Biochemical and Biophysical Research Communications **276,** 189–196 (2000)
doi:10.1006/bbrc.2000.3458, available online at http://www.idealibrary.com on IDEAL®

Glucocorticoid Resistance in Thymocytes from Mice Expressing a T Cell Receptor Transgene

Ahmad Pazirandeh,*,† Yintong Xue,* Sam Okret,† and Mikael Jondal*

**Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, SE-171 77 Stockholm, Sweden; and †Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital, Novum, SE-141 86 Huddinge, Sweden*

Received August 15, 2000

A majority of thymocytes undergo apoptosis during differentiation due to lack of survival signals provided by T cell receptor (TCR) activation. As glucocorticoids (GC) have been suggested to be involved in this process, we have investigated the GC sensitivity in thymocytes from mice expressing a transgenic selecting TCR. We now report that immature $CD4^+CD8^+$ double-positive thymocytes from these mice are comparatively more resistant to corticosterone-induced apoptosis. This is associated with reduced glucocorticoid receptor (GR) expression, increased levels of membrane CD28, increased NF-κB DNA binding activity, and increased binding to the CD28 response element in the interleukin-2 gene promoter. Analysis of NF-κB/Rel proteins from nuclear extracts demonstrated altered levels of some of these proteins. Our results suggest that TCR recognition of self major histocompatibility antigens generates intracellular signals which alter the thymocyte GC sensitivity and thereby protect them against apoptosis induced by endogenous GC.

Key Words: **cross-talk; NF-κB; glucocorticoid receptor; apoptosis; T cell development.**

T cell receptors (TCR) are essential for the survival of immature thymocytes and differentiation of these cells into mature T cells through an interaction with self MHC molecules expressed on thymic epithelial cells (TEC). Early $CD4^-$ $CD8^-$ double-negative thymocytes progress to a $CD4^+$ $CD8^+$ double-positive (DP) stage, at which the TCR determines the fate of thymocytes (1). A majority of DP thymocytes lack an appropriate TCR for recognizing self MHC and will not mature, but undergo apoptosis (1, 2). We, and others (3–5), have suggested that glucocorticoids (GC) are important in this process as they are strong inducers of apoptosis in thymocytes (6).

DO11.10 transgenic mice express a TCR transgene obtained from the DO11.10 T cell hybridoma. Expression of this transgene allows maturation of mainly $CD4^+$ T lymphocytes in these mice (7). In our earlier work we have shown that in DO11.10 TCR transgenic mice the thymus is enlarged and that there is an increased cellularity in the organ. In addition, the apoptotic activity is reduced as measured by flow cytometry, reduced mitochondrial membrane potential, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) techniques (8). In the present work we have investigated the background for this phenotype by analysing the sensitivity of the DO11.10 TCR^+ thymocytes and in particular the DP population to GC. We have also investigated intracellular GR levels, co-receptor expression and activation of transcription factor NF-κB in the transgenic thymocytes. All of these factors have been implicated in development of T cells and are functionally related to each other (9–11).

GC play an important role in the development and function of T cells (5). GC bind to GR, which is a member of the nuclear hormone receptor superfamily (12). After binding of GC, the GR dissociates from its associated proteins and translocates to the nucleus where the GR activates or represses the transcription of target genes (13). GR transcriptional activation has been shown to be important in GC-induced apoptosis of thymocytes (14). An important factor determining cellular GC sensitivity is the intracellular GR concentration (15). GR can also directly bind to some transcription factors such as NF-κB proteins and influence their transcriptional activities (16–18).

The mammalian NF-κB transcription factors include p50/p105, p52/p100, RelA (p65), c-Rel, and RelB, which play important roles in the regulation of immune and inflammatory responses, cellular proliferation, cell death, and development of the immune system (19–21). NF-κB proteins are sequestered in the cytosol of

Abbreviations used: CD28RE, CD28 response element; CS, corticosterone; DP, double positive; GC, glucocorticoids; GR, glucocorticoid receptor; MHC, major histocompatibility complex; NF-κB, nuclear factor kappa B; TCR, T cell receptor; TEC, thymic epithelial cells.

0006-291X/00 $35.00

unstimulated cells by a noncovalent interaction with inhibitory proteins (IκBs) such as IκBα and IκBβ (22). Upon activation, IκBs are degraded allowing NF-κB to translocate to the nucleus and to bind to cognate response elements in target genes (23). GR can antagonize the transcriptional function of Rel/NF-κB either by induction of IκBα (24, 25) or by direct protein-protein binding to some NF-κB components (26).

Stimulation of T cells through the TCR/CD3 complex alone is not sufficient to activate optimal cytokine production and cell proliferation. Additional costimulatory signals provided by the antigen-presenting cells (APC) are required, which are generated through interactions between B7-1 and B7-2, on the APC and the CD28 on the T cells (27). In T cells, TCR/CD3 and CD28 signaling pathways through activation of NF-κB-inducing kinase (NIK) and IκB kinase (IKK), degrade the IκB proteins (28) and enhance activation of NF-κB proteins. NF-κB binds both to the NF-κB site and the CD28 response element (CD28RE) in the promoter of some cytokine genes including interleukin-2 (IL-2) (29–31) leading to IL-2 synthesis and T cell proliferation.

In thymocytes, functional crosstalk between TCR and the glucocorticoid receptor (GR) has been reported (32). It has also been shown that TCR stimulation of thymocytes *in vitro* can rescue these cells from GC induced apoptosis (33). In this work we have found that expression of the DO11.10 TCR transgene renders immature DP transgenic thymocytes comparatively more resistant to induction of apoptosis by corticosterone (CS). This was associated with a reduced GR level, increased CD28 expression and an alteration in the activation of some NF-κB components in the DO11.10 TCR^+ thymocytes. In addition, we could demonstrate an *in vivo* physical interaction between RelA and GR in thymocytes. Based on these observations we propose molecular mechanisms contributing to the GC resistance in TCR activated thymocytes which might be crucial for their survival in thymus during differentiation.

MATERIALS AND METHODS

Mice and antibodies. The DO11.10 TCR transgenic mice strain (back-crossed to BALB/c), expressing a MHC-II restricted transgene specific for an $I\text{-}A^d$ restricted OVA 323-339 peptide, was kindly supplied by Dr. Dennis Loh (Howard Hughes Medical Institute, St. Louis, MO) (7). These mice were bred as heterozygous. To type the transgene expression, circulating T cells were stained with the F23.1 mAb and analyzed by FACScan (Becton Dickinson) (7). All mice were bred and housed under standard conditions in the animal facility at Karolinska Institutet. Four- to six-week-old transgenic mice and their control littermates were used throughout the study.

FITC-conjugated hamster anti-mouse CD28 (37.51.1 clone) mAb was bought from Nordic Biosite AB (Stockholm, Sweden). FITC-conjugated rat anti-mouse CD4 (RM4-5 clone), and PE-conjugated rat anti-mouse CD8 (53-6.7 clone) were purchased from PharMingen (San Diego, CA). OVA 323-336 peptides was synthesized by solid phase method using Fmoc chemistry in the Peptide Synthesis Facility at MTC, Karolinska Institute (Stockholm, Sweden). Corticosterone was bought from Sigma-Aldrich. Antibodies against GR (PA1-512) was bought from Affinity Bioreagents.Inc. Antibodies against RelA (sc-109), RelB (sc-226), P50 (sc-114), and c-Rel (sc-70-G) were purchased from Santa Cruz Biotechnology. Protein A Sepharose CL-4B was purchased from Pharmacia Biotech.

Isolation of whole thymocytes and DP cells. Single cell thymocytes were prepared by passage through a steel net. Thymocytes were stained with antibodies against the CD4 and CD8 molecules and washed with complete RPMI. Using a FACS sorter (Becton Dickinson), DP thymocytes were gated and collected.

Preparing nuclear and whole cell extract. Nuclear extracts were prepared as described before (34), with minor modification, as given below. Briefly, thymocytes were resuspended in 1 ml ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreithol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml aprotonin) by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 10 μl of a 10% solution of NP-40 was added and the tubes were vigorously vortexed for 10 s. The homogenate was centrifuged for 1 min at 14,000 rpm in a microfuge. The nuclear pellet was resuspended in 100 μl ice-cold buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 25% glycerol; 2 mM dithiothreithol, 0.5 mM phenylmethylsulfunyl fluoride, 5 μg/ml leupeptin) and the tubes were vigorously rocked on ice for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min at 14,000 rpm in a microfuge and the supernatant was frozen in aliquots at −70°C. For preparing whole cell protein extracts, thymocytes were washed and resuspended in 60 μl of a buffer containing 20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 25% glycerol; 0.6% NP40; 2 mM dithiothreithol, 0.5 mM phenylmethylsulfunyl fluoride, 5 μg/ml leupeptin and the tubes were vigorously vortexed for 10 s. The cells were kept on ice for 20 min while they were vigorously rocked for 15 min on a shaking platform. The extract was centrifuged for 5 min at 14,000 rpm in a microfuge and the supernatant was used for further experiments. The protein concentration in cell extracts was quantitated spectrophotometrically with the Bio-Rad protein assay kit.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides were end labeled with [γ-^{32}P]ATP (3000 Cimmol), using T4 polynucleotide kinase. Unincorporated nucleotides were removed by column chromatography on a Sephadex G50 Nick Column (Pharmacia Biotech). Nuclear extracts (4 μg) were incubated with 5 μl of 4× binding buffer (BB) (100 mM HEPES (pH 7.9), 600 mM KCl, 40% glycerol, 1 μg of poly (dI-dC), 5 mM dithiothreithol) and water up to 20 μl for 10 min at room temperature (RT). 50,000 cpm of ^{32}P-labeled oligonucleotides in the absence or presence of 100-fold excess of nonlabeled specific and nonspecific oligonucleotides was added and incubated for further 15 min at RT. Protein-DNA complex interactions were analyzed on 6% polyacrylamide Tris–glycine–EDTA (TGE) gels in 1× TGE buffer containing 40 mM Tris (pH 8.5), 200 mM glycin, and 1 mM EDTA.

The following oligos were used for analysis of NF-κB DNA binding activity by EMSA: (i) NF-κB, classical binding site (Igκ enhancer), (35), (5′) AGT TGA GGG GAC TTT CCC AGG C (3′); (ii) NF-κB binding site in IL-2 promoter, (36), (5′)AAG AGG GAT TTC ACC TAA AT (3′); (iii) CD28RE in IL-2 promoter (36), (5′) GGG GGT TTA AAG AAA TTC C (3′); (iv) Nonspecific competitor (NF-1 binding site): (5′) TTT TGG ATT GAA GCC AAT ATG ATA A (3′).

Western blotting. Twenty micrograms of nuclear or whole cell extract in SDS sample buffer was denatured for 3 min in boiling water. Samples were separated on an 8% SDS–polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane, using an electroblot apparatus. The protein concentration in cell extracts was quantitated spectrophotometrically with the Bio-Rad protein assay kit. To ensure equal loading and transfer of the protein in each well, the membrane were stained with Ponceau S (Sigma). Membranes

were blocked with 5% non-fat dry milk in PBS for 2 h at RT and then incubated with specific anti-serum in PBS containing 1% non-fat dry milk plus 0.05% Tween 20 in PBS for 1 h at RT. After washing 3 × 10 min with 0.05% Tween 20 in PBS, the membranes were incubated with a horseradish peroxidase-labeled secondary anti-serum and washed as explained above. Membranes were then stained with the ECL Western blotting detection system (Amersham) according to the manufacturer's recommendations.

Immunoprecipitation. Forty micrograms of cell extracts from normal and transgenic thymocytes was incubated with 5 μg antibodies to RelA or p50 for 1 h on ice. Then 25 μl of 50% (vv) protein A Sepharose in a buffer containing 50 mM Tris buffer, pH 7.0, 0.5 mM phenylmethylsulfunyl fluride, 5 μg/ml leupeptin, 1 μg/ml aprotonin was added. The tubes were rotated for further 1 h at 4°C. The pellets were washed 4 times with cold PBS and were resuspended in 2× SDS buffer and boiled for 4 min.

Corticosterone sensitivity. Thymi were removed from DO11.10 TCR$^+$ and DO11.10 TCR$^-$ mice. Single cell suspensions were prepared by passage through a steel mesh, washed in PBS and counted in trypan blue solution. Incubations were performed in duplicate, in 24-well plates with 10×10^6 cell per well in RPMI 1640 medium supplemented with 0.05% BSA and incubated at 37°C, 5% CO_2 for 15 h, in the absence or presence of 0.01, 0.1, or 1 μM corticosterone. After incubation, viability of the cells was determined by trypan blue staining. Treated cells were also stained with antibodies against the CD4 and CD8 molecules, and were analyzed for the percentage of living DP cells.

Flow cytometry. Thymocytes were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, biotin conjugated anti-CTLA-4 (followed by avidin-FITC), FITC-conjugated anti-CD28, and FITC-conjugated anti-LFA-1 mAbs and washed with cold PBS. 10,000 viable cells were analyzed by flow cytometry in a FACSscan (Becton Dickinson) using Cell Quest software. Viable cells were identified by gating on forward and side scatter.

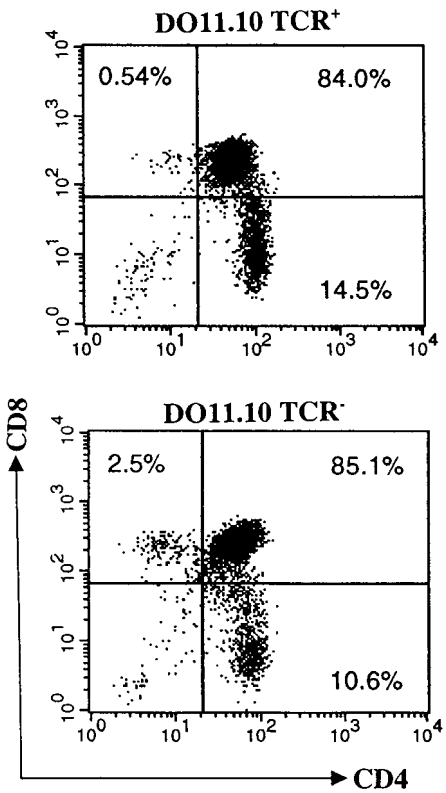

FIG. 1. Selection of CD4 SP cells in the thymus of DO11.10 TCR$^+$ mice. Freshly isolated DO11.10 TCR$^+$ and DO11.10 TCR$^-$ thymocytes were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 and analyzed by FACS.

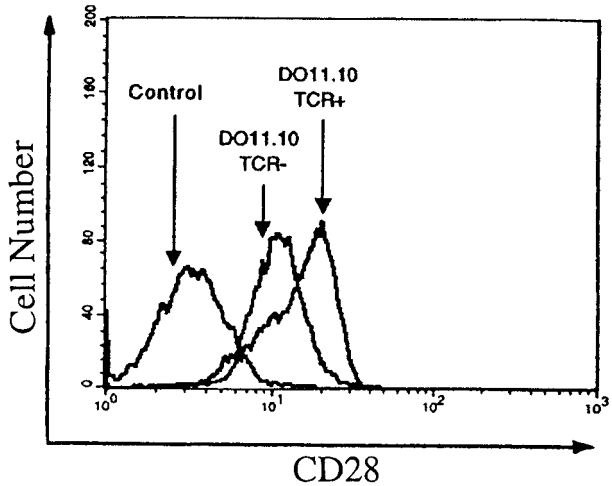

FIG. 2. Upregulation of CD28 in the thymocytes of DO11.10 TCR$^+$ mice. Freshly isolated DO11.10 TCR$^+$ and DO11.10 TCR$^-$ thymocytes were stained with FITC-conjugated anti-CD28 and analyzed by FACS. Control cells are nonstained cells.

RESULTS

Phenotype of DO11.10 TCR$^+$ Thymocytes

Thymocytes were first stained for CD4 and CD8 molecules to investigate the effect of the DO11.10 TCR transgene on different thymocyte subpopulations. As can be seen in Fig. 1, DO11.10 TCR$^+$ mice allowed for maturation of mainly CD4$^+$ cells (Fig. 1). The dominating CD4CD8 DP population had a similar size in both TCR transgenic and control mice, as has been earlier reported (37).

DO11.10 TCR$^+$ thymocytes were then phenotyped for expression of cell surface receptors, which might be associated with GC resistance including CD28, CTLA-4 (38) and LFA-1 (39). We found an increased expression of CD28 on transgenic thymocytes (Fig. 2), but no difference in the expression of CTLA-4 and LFA-1 between DO11.10 TCR$^+$ and DO11.10 TCR$^-$ thymocytes (data not shown). The increased CD28 expression occurred on the major DP cell population, as verified with CD8/CD28 staining of transgenic thymocytes (data not shown).

Decreased Glucocorticoid Sensitivity, GR Expression, and Activation in DO11.10 TCR$^+$ Thymocytes

Due to the potential importance of GC in thymocyte development, we compared the GC sensitivity between DO11.10 TCR$^+$ and DO11.10 TCR$^-$ thymocytes. Cells were exposed to increasing concentration of CS for 15 h

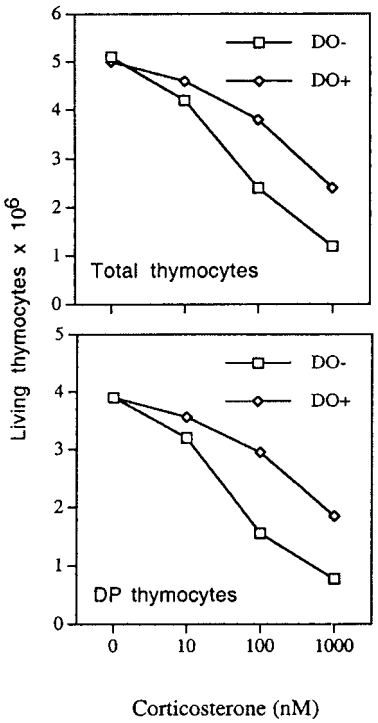

FIG. 3. GC sensitivity of DO11.10 TCR$^-$ and DO11.10 TCR$^+$ thymocytes. Cells were incubated for 15 h in the absence or presence of 0.01, 0.1, or 1 μM corticosterone. After incubation, viability of the cells was determined by trypan blue staining. Treated cells were harvested and stained with anti-CD4–FITC and anti-CD8–PE, and 10,000 viable cells were analyzed by flow cytometry for the percentage of living DP cells. Total number of living DP cells was calculated by multiplying the percentage of living DP cells by total number of living thymocytes.

in vitro. DO11.10 TCR$^+$ cells were clearly less sensitive, with almost one order of magnitude difference at physiological concentrations of the hormone (Fig. 3, upper panel). The decreased CS sensitivity of transgenic thymocytes was a property of DP cells as a similar shift in CS sensitivity in the DP population was seen (Fig. 3, lower panel). In order to further investigate the molecular mechanism behind this impaired GC response, we measured the nuclear level of GR, representing activated GR, as well as the total cellular amount of GR using whole cell protein extracts by Western blotting. Our results demonstrated a decreased nuclear level of GR, as well as reduced total cellular GR expression in whole DO11.10 TCR$^+$ thymocytes (Fig. 4A). Identical results were obtained when the nuclear extracts from isolated DP populations were analysed (Fig. 4B).

NF-κB Activation in the DO11.10 TCR$^+$ Thymocytes

NF-κB proteins play a crucial role in T cell activation and cytokine production. The importance of these transcription factors in thymocyte development has also been demonstrated in several reports (40, 41), and it has recently been shown that NF-κB induction prevents GC-induced apoptosis in thymocytes (42). In order to investigate the NF-κB activity in DO11.10 TCR$^+$ transgenic thymocytes, nuclear protein extracts from freshly isolated thymocytes were subjected to EMSA, using an oligonucleotide probe containing a classical NF-κB/Rel DNA-binding sequence. As was reported earlier, thymocytes were found to express constitutive NF-κB/Rel activity (42, 43). In the DO11.10 TCR$^+$ transgenic thymocytes an increase in the intensity of the slowly migrating NF-κB/Rel complex can be seen, as compared to DO11.10 TCR$^-$ thymocytes (Fig. 5A). In order to further characterize the NF-κB activity, the levels of different NF-κB subunits in the nuclear extracts of DO11.10 TCR$^+$ and DO11.10 TCR$^-$ thymocytes were compared. Our results showed a considerable increase in the nuclear RelA, a slight increase in the nuclear c-Rel and a decrease in the nuclear RelB levels in the thymocytes of DO11.10 TCR$^+$ transgenic mice (Fig. 5B). The considerable increase of nuclear RelA was verified in isolated DP cells (Fig. 5C). The nuclear levels of p50 in transgenic and nontransgenic thymocytes were not significantly altered.

The IL-2 promoter contains binding sites for NF-κB/Rel and the CD28 responsive complex (CD28RC). The latter also belongs to the NF-κB/Rel family of proteins (29–31). It has been demonstrated that stimulation of CD28 results in an increase in the nuclear levels of NF-κB/Rel proteins in T lymphocytes pretreated with anti-CD3, and in thymocytes treated with anti-TCR or anti-CD3 (44). This activation leads to IL-2 gene expression and activation of T cells. In order to investigate the possible contribution of CD28 upregulation, as seen in the transgenic thymocytes, with their increased NF-κB/Rel activity, we analyzed complex formation on the CD28RE (Fig. 6A) and the NF-κB/Rel binding site (Fig. 6B) from the IL-2 gene promoter. An increased binding to both the CD28RE and the NF-κB binding site was seen in the DO11.10 TCR$^+$, as compared to the DO11.10 TCR$^-$ thymocytes. This supports an increased IL-2 gene activation.

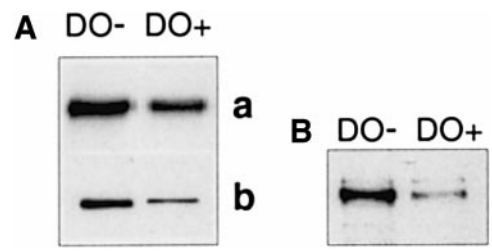

FIG. 4. Western blot analysis of GR in DO11.10 TCR$^-$ (DO−) and DO11.10 TCR$^+$ (DO+) thymocytes. Whole cell (a) and nuclear protein extract (b) from total thymocytes (A), and nuclear protein extract from the DP thymocytes (B), were analysed by Western blotting. The protein extracts from thymocytes were separated in SDS–PAGE gels and transferred to nitrocellulose membranes. Membranes were probed with specific antibodies against the mouse GR.

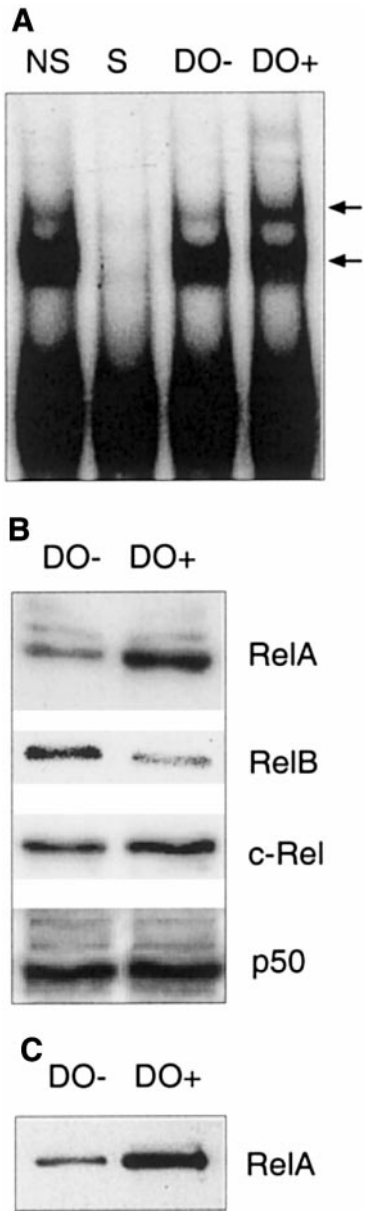

FIG. 5. EMSA for NF-κB binding activity and Western blot analysis of different NF-κB proteins. (A): NF-κB binding activity in DO11.10 TCR^- (DO−) and DO11.10 TCR^+ (DO+) thymocytes. 5 μg of nuclear extract from freshly isolated thymocytes was incubated with a ^{32}P-end-labeled oligomer containing a classical NF-κB binding site, and was analysed by EMSA. The arrows indicate the different NF-κB complexes formed. S, specific competitor; NS, nonspecific competitor. (B) Western blot analysis (as explained in the legend to Fig. 4) of nuclear protein extracts from DO11.10 TCR^- (DO−) and DO11.10 TCR^+ (DO+) thymocytes for different NF-κB proteins using specific antibodies against the RelA, RelB, c-Rel and p50 components of the NF-κB/Rel family of proteins, as shown in the figure. (C) Western blot analysis of nuclear protein extracts from DP thymocytes for RelA protein.

Direct Interaction between RelA and GR in Thymocytes

We and others have previously shown that a direct NF-κB/GR interaction leads to inhibition of the transcriptional activity of GR and NF-κB (16, 17). To determine whether a direct protein-protein interaction in normal and transgenic mice occurred, protein extracts were precipitated with antibodies against RelA or p50. The precipitates were then separated on a denaturing gel, blotted and finally probed with antibody against GR. We could demonstrate a direct binding between GR and RelA but not between GR and p50 in thymocytes (Fig. 7). The amount of GR precipitated from DO11.10 TCR^- and DO11.10 TCR^+ transgenic extracts appeared similar. However, this result has to be interpreted in relation to a decreased level of GR, but increased level of RelA in DO11.10 TCR^+ as compared to DO11.10 TCR^- thymocytes. Thus, it is likely that in transgenic cells a higher fraction of GR is involved in NF-κB/GR interaction, which might contribute to the decreased GC sensitivity in these cells.

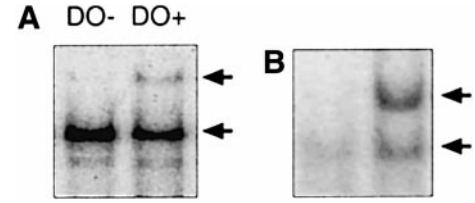

FIG. 6. EMSA for CD28 response complex and NF-κB binding activity to the mouse IL-2 promoter. Five micrograms of nuclear extract from freshly isolated DO11.10 TCR^- (DO−) and DO11.10 TCR^+ (DO+) thymocytes was incubated with a ^{32}P-end-labeled oligomer containing the NF-κB binding site (A) or CD28RE (B) from the mouse IL-2 promoter, and analysed by EMSA. The arrows indicate the different NF-κB/Rel complexes formed.

DISCUSSION

In thymus, the majority of DP thymocytes undergo apoptosis due to lack of TCR binding to MHC molecules and subsequent signaling required for survival of these

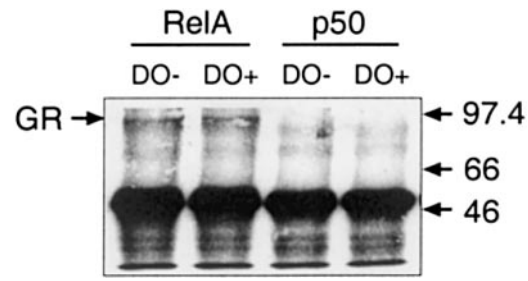

FIG. 7. GR-NF-κB interaction in DO11.10 TCR^- and DO11.10 TCR^+ thymocytes analysed by immunoprecipitation. Forty micrograms of whole cell protein extracts from DO11.10 TCR^- (DO−) and DO11.10 TCR^+ (DO+) thymocytes was immunoprecipitated with 5 μg of antibody to RelA or p50 using protein A Sepharose. The precipitates were separated on a denaturing gel, blotted and finally probed with an antibody against GR.

cells. It has been suggested that these ignored cells are removed by endogenous GC (3–5). In thymocytes a functional crosstalk between the TCR and GR has been reported (32). Stimulation of thymocytes via the TCR/CD3 complex or GR trigger apoptosis in these cells. However, simultaneous stimulation of TCR and GR results in protection of thymocytes from either apoptotic stimuli (32). TCR stimulation of thymocytes and DO11.10 T cell hybridoma has also been shown to rescue these cells from apoptosis induced by dexamethasone (33). Jamieson *et al.* have recently found that TCR mediated activation of the MEK/ERK cascade led to a reduced capacity of GR to mediate apoptosis and to an altered transcriptional activity by the receptor in T and thymocyte cell lines and in primary T cells (45). Expression of I-A^d restricted DO11.10 TCR transgene in the thymocytes leads to an increase in thymic cellularity and a decrease in thymic apoptosis (8). In the present report, we have found that expression of this TCR transgene render DP thymocytes comparatively resistant to CS. As an important factor determining cellular GC sensitivities is the intracellular GR concentration (15) we further investigated the GR levels in transgenic cells and in particular in the DP population and found that the GC resistance in these cells was associated with reduced levels of GR. Thus, the reduced GR expression in transgenic DP thymocytes may account for the relative GC resistance detected in these cells.

Gene expression induced via the TCR/CD3 is markedly enhanced by CD28-dependent signals. This is mediated by a specific element (CD28RE) that is related to an NF-κB site and is found in the promoters of the IL-2, IL-3, and GM-CSF genes (29, 46). Wagner *et al.* have shown that signaling through CD28 can rescue thymocytes from GC induce apoptosis (38). They have found that paraformaldehyde fixed B7 expressing cells protected thymocytes against killing by dexamethasone. This result directly shows that signaling through CD28 can generate intracellular signals which convert thymocytes to a relative state of GC resistance. Thus, the upregulation of CD28 that we detected on DO11.10 TCR transgenic thymocytes, may be of functional importance in the development GC resistance in these cells.

TCR stimulation leads to the rapid activation of tyrosine kinases that phosphorylate a variety of signal transducing proteins. These in turn activate signaling pathways, leading to induction of a number of transcription factors including NF-κB proteins (47). We have found that the NF-κB family members RelA and c-Rel are increased in DO11.10 TCR transgenic thymocytes. In thymocytes, both constitutive and inducible NF-κB complexes have been defined, with RelA and c-Rel activation being associated with thymocyte activation (43). NF-κB has also been shown to be important for T cell development as repression of NF-κB proteins by overexpression of IκBα in lymphocytes (40) and T cell lineage (41) resulted in impaired T cell development. Several reports have shown that NF-κB can prevent cell death, most likely by inducing the expression of anti-apoptotic genes (48–51). Interestingly, it has recently been shown that down-regulation of NF-κB in thymocytes preceded cell death, suggesting that NF-κB may be important for the survival of these cells. In addition, GC treatment of thymocytes accelerated p65/RelA down-regulation and cell death (42). Importantly, it was shown that NF-κB induction diminished GC-induced apoptosis in thymocytes (42). In DO11.10 TCR transgenic thymocytes, we found an upregulation of RelA and c-Rel. Both these transcription factors may thus contribute to GC resistance by crosstalk mechanisms that prevent apoptosis. RelA-c-Rel heterodimer binds to the CD28RE and synergistically activates the CD28RE enhancer activity (52). Increased binding to NF-κB site and CD28RE in IL-2 gene promoter may suggest an increased expression of IL-2 gene, which may also play a role in GC resistance, as IL-2 has been reported to reduce spontaneous or dexamethasone-induced apoptosis of thymocytes (53). We also observed decreased nuclear levels of RelB in transgenic thymocytes. In RelB deficient mice deletion of autoreactive thymocytes (negative selection) is inefficient (54, 55). Thus, the decreased RelB level may contribute to downregulation of thymic apoptosis that we observed in the thymocytes of DO11.10 TCR^+ mice (8).

We have previously shown that NF-κB/GR interaction impairs the transcriptional activity of GR, thus reducing the GC response (17). In line with such a possibility, we have detected a direct *in vivo* RelA-GR interaction in the thymocytes. The reduced GR expression in the I-A^d restricted DO11.10 TCR transgenic thymocytes can also explain why we did not find an increased amount of GR associated with RelA in transgenic thymocytes compared to control cells despite the higher RelA concentration in these cells. Thus, it is likely that the NF-κB/GR interaction involves higher percentage of GR in the transgenic thymocytes, which may contribute to the decreased GC sensitivity seen in these cells.

In summary, in DO11.10 TCR^+ DP thymocytes, we find an upregulation of CD28, which may be a consequence of TCR recognition of the MHC-II molecules during the development of these cells. TCR/CD28 corecognition of MHC-II/B7 molecules may then activate signaling cascades, leading to an alteration in the activation of NF-κB proteins. These combined effects may result in a reduced GR activity, either by a reduction in GR expression and/or direct inhibition of GR activity by NF-κB cross talk. These effects could account for the relative GC resistance observed in the DP DO11.10 TCR^+ thymocytes and may reflect an important mechanism protecting thymocytes during their

development. In addition, above described mechanisms could be important in the maintenance of the naive peripheral T cell pool, as GC levels in blood are significant and as naive T cells are known to require continuous MHC ligation for their survival (56).

ACKNOWLEDGMENTS

We thank Dr. Dennis Loh (Washington University, School of Medicine, St. Louis, MO) for providing us with DO11.10 mice. This work was supported by the Swedish Cancer Society (to S.O.), the Swedish Medical Research Council, and the Swedish Council for Work Life Research (to M.J. and S.O.).

REFERENCES

1. Anderson, G., Moore, N. C., Owen, J. J., and Jenkinson, E. J. (1996) Cellular interactions in thymocyte development. *Annu. Rev. Immunol.* **14,** 73–99.
2. Alam, S. M., Travers, P. J., Wung, J. L., Nasholds, W., Redpath, S., Jameson, S. C., and Gascoigne, N. R. (1996) T-cell-receptor affinity and thymocyte positive selection. *Nature* **381,** 616–620.
3. Pazirandeh, A., Xue, Y., Rafter, I., Sjovall, J., Jondal, M., and Okret, S. (1999) Paracrine glucocorticoid activity produced by mouse thymic epithelial cells. *FASEB J.* **13,** 893–901.
4. Jondal, M., Xue, Y., McConkey, D. J., and Okret, S. (1995) *Curr. Top. Microbiol. Immunol.,* 67–79.
5. Ashwell, J. D., Lu, F. W., and Vacchio, M. S. (2000) Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.* **18,** 309–345.
6. Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555–556.
7. Murphy, K. M., Heimberger, A. B., and Loh, D. Y. (1990) Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* **250,** 1720–1723.
8. Xue, Y., Chomez, P., Castanos-Velez, E., Biberfeld, P., Perlmann, T., and Jondal, M. (1997) Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression [published erratum appears in *Eur. J. Immunol.* **10,** 2748, 1997]. *Eur. J. Immunol.* **27,** 2048–2056.
9. Moore, N. C., Girdlestone, J., Anderson, G., Owen, J. J., and Jenkinson, E. J. (1995) Stimulation of thymocytes before and after positive selection results in the induction of different NF-kappa B/Rel protein complexes. *J. Immunol.* **155,** 4653–4660.
10. Cohen, J. J. (1992) Glucocorticoid-induced apoptosis in the thymus. *Semin. Immunol.* **4,** 363–369.
11. Punt, J. A., Havran, W., Abe, R., Sarin, A., and Singer, A. (1997) T cell receptor (TCR)-induced death of immature CD4+CD8+ thymocytes by two distinct mechanisms differing in their requirement for CD28 costimulation: Implications for negative selection in the thymus. *J. Exp. Med.* **186,** 1911–1922.
12. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889–895.
13. Knudsen, P. J., Dinarello, C. A., and Strom, T. B. (1987) Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U937 cells. *J. Immunol.* **139,** 4129–4134.
14. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schutz, G. (1998) DNA binding of the glucocorticoid receptor is not essential for survival [see comments]. *Cell* **93,** 531–541.
15. Okret, S., Dong, Y., Bronnegard, M., and Gustafsson, J. A. (1991) Regulation of glucocorticoid receptor expression. *Biochimie* **73,** 51–59.
16. Ray, A., and Prefontaine, K. E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA* **91,** 752–756.
17. Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J. A., and Van der Saag, P. T. (1995) Negative cross-talk between RelA and the glucocorticoid receptor: A possible mechanism for the anti-inflammatory action of glucocorticoids. *Mol. Endocrinol.* **9,** 401–412.
18. McKay, L. I., and Cidlowski, J. A. (1999) Molecular control of immune/inflammatory responses: Interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* **20,** 435–459.
19. Baeuerle, P. A., and Henkel, T. (1994) Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* **12,** 141–179.
20. Baldwin, A. S., Jr. (1996) The NF-kappa B and I kappa B proteins: New discoveries and insights. *Annu. Rev. Immunol.* **14,** 649–683.
21. Ghosh, S., May, M. J., and Kopp, B, a. E. (1998) NF-kB and REL proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.
22. Sha, W. C. (1998) Regulation of immune responses by NF-kappa B/Rel transcription factor. *J. Exp. Med.* **187,** 143–146.
23. Baeuerle, P. A., and Baltimore, D. (1988) I kappa B: A specific inhibitor of the NF-kappa B transcription factor. *Science* **242,** 540–546.
24. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., and Karin, M. (1995) Immunosuppression by glucocorticoids: Inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* **270,** 286–290.
25. Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., and Baldwin, A. S., Jr. (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* **270,** 283–286.
26. Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S., Jr. (1995) Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol. Cell. Biol.* **15,** 943–953.
27. Chambers, C. A., and Allison, J. P. (1997) Co-stimulation in T cell responses. *Curr. Opin. Immunol.* **9,** 396–404.
28. Lin, X., Cunningham, E. T., Jr., Mu, Y., Geleziunas, R., and Greene, W. C. (1999) The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and IkappaB kinases. *Immunity* **10,** 271–280.
29. Fraser, J. D., Irving, B. A., Crabtree, G. R., and Weiss, A. (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251,** 313–316.
30. Verweij, C. L., Geerts, M., and Aarden, L. A. (1991) Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-kB-like response element. *J. Biol. Chem.* **266,** 14179–14182.
31. Costello, R., Lipcey, C., Algarte, M., Cerdan, C., Baeuerle, P. A., Olive, D., and Imbert, J. (1993) Activation of primary human T-lymphocytes through CD2 plus CD28 adhesion molecules induces long-term nuclear expression of NF-kappa B. *Cell Growth Differ.* **4,** 329–339.
32. Ashwell, J. D., King, L. B., and Vacchio, M. S. (1996) Cross-talk between the T cell antigen receptor and the glucocorticoid receptor regulates thymocyte development. *Stem Cells* **14,** 490–500.

33. Iwata, M., Hanaoka, S., and Sato, K. (1991) Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3 complex: A possible in vitro model for positive selection of the T cell repertoire. *Eur. J. Immunol.* **21,** 643–648.

34. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17,** 6419.

35. Lenardo, M. J., and Baltimore, D. (1989) NF-kappa B: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58,** 227–229.

36. Chen, D., and Rothenberg, E. V. (1993) Molecular basis for developmental changes in interleukin-2 gene inducibility. *Mol. Cell. Biol.* **13,** 228–237.

37. Nakajima, H., and Leonard, W. J. (1999) Role of Bcl-2 in alpha beta T cell development in mice deficient in the common cytokine receptor gamma-chain: The requirement for Bcl-2 differs depending on the TCR/MHC affinity. *J. Immunol.* **162,** 782–790.

38. Wagner, D. H., Jr., Hagman, J., Linsley, P. S., Hodsdon, W., Freed, J. H., and Newell, M. K. (1996) Rescue of thymocytes from glucocorticoid-induced cell death mediated by CD28/CTLA-4 costimulatory interactions with B7-1B7-2. *J. Exp. Med.* **184,** 1631–1638.

39. Zhao, Y., and Iwata, M. (1995) Cross-linking of the TCR-CD3 complex with CD4, CD8 or LFA-1 induces an anti-apoptotic signal in thymocytes: the signal is canceled by FK506. *Int. Immunol.* **7,** 1387–1396.

40. Esslinger, C. W., Wilson, A., Sordat, B., Beermann, F., and Jongeneel, C. V. (1997) Abnormal T lymphocyte development induced by targeted overexpression of IkappaB alpha. *J. Immunol.* **158,** 5075–5078.

41. Boothby, M. R., Mora, A. L., Scherer, D. C., Brockman, J. A., and Ballard, D. W. (1997) Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)-kappaB. *J. Exp. Med.* **185,** 1897–1907.

42. Wang, W., Wykrzykowska, J., Johnson, T., Sen, R., and Sen, J. (1999) A NF-kappa B/c-myc-dependent survival pathway is targeted by corticosteroids in immature thymocytes. *J. Immunol.* **162,** 314–322.

43. Weih, F., Carrasco, D., and Bravo, R. (1994) Constitutive and inducible Rel/NF-kappa B activities in mouse thymus and spleen. *Oncogene* **9,** 3289–3297.

44. Ghosh, P., Tan, T. H., Rice, N. R., Sica, A., and Young, H. A. (1993) The interleukin 2 CD28-responsive complex contains at least three members of the NF kappa B family: c-Rel, p50, and p65. *Proc. Natl. Acad. Sci. USA* **90,** 1696–1700.

45. Jamieson, C. A., and Yamamoto, K. R. (2000) Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling. *Proc. Natl. Acad. Sci. USA* **97,** 7319–7324.

46. Maggirwar, S. B., Harhaj, E. W., and Sun, S. C. (1997) Regulation of the interleukin-2 CD28-responsive element by NF-ATp and various NF-kappaB/Rel transcription factors. *Mol. Cell. Biol.* **17,** 2605–2614.

47. Cantrell, D. (1996) T cell antigen receptor signal transduction pathways. *Annu. Rev. Immunol.* **14,** 259–274.

48. Beg, A. A., and Baltimore, D. (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* **274,** 782–784.

49. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) TNF- and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kappa B. *Science* **274,** 784–787.

50. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* **274,** 787–789.

51. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsura, M., Katz, D., FitzGerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. (1996) Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *EMBO J.* **15,** 4682–4690.

52. Lai, J. H., Horvath, G., Subleski, J., Bruder, J., Ghosh, P., and Tan, T. H. (1995) RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter. *Mol. Cell. Biol.* **15,** 4260–4271.

53. Kirsch, A. H., Mahmood, A. A., Endres, J., Bohra, L., Bonish, B., Weber, K., and Fox, D. A. (1999) Apoptosis of human T-cells: Induction by glucocorticoids or surface receptor ligation in vitro and ex vivo [published erratum appears in *J. Biol. Regul. Homeost. Agents* **13**(4), following 267, 1999] *J. Biol. Regul. Homeost. Agents* **13,** 80–89.

54. Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D., and Glimcher, L. H. (1996) Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* **383,** 81–85.

55. Naspetti, M., Aurrand-Lions, M., DeKoning, J., Malissen, M., Galland, F., Lo, D., and Naquet, P. (1997) Thymocytes and RelB-dependent medullary epithelial cells provide growth-promoting and organization signals, respectively, to thymic medullary stromal cells. *Eur. J. Immunol.* **27,** 1392–1397.

56. Kirberg, J., Berns, A., and von Boehmer, H. (1997) Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. *J. Exp. Med.* **186,** 1269–1275.