Modulation of pro- and anti-apoptotic molecules in double positive (CD4⁺CD8⁺) thymocytes following dexamethasone treatment

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Non-standard abbreviations:
Degs1, dihydroceramide desaturase, isoform 1;
Dex, dexamethasone;
DPT, CD4+CD8+ double positive thymocytes;
Dusp2, dual-specificity phosphatase 2;
Gapdh, glyceraldehyde-3-phosphate dehydrogenase;

GC, glucocorticoid hormone;

GO, Gene Ontology Consortium;

Gpr65, G-protein-coupled receptor 65;

Id3, Inhibitor of DNA binding 3;

Idh2, NADP⁺-dependent isocitrate dehydrogenase;

Il6st, glycoprotein 130;

Nedd4L, neural precursor cell expressed, developmentally down-regulated gene 4-like;

NF-κB, Nuclear factor κB;

Nr3c1, GC receptor;

Plzf, Promyelocytic leukemia zinc finger gene;

ROS, reactive oxygen species;

Rpt801, Regulated in development and damage response 1 gene;

Runx1, Runt related transcription factor 1;

SAM, Significance Analysis of Microarrays;

Sgpp1, sphingosine 1-phosphate phosphatase;

Socs1, Suppressor of cytokine signaling 1;

Tnfaip3, Tumor necrosis factor alfa induced protein 3;

Txnip, thioredoxin reductase inhibitor;

Txnrd1, thioredoxin reductase;

Ugcg, UDP-glucose ceramide glucosyltransferase;

Usp18, ubiquitin specific protease 18;

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ABSTRACT

Glucocorticoids play a role in regulation of T lymphocytes homeostasis and development. In particular, glucocorticoid treatment induces massive apoptosis of CD4⁺CD8⁺ double positive (DP) thymocytes. This effect is due to many mechanisms, mainly driven by modulation of gene transcription. To find out which genes are modulated, we analyzed DP thymocytes treated for 3 hours with dexamethasone (a synthetic glucocorticoid), by global gene expression profiling. Results indicate modulation of 163 genes, also confirmed by either RNAse protection assay or Real Time PCR. In particular, dexamethasone caused down-regulation of genes promoting DP thymocyte survival (e.g. Notch1, Suppressor of cytokine signaling 1-Socs1 and Inhibitor of DNA binding 3-Id3) or modulation of genes activating cell death through the ceramide pathway (UDP-glucose ceramide glucosyltransferase-Ugcg, sphingosine 1-phosphate phosphatase-Sgpp1, dihydroceramide desaturase, isoform 1-Degs1 and G-protein-coupled receptor 65-Gpr65) or through the mitochondrial machinery. Among the latter, there are Bcl-2 family members (Bim, Bfl-1, Bcl-xL and Bcl-xβ), genes involved in the control of redox status (thioredoxin reductase, thioredoxin reductase inhibitor-Txnip and NADP⁺-dependent isocitrate dehydrogenase-Idh2) and genes belonging to Tis11 family which are involved in mRNA stability. Our study suggests that dexamethasone treatment of DP thymocytes modulates several genes belonging to apoptosis-related systems that can contribute to their apoptosis.
INTRODUCTION

Regulation of T cell survival is important as a physiological mechanism involved in determining the development of the immune response. Moreover, it is well accepted that apoptosis plays a relevant role in the thymus where massive cell death occurs in continuous selection process (Vacchio and Ashwell, 2000; Jondal et al., 2004; Lepine et al., 2005). Among different signals and stimuli, glucocorticoid hormones (GCs) have been shown to regulate apoptosis of T cells and thymocytes (Kong et al., 2002) among which CD4⁺CD8⁺ double positive thymocytes (DPTs) are the major target of GC-induced apoptosis (Vacchio and Ashwell, 2000; Kong et al., 2002).

Apoptosis involves binding of GCs to their receptor, whose activation causes genomic and non-genomic effects (Goulding, 2004; Lepine et al., 2005). In thymocytes, modulation of gene transcription is crucial for the pro-apoptotic effect so that inhibition of mRNA or protein synthesis inhibits apoptosis (Distelhorst, 2002; Lepine et al., 2005). In the attempt to find out those genes whose modulation plays a crucial role in the GC-induced apoptosis, some studies have focused their attention only on a single gene modulated by GC treatment (such as Bim and Gpr65), whose over-expression has a pro-apoptotic effect (Tosa et al., 2003; Wang et al., 2003a). However, despite the valuable meaning of these studies, other studies using mice in which those genes were individually ablated, demonstrated that these single genes were dispensable for the GC-induced apoptosis (Bouillet et al., 1999; Radu et al., 2006). On the contrary, the forced over-expression of anti-apoptotic genes (such as Bcl-2 and Notch1) protects from GC-induced apoptosis and their elimination causes an increased sensitivity to GC-induced apoptosis (Izon et al., 2002; Ma et al., 1995), possibly suggesting that the balance between pro- and anti-apoptotic genes play a role in the response to GCs (network hypothesis).
In the very recent past, studies using microarray techniques focused on cell lines undergoing apoptosis following GC treatment. By this way, it was possible to study the GC effects following several hours and some days of treatment, identifying genes potently modulated by GCs. However, it is likely that cell lines respond differently from un-transformed cells to GC treatment.

Two more studies used microarray techniques to describe GC effects on primary culture of tumoral cells undergoing apoptosis, but reached quite different conclusions (Chauhan et al., 2002; Schmidt et al., 2005). Indeed, a meta-analysis of all microarray studies dealing with GC-induced apoptosis revealed that only few genes appeared in three or more systems (Schmidt et al., 2004), strongly suggesting that the effects of GC treatment are different, depending on differentiation and functional status of the cells, and level of expression of other transcription factors and apoptosis regulators, so that different apoptotic genes are important in a cell sub-population but not in others.

For these reasons, we studied the modulation of gene transcription following GC treatment using exclusively DPTs, a prototype of GC sensitive cells. In particular, in this study we show the results of a global gene expression profiling of DPTs undergone to short-time treatment with Dex, a synthetic GC. Upon statistical validation, data bank queries and evaluation of known gene functions in T lymphocytes, we were able to group modulated genes in 5 systems that could cooperate in the induction of apoptosis in DPTs.
MATERIALS AND METHODS

**CD4^+CD8^+ double positive thymocyte (DPT) purification.**

Thymi from 4 week old C3H/HeN mice were teased in culture medium (RPMI supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin, 10 mM Hepes, 0.1% non-essential amino acids, 1 mM sodium piruvate and 50 μM 2-ME). After staining cells with FITC-conjugated anti-CD8 mAb (BD Pharmingen) and MACS MultiSort anti-FITC microbeads (Miltenyi Biotech), CD8^+ cells were isolated by using the MidiMACS LS^+ positive selection column. Before elution of fixed cells, columns were washed twice. To wash away microbeads from selected cells, eluted cells were incubated with MACS MultiSort release reagent (Miltenyi Biotec) following manufacturer’s instructions. Then, CD8^+ cells were stained with anti-CD4 microbeads and passed through a MidiMACS LS^+ positive selection column as above described. DPTs were considered pure population when cells resulted >98% CD4^+CD8^+ cells upon flow cytometry.

**Dex treatment and apoptosis analysis.**

DPTs were plated in 6 well plates in 3 ml of the culture medium at a concentration of 5x10^6 cell/ml together with 10^-7 M Dex or a corresponding volume of PBS. DPTs were kept at 37°C, 5% CO₂ in a humidified atmosphere for 1, 3, 6 or 9 h. Apoptosis was analysed with FacScan flow cytometry (Becton Dickinson) and LYSIS II software after treating cells with hypotonic propidium iodide (Cifone et al., 1999). Phenotyping of treated thymocytes was performed by analysed with FacScan flow cytometry (Becton Dickinson) and LYSIS II software after staining with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAb (BD Pharmingen).
Preparation of complementary RNA (cRNA) and chip hybridisation.

The cRNA from 3 h (medium or Dex) treated DPTs, to be used in hybridisation of GeneChip (Affymetrix), was synthesized starting from total RNA. Briefly, preparation includes: synthesis of double-stranded DNA, purification of double-stranded DNA, synthesis of full-length cRNA, non-enzymatic fragmentation of cRNA.

Total RNA was isolated with TRIzol LS reagent (Invitrogen, Life Technologies), following the manufacturer’s instructions.

Double-stranded DNA was synthesized with the GIBCO/BRL Superscript Choice System kit (GIBCO/BRL, 18090-019). Briefly, total RNA was used in a reverse transcription reaction to synthesize cDNA with a primer containing 24 thimidine residues and T7 RNA polymerase promoter sequences (T7-dT). T7-dT primer (1 µl) was added to 10 µg total RNA and DEPC-H2O to a final volume of 11 µl. Then samples were heated for 10 min at 70°C, spun and kept on ice. A second mix (5x first strand cDNA buffer, 4 µl; 0.1 M DTT, 2 µl and 10 mM dNTP mix, 1 µl) was added to the sample and then heated at 42°C for 2 min. After spinning, 2 µl of SSII RT enzyme were added and samples (20 µl final volume) were kept at 42°C for 1 h. To synthesize double-stranded cDNA, 150 µl of a third mix (DEPC-H2O, 91 µl; 5x second strand reaction buffer, 30 µl; 10 mM dNTP mix, 3 µl; 10 U/µl DNA ligase, 1 µl; 10 U/µl DNA polymerase I, 1 µl; 2 U/µl RNase H, 1 µl) were added and samples were incubated for 2 h at 16°C. After the addition of 10 U T4 DNA polymerase (2 µl) incubation was prolonged for further 5 min. Finally, reaction was stopped by adding 10 µl of 0.5 M EDTA.

Resulting double strand cDNA was cleaned up with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated upon centrifugation (20 min) using 0.5 volumes of 7.5 M NH4Ac and 2.5 volumes of 100% ethanol. Pellet was washed with ice-cold 80% ethanol (500 µl). Dry pellet was resuspended in 12 µl of DEPC-H2O. cDNA was checked for appropriate length through agarose gel electrophoresis.
Synthesis of cRNA was performed with Bioarray High Yield RNA Transcription Kit (ENZO Diagnostics). At first we made a mix containing: of 10x HY reaction buffer, 4 µl; 10x Biotin labeled ribonucleotides, 4 µl; 10x DTT, 4 µl; 10x RNase inhibitor mix, 4 µl; 20x T7 RNA polymerase, 2 µl; DEPC-H₂O, 17 µl. The mix was added to 5 µl double stranded cDNA (40 µl final volume) and samples were heated at 37°C for 6 h. Then RNAeasy spin columns (Qiagen) were used to clean up cRNA following the manufacturer’s instructions and cRNA was first checked for quality through agarose gel electrophoresis and then quantified at the spectrophotometer. The quantification of cRNA was adjusted with the rule: adjusted cRNA yield = microgram of cRNA measured – 4.2.

The non-enzymatic fragmentation of cRNA was performed by mixing 8 µl of 5x fragmentation buffer (1 M Tris acetate, pH 8.1, 2.5 µl; 256 mg MgOAc; 392 mg KOAc; final volume 8 µl with DEPC-H₂O) with 12 µl of DEPC-H₂O and 20 µl of cRNA (1 µg/µl) and incubation at 94°C for 35 min.

Hybridization of 5 µg of cRNA, appropriate washing and reading of MGU74Av2 GeneChip was performed using Affymetrix devices following manufacturers’ instructions in the Biopolio laboratories (Milan). In the same laboratories, the data from GeneChip reading were processed using MAS 5.0 software in excell sheets with expression and modulation data.

**Data and statistical analysis.**

Two independent cRNA from 3 h cultured DPTs (sample A and B) and 2 from 3 h 10⁻⁷ M Dex-treated DPTs (sample C and D) were analysed using MAS 5.0 software (Affymetrix) and Significance Analysis of Microarrays (SAM; Stanford University, Stanford, CA)(Tusher et al., 2001). Only genes resulting modulated in both methods were considered. Moreover, a cut-off of 1.7 fold for up-regulation and of 1.4 fold for down-regulation was adopted. A detail of statistical analysis is reported below.
Through MAS 5.0 Software, upon scaling, sample A was compared to sample C (comparison 1), sample B to C (comparison 2), sample A to D (comparison 3) and sample B to D (comparison 4) in the Biopolo laboratories (Milan). For each comparison, the software provides the fold change between untreated and treated samples (expressed as logarithm on 2 basis – log2) and the absolute difference (I= increase, MI= marginal increase, NC= no change, MD= marginal decrease and D=decrease). We considered modulated the probe sets (i.e. genes) concordant (only I+MI or D+MD) with NC label no more than once in the 4 comparisons. Such method gave 506 modulated genes (see supplemental data Figure 1s).

Through SAM Software, upon scaling, the expression data (called “average difference” by MAS 5.0 software) of each probe set in each array (A, B, C and D) were considered. As input information, it was specified that A and B expression data derived from untreated samples and C and D expression data from Dex-treated samples. Comparison was performed using the two classes unpaired data analysis, choosing a Δ of 0.88 (corresponding to a false discovery rate-FDR of 30.7%). As a consequence of the high FDR, probe sets resulting modulated were 2013 (see supplemental data Figure 1s). However, the SAM analysis was useful to discard 121 probe sets accepted by MAS 5.0 analysis (Figure 1s).

To evaluate the value of the increased or decreased expression, the mean of the fold modulation (in the four comparisons) deriving from MAS 5.0 software expressed as log2 was calculated. Then the antilog of the absolute log2 value was calculated. To further decrease the false positive rate and discard probe sets whose modulation was too little to represent a modulation with a biological meaning, only probe sets with a mean increase change equal or higher than 1.7 fold or a mean decrease change equal or higher than 1.4 fold were considered for further analysis. A lower cut-off for decreased gene expression was chosen considering the short incubation time and the half-live of several mRNA species, far beyond 3 h. For example, a gene with a mRNA half-life of 6 h and whose transcription has been completely inhibited by GC treatment shows a theoretic decreased expression of about 1.4 fold. On the
contrary, increased expression is much less dependent on mRNA half-life. Our reasoning is somehow confirmed by data we obtained. In fact, probe sets with an increased expression equal or more than 2 fold were 51 while probe sets with a decreased expression equal or more than 2 fold were only 10. The use of fold change cut-off discarded further 209 probe sets out of 385 probe sets resulting modulated.

Gominer software (Zeeberg et al., 2003) was used to investigate the meaning of gene resulting modulated and participating to a similar function or cell structure. All gene information were related with each other using a compiled perl program.

**Real Time PCR**

Total RNA was isolated with TRIzol LS reagent (Invitrogen, Life Technologies), following the manufacturer’s instruction. cDNA was prepared by using 2.5 µg M-MLV RT (Invitrogen, Life Technologies) and, at the end of the reaction, 80 µl of water were added.

Real Time PCR was performed in a Chromo4 thermal cycler (Biorad). Briefly, investigated gene and the house-keeping gene Gapdh were amplified in the same tube in the presence of a FAM labelled TaqMan probe (investigated gene) and a VIC labelled TaqMan probe (Gapdh). Primers, probes and master mix were purchased from Applied Biosystems (TaqMan Gene expression Assay). Reaction was performed in 20 µl following the manufacturer’s instructions. To minimize variability, every time point was investigated with 4 replicates and the amplification of 2 independent treatments was performed. The $\Delta\Delta^Ct$ method was used to determine modulation of the genes of interest (Livak and Schmittgen, 2001).

**RNase Protection Assay (RPA)**

To perform RPA the following probes were prepared: Bcl-x (U10101, 105-504 bp), Bim (NM_207680, 611-797 bp), Bfl-1 (NM_009742, 198-435 bp), Bcl-x to investigate Bcl-x alternative isoforms (U51279, 445-919 bp). Moreover, probe for the house
keeping gene Gapdh (Ambion) was used. DNA-free RNA was prepared according to 
the manufacturer’s instructions (Ambion). RPA was performed using the RPAIII kit 
(Ambion) as previously described (Vecchini et al., 2005). Cpm s from protected 
fragment and the picture presented in Figure 3 were obtained using Instant Imager 
autoradiography system (Packard BioScience).
RESULTS

**Dex modulates the expression of 163 genes in CD4^+CD8^+ thymocytes (DPTs) within the first 3 hours of contact.**

Thymocytes are very sensitive to GC treatment and DPTs are the most sensitive thymus sub-population to GC-induced apoptosis (Vacchio and Ashwell, 2000; Jondal et al., 2004). In fact, following in vitro Dex treatment, the percentage of DPTs decreased, whereas percentage of the other populations showed a relative increase (Figure 1A). Therefore, to study the pro-apoptotic effect of Dex, we used DPTs.

It is well known that Dex-induced apoptosis requires gene transcription and protein synthesis (Distelhorst, 2002; Lepine et al., 2005). In fact, when an inhibitor of RNA synthesis (actinomycin D) was added to the thymocyte culture together with Dex, apoptosis was completely prevented (Figure 1B). Apoptosis inhibition occurred when actinomycin D (Act D) was added 2 but not 3 h after the addition of Dex (Figure 1B), suggesting most of the mRNA expression sufficient for the induction of apoptosis is regulated within the first 3 h of Dex-cell contact. Therefore, we studied DPTs treated for 3 h with Dex comparing gene expression of Dex- and medium-treated DPTs.

Gene expression of DPTs was evaluated by using Affymetrix GeneChip microarray. Data were analyzed by using both the output of the MAS 5.0 software and by comparing the expression data through SAM; the probe sets (i.e. genes and gene families) selected by both analyses were considered modulated. By this criteria, 385 probe sets resulted to be significantly modulated (Figure 1s and Table 1s in Supplemental data). However, when a cut-off for up-regulation was set to 1.7 factor and for down-regulation to 1.4 factor, only 176 probe sets (corresponding to 163 genes) resulted to be regulated, among which 59 up-regulated and 104 down-regulated (Table 2s in Supplemental data). Presently, for 126 of those genes a biological meaning has been conferred by the Gene Ontology Consortium, as suggested by the assignment of 1
or more Gene Ontology identification number (GO ID). On the basis of GO ID, the modulated genes can be grouped for their presence in one or more cellular components (Table 1), as having one or more molecular functions (Table 2) and as participating to one or more biological processes (Table 3). By interrogating GO database through GoMiner software, we evaluated how many genes, belonging to each GO ID, were modulated by Dex in comparison to all genes which were included in the same GO ID and were investigated with the GeneChip we used. Then, we evaluated if the number of genes modulated in each GO ID was above the expected value and calculated the relative enrichment as shown in Tables 1, 2 and 3. In particular, genes present in each cellular component were modulated by Dex treatment with a certain preference for cytoskeletal proteins and proteins localized in the nucleoplasm and nuclear membrane (Table 1). Surprisingly, mitochondrial proteins were less frequently modulated. Furthermore, Dex modulated preferentially the expression of genes encoding enzymes (including those with oxidoreductase activity), transporter with particular reference to the solute carrier family members, protein characterized by regulatory activity and transcription factors (Table 2). In addition, Dex modulated genes involved in steroid metabolism, ion transport, microtubule-based processes, protein processing, signaling and transcription regulation (Table 3). As a consequence, genes involved in cell proliferation, development, differentiation and apoptosis resulted widely modulated (Table 3). Our attention was focused on genes whose modulation contributes to explain the pro-apoptotic effect of Dex in DPTs, chosen on the basis of GO ID and on the function of modulated genes in T cells and thymocytes. By this way, we identified 25 genes belonging to apoptosis-related systems, active in GC-treated DPTs and potentially responsible for GC-induced apoptosis (Table 4).
Up-regulation of genes with pro-apoptotic function and down-regulation of genes with a protective activity.

Alteration in the balance of gene expression of the Bcl-2 family members. The Bcl-2 family members are crucial for cell survival and death in several tissues. In DPTs, Bcl-xL and Bim are the main players since Bcl-xL is the dominant pro-survival member (Ma et al., 1995) and Bim is required for apoptosis of auto-reactive thymocytes (Bouillet et al., 2002).

In Dex-treated DPTs, we found Bim and Bcl-x gene expression significantly up- and down-regulated, respectively. Moreover, Bfl-1/A1 was also slightly but significantly down-regulated (1.2 fold, under the cut-off)(Table 4). To confirm these results, we performed an RNAse protection assay on Dex-treated DPTs (1-6 h) using Bim, Bcl-x and Bfl1/A1 probes. For each probe, the ratio between the values expressed as count per min of the fragment protected by the chosen probe and that protected by glyceraldehyde-3-phosphate dehydrogenase (Gapdh), a house keeping gene that is not modulated in our system, was compared to untreated control. Results shown in Figure 2 demonstrate that, upon Dex treatment, level of expression of Bim powerfully increases, while levels of Bcl-xL and Bfl1/A1 decrease significantly, confirming the GeneChip results.

It is known that Bcl-x gene is expressed in 5 isoforms, 3 of which (Bcl-xL, Bcl-xγ and Bcl-xΔTM) are anti-apoptotic and 2 (Bcl-xS and Bcl-xβ) are pro-apoptotic (Yang et al., 1997). Since we found that in DPTs, level of Bcl-xβ expression is quite high while that of Bcl-xS is low (not shown), we investigated the modulation of Bcl-xβ and the anti-apoptotic Bcl-x isoforms by RNAse protection assay, using a probe which detects the anti-apoptotic splice variants into one single fragment and Bcl-xβ into another one. Then, the ratio between the protected fragment of Bcl-xβ and anti-apoptotic splice
variants was evaluated in untreated and Dex-treated samples. Figure 3 shows that the ratio Bcl-xβ/anti-apoptotic splice variants after 1-6 h of Dex treatment increases. Thus, Dex modulates both Bcl-x gene and splice variant expression.

**Alteration in expression of redox status regulating genes.** One of the mechanisms responsible for GC-induced death of thymocytes has been identified in increased presence of reactive oxygen species (ROS) (Lepine et al., 2005). According to our study, 3 genes participating to the control of ROS are modulated in Dex-treated DPTs thus potentially contributing to increased ROS presence. In particular, expression of thioredoxin reductase (Txnrd1) and NADP+-dependent isocitrate dehydrogenase (Idh2) decreased and expression of Txnrd1 inhibitor (Txnip) increased (2.1 fold)(Table 4).

To confirm data from GeneChip array, we performed Real Time PCR of selected genes, such as Txnrd1 and Txnip, evaluating also different treatment time points (1, 3, 6 and 9 h). For normalization of reactions and RNA quality, we used the house-keeping gene Gapdh that was amplified contemporary to the investigated gene. Real Time PCR confirmed the regulation of both Txnrd1 and Txnip. Txnrd1 reached the maximum decrease level after 6 h of Dex treatment (Figure 4). On the contrary, Txnip kept on increasing, but if we consider that even medium treatment increased the expression of Txnip, the maximum effect of Dex is seen after 3 h (Figure 4). The role in the redox imbalance of Txnrd1, Txnip and Idh2 is summarized in Figure 5.

**Alteration in expression of sphingomyelin pathway genes.** It is well known that ceramide and sphingosine are involved in the GC-induced apoptosis of thymocytes (Cifone et al., 1999; Lepine et al., 2004; Lepine et al., 2005). We found 3 enzymes participating in the sphingomyelin pathway are modulated in Dex-treated DPTs thus potentially contributing to an increased level of ceramide and sphingosine. In particular,
UDP-glucose ceramide glucosyltransferase (Ugcg), that decreases the intracellular ceramide levels via glycosylation of ceramide, was down-regulated by a 1.9 factor (Table 4). Real Time PCR demonstrated that level of Ugcg increased during Dex treatment (Figure 4). However, since Ugcg expression increased even more in untreated DPTs during medium treatment, the overall effect of Dex treatment is Ugcg down-regulation (Figure 4).

Expression of sphingosine 1-phosphate phosphatase (Sgpp1), an enzyme catalyzing the transformation of sphingosine-1-phosphate (a lipid with anti-apoptotic activity) in sphingosine (Lepine et al., 2005), was increased by a 1.8 factor. Real Time PCR shown in Figure 4 demonstrates that increased mRNA levels of Sgpp1 was evident already after 1 h treatment and reached more than 8 fold-increase at 9 h time point. The increased intracellular concentration of ceramide seen during thymocyte apoptosis may be also favored by the significant but slight up-regulation (1.4 fold, under the cut-off) of dihydroceramide desaturase, isoform 1 (Degs1) participating to the de novo synthesis of ceramide (Table 4).

Finally, we found an increased expression of G-protein-coupled receptor 65 (Gpr65 or Tdag8), a proton-sensing and lysolipid-sensitive receptor (Radu et al., 2006), that may contribute to the apoptosis caused by the activation of ceramide pathway. In fact, its ligand psycosine is a sphingosine metabolite. The role of Ugcg, Sgpp1, Degs1 and Gpr65 in favoring the pro-apoptotic activity of the ceramide pathway products is summarized in Figure 5.

**Modulation of genes involved in the control of mRNA stability and protein synthesis.**

The immediate early protein Tis11d (Zfp36l2) is a member of Tis11 family, responsible for apoptotic cell death that occurs starting from the mitochondrial death machinery and involved in mRNA destabilization (Ciais et al., 2004). Following Dex treatment,
Tis11d was up-regulated 2.9 or 6.1 fold depending on the set probe considered (Table 4). Real Time PCR shown in Figure 4 demonstrates that the increased mRNA levels of Tis11d was already evident after 1 h of treatment and kept on increasing during the treatment. The overall effect of Dex is even higher if we consider that Tis11d is down-regulated following incubation with medium alone. On the contrary, Tis11 is slightly down-regulated as confirmed by the overall effect of Dex investigated by Real Time PCR (Figure 4).

The Regulated in development and damage response 1 gene (Rpt801, Ddit4 or Redd1) is an inhibitor of mTOR, a serine/threonine kinase working as a central regulator of protein synthesis (Brugarolas et al., 2004). Rpt801 was up-regulated in treated DPTs (Table 4) and in other T cells (Wang et al., 2003b). Functional meaning of Rpt801 up-regulation is uncertain. In fact, while its up-regulation upon stresses such as hypoxia and GC-treatment makes likely the conclusion that RPT801 is a pro-apoptotic gene (Brugarolas et al., 2004), other data suggest that it may play an anti-apoptotic role (Schwarzer et al., 2005).

**Regulation of genes involved in maturation of DPTs.** Several genes participating to maturation and differentiation of DPTs demonstrated a decreased expression in Dex-treated DPTs (Table 4). Among them, we found: Runt related transcription factor 1 (Runx1 or AML1), whose functional knocking makes thymocytes more sensitive to anti-CD3-induced apoptosis; the transcription factor Ets2, whose functional knocking makes thymocytes more sensitive to glucocorticoid-induced apoptosis; the Suppressor of cytokine signaling 1 (Socs1), whose lack determines a decreased development and an increased apoptosis of thymocytes (Starr et al., 1998). Moreover, glucocorticoid treatment may indirectly decrease the activity of other anti-apoptotic genes (NF-κB, Inhibitor of DNA binding 3 and Notch1) as briefly summarised below.
The expression of Tumor necrosis factor alfa induced protein 3 (Tnfaip3 or A20), a specific inhibitor of NF-κB, increased 2 fold. As a consequence, the activity of NF-κB (a powerful anti-apoptotic factor) can decrease.

The expression of the Inhibitor of DNA binding 3 (Id3), a repressor transcription factor crucial in thymocyte development and fate (Rivera et al., 2000), decreased. Real Time PCR demonstrated that the level of Id3 powerfully decreased after 1 h of contact and peaked after 3 h, reaching a 16 fold decrease (Figure 4) which is in line with, but quantitatively different to the value obtained with the GeneChip array (Table 4). Decreased expression of Id3 may be further amplified by the increased expression of dual-specificity phosphatase 2 (Dusp2, Pac1), a negative modulator of ERK pathway involved in the control of Id3 (Bain et al., 2001). Real Time PCR confirmed over-expression of Dusp2 that peaked at 6 h time point. Figure 5 summarizes the effect of Dex treatment on Id3-induced apoptosis.

Notch pathway participates to lineage commitment, maturation and survival in thymus and inhibits GC-induced apoptosis upon over-expression (Izon et al., 2002). Therefore, down-regulation of Notch1 and glycoprotein 130 (Il6st), a receptor subunit potentiating Notch1 pathway, seems particularly relevant for induction of DPT apoptosis (Figure 5). Real Time PCR experiments confirmed the GeneChip data and demonstrated that Dex-induced Notch1 down-regulation peaked as early as 1 h of contact (Figure 4).

**Treatment with Dex regulates the expression of several genes playing a rescuing activity in Dex-treated DPTs.**

Although Dex treatment has a pro-apoptotic effect on DPTs, it regulates expression of genes potentially counteracting GC-induced apoptosis. First of all activity of GC receptor is regulated through over-expression (3.6 fold) of FKBP5 immunophillin, an inhibitor of the interaction between GCs and their receptor (Table 5). This effect was
countered, at least in part, by the increase of GC receptor (Nr3c1) itself (2.0 fold) (Table 4). Moreover, modulation of several genes potentially exerting anti-apoptotic effects seems to counteract the pro-apoptotic effect of Dex (Table 5). Of note is the modulation of Promyelocytic leukemia zinc finger gene (Plzf or Zbtb16), a transcription repressor promoting stem cell growth. It resulted powerfully up-regulated following DPT treatment (10.7 fold) and virtually absent in untreated DPTs (not shown). Another gene with anti-apoptotic functions is the IL-7 receptor gene (Franchimont et al., 2002) that resulted up-regulated (2.3 fold) in Dex-treated DPTs.

We did not performed Real Time PCR to confirm the modulation of the above reported genes and of those listed in Table 5 since the regulation of most of them has been already described by other studies, cited in the Table 4s of the Supplemental material.
DISCUSSION

GCs mediate DPTs fate mainly through modulation of gene transcription as suggested by the inhibition of apoptosis during simultaneous exposure to Dex and actinomycin D (Figure 1)(Distelhorst, 2002). Our analysis, indeed, revealed several apoptosis-related genes whose transcription is modulated in DPTs following Dex contact for 3 h (Figure 4). This time is sufficient to trigger apoptosis. In fact, actinomycin D does not inhibit apoptosis when added 3 h after Dex. Three hours treatment is short enough to minimize the possibility of indirect transcriptional effects. Therefore, we suppose that most of the described genes are directly regulated by Dex. Moreover, at this time, apoptosis process has not been executed yet, and the cell components, including mRNA species, are still intact, avoiding a technical bias.

About one fifth of the modulated genes can be grouped in 5 systems that appear to contribute to Dex-induced apoptosis of DPTs (Table 4 and Figure 5). First of all, 9 genes participating to lineage commitment, maturation and survival of thymocytes are regulated. Even if the modulation tends towards normal levels after 6-9 h of treatment (Id3 and Notch1) or is not impressive from the quantitative point of view (e.g. Socs1 and Il6st), the overall decrease of anti-apoptotic messages during the first hours of Dex contact could make DPTs more sensitive to pro-apoptotic signals, thus favoring apoptosis.

We also described the modulation of several genes somehow related to mitochondrion: Bcl-2 and Tis11 family members, and genes involved in ROS detoxification. These genes may be responsible for the early mitochondrial alteration leading to the death decision (Lepine et al., 2005). Another group of Dex-regulated genes participates in the ceramide pathways and modulates apoptosis without being linked to mitochondrial machinery (Cifone et al., 1999; Lepine et al., 2004).
We were able to link the above described genes to apoptosis on the basis of what is known so far, but very likely future functional studies will shed light on other genes modulated by Dex in DPTs (Table 2s in Supplemental material) contributing to their apoptosis. For example, the proteins of cytoskeleton and those involved in protein depolimerization, including ubiquitin cycle, are frequently-modulated genes (Table 1 and 3), representing new fields of investigation.

Data concerning Bcl-2 family may suggest that GC effect on thymocytes is due to an overall imbalance more than to the regulation of a single gene. A support to this hypothesis is a study demonstrating that mature T cells of Bim-deficient mice are resistant to SEB-induced deletion (Hildeman et al., 2002). This observation suggested that Bim modulation was crucial for the induction of cell death in this system. However, following SEB injection, Vβ8+ wild type cells expressed levels of Bim similar to those of untreated cells, demonstrating that regulation of Bim expression is not involved in SEB-induced apoptosis. On the contrary, decreased levels of Bcl-2 were detected, suggesting that Bcl-2 down-regulation facilitates the pro-apoptotic effect of Bim in those cells (Hildeman et al., 2002).

Concerning GC effects on T cells, despite Bim modulation may appear crucial for GC-induced apoptosis, as already reported (Wang et al., 2003a), Bim-deficient thymocytes are only partially resistant to Dex (Bouillet et al., 1999), suggesting that Bim modulation alone is not sufficient to explain thymocyte apoptosis. The hypothesis of Bcl-2 family members imbalance (Bcl-2, Bcl-xL and Bax) has been proposed to explain the GC-induced apoptosis in granule cells of the hippocampus (Almeida et al., 2000), which is in line with our data but suggests that GC-induced gene regulation is peculiar to each tissue.

We also demonstrate that the ratio between the Bcl-x anti-apoptotic splice variants and the pro-apoptotic splice variant Bcl-xβ changes upon Dex treatment. Modulation of Bcl-
gene splice variants has been described in endometrial cells treated with progestins, where it plays a determinant protective role (Pecci et al., 1997), suggesting that splice variant modulation is a possible mechanism for the induction of apoptosis.

Treatment of DPTs with Dex has a clear pro-apoptotic effect, as discussed. Nevertheless, some studies, including ours, demonstrate that GCs regulate the expression of genes counteracting apoptosis (Table 5)(D’Adamio et al., 1997; Franchimont et al., 2002; Schmidt et al., 2005). This paradoxical phenomenon needs to be explained. In some cases, it is possible that genes do not play the same role in thymocytes and other cells, including lymphocytes. In fact, functional role of a protein may differ in cells from various origins and within the same cells in different microenvironmental contexts, depending on the cell state of activation/differentiation. In particular, cell death may follow activating stimuli as can be seen in the activation-induced cell death of mature T lymphocytes. This may apply to Dex-treated DPTs in which several pro-apoptotic systems have been triggered. A known example of a different role played by a gene involved in the activation process is represented by Socs1, whose lack means higher activation in mature T cells and higher apoptosis level in thymocytes (Starr et al., 1998; Chong et al., 2005). Moreover, several lines of evidence suggest that GCs cover a multifaceted function in thymus development, promoting thymocyte expansion more than apoptosis when present at physiological concentrations (Jondal et al., 2004). Therefore, it can be hypothesized that those genes whose modulation appear to have an anti-apoptotic action may favor thymocyte survival when low concentrations of GCs are present in the context of the thymic stroma.

The list of genes modulated by GCs has been continuously updated so far, and hundreds of genes are known to be modulated by GCs (Smith and Herschman, 2004; Ploner et al., 2005). Also about one third of the apoptosis-related genes that we found to be modulated in Dex-treated DPTs were already known to be regulated by GCs (see
Tables 4 and 5). Considering the number of studies dealing with GCs and T cells, the finding of several genes never described as modulated may sound bizarre if we hypothesize that the mechanism of action of GC is similar in different cell types. However, this hypothesis may be questionable. In fact, composition and proportion of individual isoforms of GC receptor expressed in particular cellular contexts may change GC receptor function (Zhou and Cidlowski, 2005). Moreover, we know that the GC receptor modulates gene transcription by several ways, including protein-protein interaction with other transcription factors and co-regulators (Reichardt et al., 1998; Zhou and Cidlowski, 2005). Therefore, it is likely that the effect of GC treatment is different according to the functional status of the cells, determined, in turn, by the type and the amount of transcription factors and coactivators which are active at a specific time. This is clearly exemplified by cells, such as endometrial cells, in which GC treatment exerts an anti-apoptotic effect (Pecci et al., 1997).

This reasoning may explain, at least in part, why different studies using global gene profiling, including ours, found out different modulated genes. In our opinion, this depends on the cells used and their activation and/or differentiation status, more than on technical bias. In fact, in the attempt to find the core gene pattern responsible for the pro-apoptotic effect of GC in lymphocytes, we tested some primary cultures from human malignant lymphocytes and a murine thymus-derived cell line. However, analysis of data revealed that only few modulated genes were common to the different systems tested (data not shown).

Another reason explaining discrepancies between our study and others is the choice of a short time of GC-cell contact. In fact, while some genes increase their levels of modulation along with the time of contact, others (e.g. Id3 or Notch1) recover to normal expression levels soon after modulation. Since the death machinery is activated during the first hours of GC-DPT contact, these genes play a role similar to those increasingly
modulated but are barely considered by the studies using a long time of treatment. Furthermore, studying cells treated for several hours with GCs leads to the identification of genes which are modulated by the transcription factors regulated by GC (i.e. studying secondary effects of GC). Finally, we used medium-treated cells as control, instead of untreated cells. In our opinion, this is the best way to discriminate the GC effects from those of the stress occurring before and during incubation. If an untreated sample is used as a control, one may run the risk to under-estimate the results (see Tis11d, Figure 4) or even reach wrong conclusions (see Ugcg, apparently up-regulated upon Dex treatment, Figure 4).

In recent and less recent past, several groups (including ours) have spent their efforts in the attempt to find the crucial pro-apoptotic event (including increase or decrease of gene expression) responsible for the GC-induced apoptosis. Our study indicates that several genes, participating in different cellular functions, are modulated by the GC treatment. Even if it does not formally demonstrate the network hypothesis, it is in accordance with it, further suggesting that several genes cooperate in inducing GC-induced apoptosis of T cells.
REFERENCES


Dietary PUFA modulate the expression of proliferation and differentiation markers
in Morris 3924A hepatoma cells. *Biochim Biophys Acta* **1737**:138-144.

analysis uncovers the induction of the proapoptotic BH3-only protein Bim in

Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced

Yang XF, Weber GF and Cantor H (1997) A novel Bcl-x isoform connected to the T
cell receptor regulates apoptosis in T cells. *Immunity* **7**:629-639.

DW, Reinhold WC, Lababidi S, Bussey KJ, Riss J, Barrett JC and Weinstein JN

Footnotes

* R.B. and G.N. equally contributed

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Reprint:

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FIGURE LEGENDS

Figure 1
The high sensitivity of CD4⁺CD8⁺ double positive thymocytes (DPTs) to Dex-induced apoptosis is dependent on protein synthesis. (A) Murine thymocytes were incubated for 18 h with or without Dex (10⁻⁷ M). On the left panel, nuclei processed for DNA content analysis by hypotonic propidium iodide staining were analyzed with a FACScan flowcytometer and percentages of hypodiploid nuclei and/or with high SSC (apoptotic cells in the box) are shown for each condition. On the right panel, the phenotype of alive thymocytes incubated for 18 h with or without Dex (10⁻⁷ M) is shown. Percentage of CD4⁺, CD8⁺, CD4⁺CD8⁺ double positive (DPTs) and CD4⁻CD8⁻ double negative thymocytes show a decreased amount of DPTs in the Dex-treated thymocytes. (B) Percentages of thymocytes undergoing apoptosis following 18 h culture with or without Dex (10⁻⁷ M) are shown. When specified, actinomycin D was added to thymocytes together with Dex following the specified lag time. Actinomycin D alone did not show a significant toxicity.

Figure 2
The expression of Bim, Bfl-1 and Bcl-x gene was evaluated by RNAse protection assay in DPTs treated for 1-6 h with Dex. Expression data were normalized by using an internal probe for Gapdh (that is not modulated in DPTs) and compared to untreated DPTs.

Figure 3
Expression of the splicing variants of Bcl-x gene in Dex-treated DPTs. The probe, shown on the left, was chosen so that the antiapoptotic splice variants of Bcl-x gene
(Bcl-xL, Bcl-xγ and Bcl-xδ) were detected together, giving only one protected fragment (shown on the right panel); conversely, the pro-apoptotic splice variant Bcl-xβ was detected separately. The gel was subjected to quantitative analysis using an Instant Imager autoradiography system. The Table and the graph show respectively the cpms of the protected fragments and the ratio between the fragment from Bcl-xβ and the anti-apoptotic (AP) splice variants.

**Figure 4**

Expression of thioredoxin reductase (Txnrd1), thioredoxin interacting protein (Txnip), UDP-glucose ceramide glucosyltransferase (Ugcg), sphingosine 1-phosphate phosphatase (Sgpp1), zinc finger protein 36 (Tis11), zinc finger protein 36 C3H type-like 2 (Tis11d), Notch gene homolog 1 (Notch1), dual-specificity phosphatase (Dusp2), and Inhibitor of DNA binding 3 (Id3) following treatment of DPTs for 1, 3, 6, 9 h, investigated by Real Time PCR. Gene expression was evaluated upon medium- (line-linked open circle) or Dex- (line-linked full circle) treatment of DPTs and plotted relatively to the expression of untreated DPTs. To evaluate the overall regulation by Dex, the ratio between gene expression of Dex-treated DPTs and gene expression of medium-treated DPTs at the same time-points was plotted (dashed line-linked full triangle).

**Figure 5**

Summary of the genes responsible for the transcription-dependent apoptosis of DPTs following Dex treatment. Genes whose transcription was up-regulated and those whose transcription was down-regulated are shown as specified in the legend inside the figure. Lines with an arrowhead indicate activation, lines ending with a small line indicate inhibition. Dotted lines with an arrowhead indicate enzymatic reactions.
Nonboxed genes belong to a pathway affected by Dex treatment but their expression is not modulated in Dex-treated DPTs.
**Table 1.** The most modulated gene categories (restricted to classification of cellular localization) following Dex treatment of CD4⁺CD8⁺ double positive thymocytes (DPTs). Only Gene Ontology (GO) IDs with a relative enrichment higher than 1.4 or lower than 0.7 are reported. GO IDs with less than 3 modulated genes were not considered.

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Cellular Component</th>
<th>Modulated / Tested Genes b</th>
<th>Relative Pauperization c</th>
</tr>
</thead>
<tbody>
<tr>
<td>5576</td>
<td>Extracellular region</td>
<td>0.9 %</td>
<td>0.5</td>
</tr>
<tr>
<td>5829</td>
<td>Cytosol</td>
<td>1.0 %</td>
<td>0.6</td>
</tr>
<tr>
<td>5739</td>
<td>Mitochondrion</td>
<td>0.8 %</td>
<td>0.5</td>
</tr>
<tr>
<td>30054</td>
<td>Cell junction</td>
<td>3.7 %</td>
<td>2.1</td>
</tr>
<tr>
<td>5783</td>
<td>Endoplasmic reticulum</td>
<td>2.7 %</td>
<td>1.5</td>
</tr>
<tr>
<td>12505</td>
<td>Endomembrane system</td>
<td>3.6 %</td>
<td>2.1</td>
</tr>
<tr>
<td>15630</td>
<td>Microtubule cytoskeleton</td>
<td>2.7 %</td>
<td>1.5</td>
</tr>
<tr>
<td>15629</td>
<td>Actin cytoskeleton</td>
<td>3.3 %</td>
<td>1.9</td>
</tr>
<tr>
<td>5654</td>
<td>Nucleoplasm</td>
<td>2.8 %</td>
<td>1.6</td>
</tr>
<tr>
<td>5635</td>
<td>Nuclear membrane</td>
<td>6.7 %</td>
<td>3.9</td>
</tr>
</tbody>
</table>

a Gene Ontology number;
b percentage of the genes resulting modulated (up-regulated or down-regulated) out of those investigated with the GeneChip, belonging to the specified GO ID;
c the genes modulated by Dex with an assigned GO ID were 126 vs 7273 investigated with our chip (1.7%). When the percentage of genes modulated and belonging to a GO ID was around 1.7 % (> 1.1 % and < 2.5 %) no enrichment/pauperization was stated. If the percentage of genes modulated and belonging to a GO ID was significantly low (<1.1 %), pauperization was stated and relative pauperization was calculated by dividing the percentage found by 1.7 %. If percentage of modulated genes belonging to a GO ID was significantly high (> 2.5 %), enrichment was stated and relative enrichment was calculated by dividing the percentage found by 1.7 %. A relative enrichment of 2 means that the number of genes belonging to the specified ID is twice than that expected and a relative pauperization of 0.5 means that the number of genes belonging to the specified ID is half than that expected.
Table 2. The most modulated gene categories (restricted to classification of molecular functions) following Dex treatment of CD4⁺CD8⁺ double positive thymocytes (DPTs). Only Gene Ontology (GO) IDs with a relative enrichment higher than 1.4 are reported. GO IDs with less than 3 modulated genes were not considered.

<table>
<thead>
<tr>
<th>GO IDᵃ</th>
<th>Molecular Function</th>
<th>Modulated / Tested Genesᵇ</th>
<th>Relative Enrichmentᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>4497, 15036, 16614, 16627</td>
<td>Enzymatic activities Oxidoreductase activities</td>
<td>5.2 % 3.0</td>
<td></td>
</tr>
<tr>
<td>16757, 16746</td>
<td>Transferase activities</td>
<td>2.9 % 1.7</td>
<td></td>
</tr>
<tr>
<td>42578</td>
<td>Phosphoric ester hydrolase activity</td>
<td>4.0 % 2.3</td>
<td></td>
</tr>
<tr>
<td>16829</td>
<td>Lyase activity</td>
<td>3.8 % 2.2</td>
<td></td>
</tr>
<tr>
<td>16853</td>
<td>Isomerase activity</td>
<td>6.0 % 3.4</td>
<td></td>
</tr>
<tr>
<td>15290</td>
<td>Electrochemical potential-driven transporter activity</td>
<td>4.9 % 2.8</td>
<td></td>
</tr>
<tr>
<td>5515</td>
<td>Protein binding (including transcription factor binding)</td>
<td>2.6 % 2.1</td>
<td></td>
</tr>
<tr>
<td>46914</td>
<td>Transition metal ion binding</td>
<td>3.0 % 1.7</td>
<td></td>
</tr>
<tr>
<td>8047</td>
<td>Enzyme activator activity</td>
<td>4.3 % 2.5</td>
<td></td>
</tr>
<tr>
<td>19207</td>
<td>Kinase regulator activity</td>
<td>6.8 % 3.9</td>
<td></td>
</tr>
<tr>
<td>30695</td>
<td>GTPase regulator activity</td>
<td>2.7 % 1.5</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Gene Ontology number;
ᵇ percentage of the genes resulting modulated (up-regulated or down-regulated) out of those investigated with the GeneChip, belonging to the specified GO ID;
ᶜ the genes modulated by Dex with an assigned GO ID were 126 vs 7273 investigated with our chip (1.7%). When the percentage of genes modulated and belonging to a GO ID was aroung 1.7 % (> 1.1 % and < 2.5 %) no enrichment/pauperization was stated. If percentage of modulated genes belonging to a GO ID was significantly high (> 2.5 %), enrichment was stated and relative enrichment was calculated by dividing the percentage found by 1.7 %. A relative enrichment of 2 means that the number of genes belonging to the specified ID is twice than that expected.
Table 3. The most modulated gene categories (restricted to classification of biological processes) following Dex treatment of CD4⁺CD8⁺ double positive thymocytes (DPTs). Only Gene Ontology (GO) IDs with a relative enrichment higher than 1.4 are reported. GO IDs with less than 3 modulated genes were not considered.

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Biological Process</th>
<th>Modulated / Tested genes</th>
<th>Relative Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6629</td>
<td>Lipid metabolism</td>
<td>5.4 %</td>
<td>3.1</td>
</tr>
<tr>
<td>8202</td>
<td>Steroid metabolism</td>
<td>11.5 %</td>
<td>6.7</td>
</tr>
<tr>
<td>6066</td>
<td>Alcohol metabolism</td>
<td>7.5 %</td>
<td>4.3</td>
</tr>
<tr>
<td>51261</td>
<td>Protein depolymerization</td>
<td>18.2 %</td>
<td>10.5</td>
</tr>
<tr>
<td>6512</td>
<td>Ubiquitin cycle</td>
<td>3.3 %</td>
<td>1.9</td>
</tr>
<tr>
<td>9101</td>
<td>Glycoprotein biosynthesis</td>
<td>6.0 %</td>
<td>3.5</td>
</tr>
<tr>
<td>9966</td>
<td>Regulation of signal transduction</td>
<td>7.1 %</td>
<td>4.1</td>
</tr>
<tr>
<td>16311</td>
<td>Dephosphorylation</td>
<td>5.7 %</td>
<td>3.3</td>
</tr>
<tr>
<td>7219</td>
<td>Notch signal pathway</td>
<td>42.9 %</td>
<td>24.7</td>
</tr>
<tr>
<td>6811</td>
<td>Ion transport</td>
<td>2.8 %</td>
<td>1.6</td>
</tr>
<tr>
<td>7017</td>
<td>Microtubule-based process</td>
<td>3.8 %</td>
<td>2.2</td>
</tr>
<tr>
<td>16568</td>
<td>Chromatin modification</td>
<td>8.8 %</td>
<td>5.1</td>
</tr>
<tr>
<td>6366</td>
<td>Transcription from RNA polymerase II promoter</td>
<td>3.4 %</td>
<td>2.0</td>
</tr>
<tr>
<td>42127</td>
<td>Regulation of cell proliferation</td>
<td>2.8 %</td>
<td>1.6</td>
</tr>
<tr>
<td>51301</td>
<td>Cell division</td>
<td>5.2 %</td>
<td>3.0</td>
</tr>
<tr>
<td>9790</td>
<td>Embryonic development</td>
<td>2.9 %</td>
<td>1.7</td>
</tr>
<tr>
<td>50793</td>
<td>Regulation of development</td>
<td>3.8 %</td>
<td>2.2</td>
</tr>
<tr>
<td>45595</td>
<td>Regulation of cell differentiation</td>
<td>5.1 %</td>
<td>2.9</td>
</tr>
<tr>
<td>7389</td>
<td>Pattern specification</td>
<td>2.9 %</td>
<td>1.7</td>
</tr>
<tr>
<td>35295</td>
<td>Tube development</td>
<td>6.1 %</td>
<td>3.5</td>
</tr>
<tr>
<td>910</td>
<td>Cytokinesis</td>
<td>3.4 %</td>
<td>2.0</td>
</tr>
<tr>
<td>6915</td>
<td>Apoptosis</td>
<td>3.7 %</td>
<td>2.1</td>
</tr>
<tr>
<td>43065</td>
<td>Positive regulation of apoptosis</td>
<td>5.8 %</td>
<td>3.4</td>
</tr>
<tr>
<td>6955</td>
<td>Immune response</td>
<td>2.8 %</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a Gene Ontology number;
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c the genes modulated by Dex with an assigned GO ID were 126 vs 7273 investigated with our chip (1.7%). When the percentage of genes modulated and belonging to a GO ID was around 1.7% (> 1.1% and < 2.5%) no enrichment/pauperization was stated. If percentage of modulated genes belonging to a GO ID was significantly high (> 2.5%), enrichment was stated and relative enrichment was calculated by dividing the percentage.
found by 1.7%. A relative enrichment of 2 means that the number of genes belonging to the specified ID is twice than that expected.
Table 4.

Genes whose transcriptional regulation contributes to explain the pro-apoptotic effect of Dex in CD4+CD8+ double positive thymocytes (DPTs).

<table>
<thead>
<tr>
<th>EMBL ID</th>
<th>Gene name</th>
<th>Gene symbol (Hugo ID)</th>
<th>Fold Modulation</th>
<th>original data</th>
<th>Transcriptional regulation upon GC treatment in cell types other than thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>L35049</td>
<td>Bcl2-like 1 (Bcl-x)</td>
<td>Bcl2l1</td>
<td>-1.4</td>
<td>Yes</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>AI324342</td>
<td>B-cell leukemia/lymphoma 2 related protein A1 (BII-1)</td>
<td>Bcl2a1</td>
<td>-1.2</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AF032459</td>
<td>BCL2-like 11 (Bim)</td>
<td>Bcl2l11</td>
<td>2.3 / 2.3*</td>
<td>No</td>
<td>Pre-B ALL and cell lines</td>
</tr>
<tr>
<td>Y08460</td>
<td>dihydroceramide desaturase (Degs1)</td>
<td>Degs1</td>
<td>1.4*</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AI849939</td>
<td>regulated in development and dna damage responses 1 (dig-2, RTP801)</td>
<td>Ddit4</td>
<td>3.4*</td>
<td>No</td>
<td>T cell lines</td>
</tr>
<tr>
<td>U09268</td>
<td>dual specificity phosphatase 2 (PAC1)</td>
<td>Dusp2</td>
<td>1.9</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>J04103</td>
<td>E26 avian leukemia oncogene 2</td>
<td>Ets2</td>
<td>-1.5</td>
<td>No</td>
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<tr>
<td>U39827</td>
<td>G protein coupled receptor 65 (TDAG8)</td>
<td>Gpr65</td>
<td>5.8</td>
<td>Yes</td>
<td>Lymphoma cell lines</td>
</tr>
<tr>
<td>M60523</td>
<td>inhibitor of DNA binding 3</td>
<td>Id3</td>
<td>-4.0</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>U51167</td>
<td>NADP-dependent isocitrate dehydrogenase 2</td>
<td>Idh2</td>
<td>-1.4</td>
<td>Yes</td>
<td></td>
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<tr>
<td>AI843709</td>
<td>interleukin 6 signal transducer (glycoprotein 130)</td>
<td>Il6st</td>
<td>-1.4</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AI837528</td>
<td>Janus kinase 1</td>
<td>Jak1</td>
<td>1.8</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Z11886</td>
<td>Notch gene homolog 1</td>
<td>Notch1</td>
<td>-1.6</td>
<td>Yes</td>
<td>Osteoblast, osteosarcoma</td>
</tr>
<tr>
<td>X04435</td>
<td>nuclear receptor subfamily 3, group C, member 1 (GC receptor)</td>
<td>Nr3c1</td>
<td>2.0</td>
<td>No</td>
<td>lymphoma and myeloma cell lines</td>
</tr>
<tr>
<td>M60909</td>
<td>retinoic acid receptor, alpha</td>
<td>Rara</td>
<td>-2.1</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>D26532</td>
<td>runt-related transcription factor 1 (AML1)</td>
<td>Runx1</td>
<td>-1.6</td>
<td>Yes</td>
<td></td>
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<tr>
<td>AI835784</td>
<td>sphingosine-1-phosphate phosphatase 1</td>
<td>Sgpp1</td>
<td>1.8</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>U88325</td>
<td>suppressor of cytokine signaling 1</td>
<td>Socs1</td>
<td>-1.6</td>
<td>Yes</td>
<td>Opposite in T-ALL and B-ALL</td>
</tr>
<tr>
<td>AV376312</td>
<td>spleen/ryanodine receptor domain and SOCS box containing 1</td>
<td>Spsb-1</td>
<td>-1.6</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>U19463</td>
<td>Tumor necrosis factor-alpha-induced protein 3 (A20)</td>
<td>Tnfaip3</td>
<td>2.0*</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>AI839138</td>
<td>thioredoxin interacting protein</td>
<td>Txnip</td>
<td>2.1*</td>
<td>No</td>
<td>Lymphoma and myeloma cell lines</td>
</tr>
<tr>
<td>AB027565</td>
<td>thioredoxin reductase 1</td>
<td>Txnrd1</td>
<td>-1.6</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>D98966</td>
<td>UDP-glucose ceramide glucosyltransferase</td>
<td>Ugcg</td>
<td>-1.9</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>X14678</td>
<td>zinc finger protein 36 (Tis11, Tris-Tetraprolin)</td>
<td>Zfp36</td>
<td>-1.7</td>
<td>Yes</td>
<td>Activated macrophages</td>
</tr>
<tr>
<td>M58564</td>
<td>zinc finger protein 36, C3H type-like 2 (Tis11d, BRF2, ERF2)</td>
<td>Zfp36l2</td>
<td>2.9 / 6.1*</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
a Modulation resulting from GeneChip array. When more than 1 Affymetrics set probe investigated the same gene, more than 1 value is given.
b For each gene listed, we verified whether transcriptional regulation upon GC treatment of thymocytes (even if on the whole thymic population) had been already published in PubMed data bank. If our finding was not original, No was stated. In a similar table (Table 3s) of the supplemental material the reference of the proper publication is given.
c Cell type.
d Gene expression is significantly modulated but under the chosen cut-off values. It was included because belonging to a pathway in which other genes are modulated and may contribute to the overall modulation of the signaling.
### Table 5. Genes whose transcriptional regulation may play a rescue activity in Dex-treated CD4⁺CD8⁺ double positive thymocytes (DPTs).

<table>
<thead>
<tr>
<th>EMBL ID</th>
<th>Gene name</th>
<th>Gene symbol (Hugo ID)</th>
<th>Fold Modulationa</th>
<th>Original data b</th>
<th>Transcriptional regulation upon GC treatment in cell types other than thymocytes c</th>
</tr>
</thead>
<tbody>
<tr>
<td>X05719</td>
<td>cytotoxic T-lymphocyte-associated protein 4</td>
<td>Ctl4</td>
<td>1.9 f</td>
<td>Yes</td>
<td>Activated T cells</td>
</tr>
<tr>
<td>AW061330</td>
<td>Embigin</td>
<td>Emb</td>
<td>2.7 f</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>U169599</td>
<td>FK506 binding protein 5</td>
<td>Fkbp5</td>
<td>3.6 f</td>
<td>Yes</td>
<td>Lymphocyte-derived cell lines</td>
</tr>
<tr>
<td>M29697</td>
<td>interleukin 7 receptor</td>
<td>Il7r</td>
<td>2.3 f</td>
<td>No</td>
<td>T cells</td>
</tr>
<tr>
<td>X84896</td>
<td>purinergic receptor P2X, ligand-gated ion channel, 1</td>
<td>P2rx1</td>
<td>-1.5 f</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>AF021335</td>
<td>TCR gamma</td>
<td>TRGγ</td>
<td>1.9-2.4 f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U15635</td>
<td>T-cell specific GTPase (Mg21 or TGTP)</td>
<td>-</td>
<td>1.9 f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF019048</td>
<td>Tumor necrosis factor (ligand) superfamily, member 11 (RANKL, OPGL or TRANCE)</td>
<td>Tnfsf11</td>
<td>9.6 f absent → present</td>
<td></td>
<td>Stromal cells</td>
</tr>
<tr>
<td>AF004100</td>
<td>solute carrier family 30 (zinc transporter), member 4</td>
<td>SLC30A4</td>
<td>2.4 f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AI553024</td>
<td>zinc finger and BTB domain containing 16 (PLZF)</td>
<td>Zbtb16</td>
<td>10.7 f absent → present</td>
<td>No</td>
<td>Several cell types</td>
</tr>
</tbody>
</table>

*a Modulation resulting from GeneChip array. When more than 1 Affymetrics set probe investigated the same gene, more than 1 value is given. When the gene resulted virtually non-expressed in medium-treated DPTs and expressed in Dex-treated DPTs, absent → present is annotated.*

*b we verified whether transcriptional regulation upon GC treatment of thymocytes (even if on the whole thymic population) had been already published in PubMed data bank. If our finding was not original, No was stated. In a similar table (Table 4s) of the supplemental material the reference of the proper publication is given.*

*c cell type.
Figure 1

A

Medium-treated

SSC

PI

CD8+

CD4+

7% 14%

71%

Dex-treated

SSC

PI

CD8+

CD4+

89% 27%

23%

18% 31%

B

% apoptosis

Dex and Act D addition (h)

Lag time between Dex and Act D addition (h)
Figure 2
Figure 3
Figure 4