (T-bet). After 16 h in culture medium, cells were treated for 1 or 6 h with dexamethasone (Dex, 100 nM) under activated conditions using 1 μM ionomycin and 0.1 μM PMA for T-bet activation. For ChIP analysis, EL-4 cells were fixed with formaldehyde for 10 min. Cell lysates were immunoprecipitated (IP) with anti-T-bet antibody (1 μg/μl) in combination with protein A sepharose beads. Input loading controls carried out without the immunoprecipitation step were also performed. The extracted DNA was used for PCR analysis. Mock control conditions were carried out without antibodies, and also a nonspecific IgG_1 antibody was added as control. *C)* A semiquantitative analysis of the PCR product normalized by the input-loading controls and β-actin is shown. One out of three independent experiments with similar results is shown.

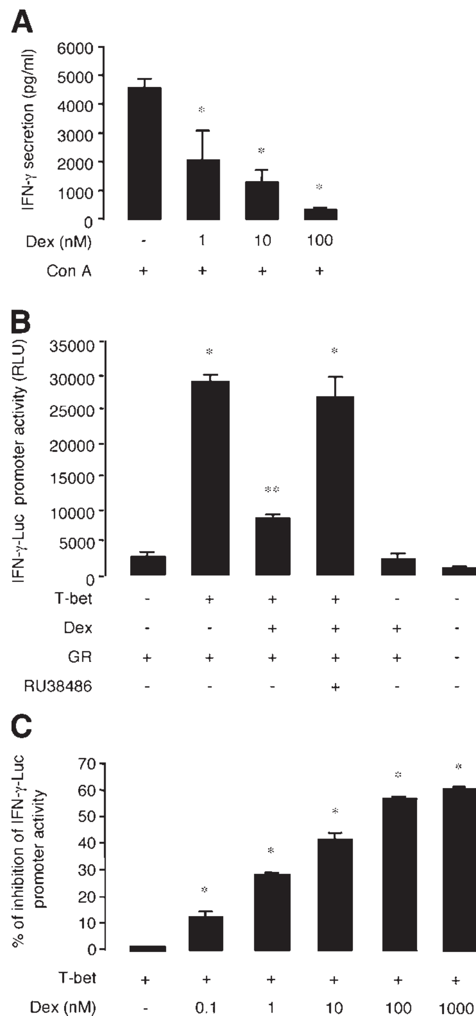

Figure 7. Glucocorticoids inhibit IFN-γ protein and promoter activity under T-bet stimulation. *A)* Purified splenocyte cultures were activated with Con A (2.5 μg/ml) during 24 h in the presence or absence of the glucocorticoid dexamethasone (Dex, 1, 10, 100 nM) during the 24 h. Then, supernatants from splenocyte cultures were used to measure mouse IFN-γ according to the manufacturer's instructions by ELISA. Results are expressed as mean ± SEM (n=4, *A*: * P<0.001 *vs.* Con A). One out of four independent experiments with similar results is shown. *B)* EL-4 cells were cotransfected with 9 μg of IFN-γ promoter-driven luciferase plasmid (IFN-γ-Luc) plus 9 μg of the CMVhGR glucocorticoid receptor expression vector (GR), in the presence (+) or absence (–) of 9 μg of pcDNA3-T-bet expression vector (T-bet). After 16 h in culture medium, cells were stimulated for 8 h with (+) or without (–) dexamethasone (Dex, 100 nM) and with (+) or without (–) the glucocorticoid receptor specific antagonist RU38486 (1 μM). Results are given as RLU, normalized to β-galactosidase activity, and are expressed as mean ± SEM (n=4, *B*: *P<0.001 *vs.* basal without T-bet, **P<0.001 *vs.* T-bet without Dex). One out of four independent experiments with similar results is shown. *C)* EL-4 cells were cotransfected with 9 μg of IFN-γ promoter-driven luciferase plasmid (IFN-γ-Luc) plus 9 μg of the CMVhGR GR expression vector, in the presence (+) or absence (–) of 9 μg of pcDNA3-T-bet expression vector (T-bet). After 16 h in culture medium, cells were stimulated for 8 h with (+) or without (–) dexamethasone (Dex, 0.1, 1, 10, 100, or 1000 nM). Each value was normalized to β-galactosidase activity. Results are expressed as % of inhibition of IFN-γ promoter stimulated by T-bet (n=4, *C*: *P<0.001 *vs.* T-bet without dexamethasone=0%). One out of four independent experiments with similar results is shown.

expression vector and the entire IFN-γ locus, results in IFN-γ cytokine production (26).

Protein-protein interactions as a mechanism for the regulatory actions of T-bet have recently been reported in two other systems. T-bet can not only function as an activator of IFN-γ gene expression through its direct interaction with IFN-γ promoter (61), but T-bet can also interact with the GATA-3 TF, inhibiting Th2 cytokine gene expression (62) and with RelA (NF-κB), regulating the binding of RelA to the IL-2 promoter, thereby repressing IL-2 production (63). To rule out the possibility that the inhibition of T-bet is mediated by competition between GR and T-bet for NF-κB TF, we transfected EL-4 cells with a dominant-negative I kappa B protein (64) and confirmed the inhibition of T-bet-dependent transcription by GCs without interference of NF-κB (data not shown), demonstrating that NF-κB does not play a role in T-bet transcriptional inhibition by GCs. Our data suggest that the inhibition of T-bet by GCs plays an important role in mediating the inhibition of IFN-γ gene expression and contributes to the understanding of the immunosuppressive action of GCs on the Th1 cellular immune response.

Dex treatment of T cell cultures causes a significant reduction not only of T-bet mRNA, but also protein expression. In this regard, Western blot assays were performed also under Th1-differentiating conditions confirming inhibition of T-bet protein expression (data not shown). To rule out the effect of GCs on T-bet mRNA and protein expression, transfection experiments were performed by overexpression of T-bet, which also renders the reporter genes exclusively dependent on T-bet (or GR) activity. Interestingly, the biological function of the GCs to inhibit T-bet apparently makes use of the synergism of reduced transcriptional function and expression of T-bet.

The mutual transcriptional inhibitory action between GR and T-bet may have important consequences in the neuroendocrine regulation of the immune system. Particularly the action of cytokines on the hypothalamic-pituitary-adrenal axis may be affected and thus impact on pathologies (*i.e.*, autoimmune diseases) in which these circuits are involved.

GCs have been used for the last 60 years as immunosuppressants in the therapy of Th1 inflammatory and autoimmune diseases. The antiinflammatory/immunosuppressive role of GCs is well characterized, mediated by interaction between GR and members of families of TFs, such as Rel A (NF-κB), AP-1, or CREB (12, 16, 17, 20, 65). Despite the effectiveness of GC treatment for inflammatory disorders, explaining their widespread clinical usage, an important drawback of long-term treatment with GCs involves the occurrence of a range of debilitating side effects, including diabetes, cataract, muscle wasting, *etc.* The pharmaceutical industry is therefore interested in alternative therapies, which would offer a better side effect profile. To reach this goal, a detailed understanding of the molecular mechanism underlying the antiinflammatory effects of GCs is a prerequisite. Our results indicate that T-bet might be useful as a target for GC pharmacological treatment of

immune disorders. The understanding of T-bet regulation may ultimately help to improve Th2-asthma, as well as other inflammatory and Th1-predominant autoimmune diseases treatments by GCs (8). So called "dissociative" GCs favoring transrepression mechanisms are under development (17). Blocking cytokine signaling has become important for the development of novel immunosuppressants (66). On Th1, besides the effects through AP-1, NF-κB, and NFAT, GCs have a specific action through the inhibition of STAT-4 phosphorylation (40) and T-bet. The knowledge that T-bet is inhibited by this transrepression mechanism of GCs could open new avenues for the design of novel therapy strategies.

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