



**HAL**  
open science

## Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor-mediated transcription

Zsuzsa Szondy, Katalin Toth, Zsolt Sarang, Beata Scholtz, Laszlo Fesus, Peter Brazda, Norbert B. Ghyselinck, Pierre Chambon

### ► To cite this version:

Zsuzsa Szondy, Katalin Toth, Zsolt Sarang, Beata Scholtz, Laszlo Fesus, et al.. Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor-mediated transcription. *Cell Death and Differentiation*, 2010, 10.1038/cdd.2010.136 . hal-00592290

**HAL Id: hal-00592290**

**<https://hal.science/hal-00592290>**

Submitted on 12 May 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor- mediated transcription**

Katalin Tóth<sup>1</sup>, Zsolt Sarang<sup>1</sup>, Beáta Scholtz<sup>1</sup>, Péter Brázda<sup>1</sup>, Norbert Ghyselinck<sup>2</sup>, Pierre Chambon<sup>2</sup>, László Fésüs<sup>1</sup>, and Zsuzsa Szondy<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, Apoptosis and Genomics Research Group, Hungarian Academy of Sciences, University of Debrecen, Debrecen, Hungary

<sup>2</sup>Département de Biologie Cellulaire and Développement, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Strasbourg, France

Running title: Retinoids and glucocorticoids in T cell apoptosis

Corresponding author:  
Zsuzsa Szondy, M.D., Ph.D., D.Sc.  
Department of Biochemistry and Molecular Biology  
University of Debrecen  
Nagyerdei krt.98.  
H-4012. Debrecen  
Hungary  
Tel: +36 52 416432 or +36 52 512900/64134  
Fax: +36 52 314989  
e-mail: [szondy@indi.dote.hu](mailto:szondy@indi.dote.hu)

## **Keywords**

Apoptosis, glucocorticoid, retinoid, T cells

## **Abbreviations**

ATRA, all-*trans* retinoic acid; 9cRA, 9-*cis* retinoic acid; GR, glucocorticoid receptor; PI-PLC, phosphatidylinositol-specific phospholipase C; RAR, retinoic acid receptor; RXR, retinoid X receptor

## **Abstract**

Glucocorticoid-induced apoptosis of thymocytes is one of the first recognized forms of programmed cell death. It was shown to require gene activation induced by the glucocorticoid receptor (GR) translocated into the nucleus following ligand binding. In addition, the necessity of the glucocorticoid-induced, but transcription-independent phosphorylation of phosphatidylinositol-specific phospholipase C (PI-PLC) has also been demonstrated. Here we report that retinoic acids, physiological ligands for the nuclear retinoid receptors, enhance glucocorticoid-induced death of mouse thymocytes both *in vitro* and *in vivo*. The effect is mediated via retinoic acid receptor (RAR) alpha/retinoid X receptor (RXR) heterodimers, and occurs when both RAR $\alpha$  and RXR are ligated by retinoic acids. We demonstrate that the ligated RAR $\alpha$ /RXR interacts with the ligated GR resulting in an enhanced transcriptional activity of the GR. The mechanism through which this interaction promotes GR-mediated transcription does not require DNA binding of the retinoid receptors and does not alter the phosphorylation status of Ser212, known to regulate the transcriptional activity of GR. Phosphorylation of PI-PLC was not affected. Besides thymocytes, retinoids also promoted glucocorticoid-induced apoptosis of various T cell lines suggesting that they could be used in the therapy of glucocorticoid sensitive T cell malignancies.

## Introduction

Glucocorticoids are a group of steroid hormones that possess a wide range of anti-inflammatory, immunosuppressive, and antitumor activities, including the ability to induce apoptosis in T and B lymphocytes<sup>1</sup>. Glucocorticoid-induced apoptosis of thymocytes is one of the first recognized forms of programmed cell death<sup>2</sup>. For this effect glucocorticoids passively diffuse into the cell and bind to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily<sup>3</sup>. Subsequently, the hormone-receptor complex translocates into the nucleus, where it modulates gene expression either by direct binding to its cognate response element or via interaction with other transcription factors. In the case of thymocyte apoptosis gene activation is essential to the process, since in thymocytes expressing a mutated GR capable of interacting with other transcription factors but not of transactivating genes following ligand binding, dexamethasone-induced apoptosis was impaired<sup>4</sup>. During the past decades many of the genes involved have been identified<sup>5-10</sup>. Increasing evidence suggests that the effector phase of glucocorticoid-induced apoptosis is mediated via the mitochondrial pathway involving Bcl-2 family members<sup>11-15</sup>. The role of mitochondria is further supported by the findings that caspase-9 as well as Apaf-1-deficient mice are impaired in dexamethasone-induced thymocyte cell death<sup>16,17</sup>.

In addition to the genomic effects, glucocorticoids induce a rapid src-dependent phosphorylation of the phosphatidylinositol-specific phospholipase C (PI-PLC)<sup>18,19</sup>, leading to the subsequent activation of the acidic sphingomyelinase which results in the production of ceramide and sphingosine. These compounds have no effect on the mitochondria, but contribute to caspase-8 activation and thus accelerate the glucocorticoid-induced cell death program<sup>18,20,21</sup>.

Besides glucocorticoids, all-*trans* (ATRA) and 9-*cis* retinoic acid (9cRA), possible physiological ligands for retinoic acid receptors (RAR)s and retinoid X receptors (RXRs)<sup>22</sup>,

were also shown to modulate thymocyte apoptosis<sup>23-29</sup>. They were reported to induce apoptosis in immature thymocytes<sup>23-25</sup>, inhibit negative selection<sup>26-29</sup>, and promote glucocorticoid-induced death of thymocytes<sup>23,28</sup>. Retinoid receptors similarly to the GR belong to the steroid/thyroid/retinoid nuclear receptor family<sup>4</sup>. ATRA and 9cRA are equipotent in activating RAR, while activation of RXR by ATRA is 50-fold less than by 9cRA<sup>22</sup>. In the presence of RAs retinoid receptors function in the form of RAR/RXR heterodimers or RXR/RXR homodimers<sup>30</sup>, and modulate gene expression either by direct binding to their cognate response elements or via interaction with other transcription factors. Recently we found that similarly to glucocorticoids<sup>31</sup>, retinoids are also produced by the thymic epithelial cells<sup>32</sup> suggesting that they might indeed have a physiological role in regulating thymocyte differentiation and apoptosis.

While previously we have studied the involvement of retinoids in the regulation of negative selection<sup>27-29</sup> and in the cell death induction of thymocytes<sup>25</sup>, so far the mechanisms by which retinoids promote glucocorticoid-induced apoptosis of thymocytes have not been investigated yet.

## Results

**Ligation of both RAR $\alpha$  and the RXRs promotes dexamethasone-induced death of mouse thymocytes.** Increasing concentrations of dexamethasone induced a significant degree of apoptosis in mouse thymocytes already at 6h following addition, detected by determining the amount of DNA degradation (Figure 1a). As it was reported previously<sup>23,26</sup>, increasing concentrations of RAs further enhanced glucocorticoid-induced apoptosis of thymocytes in a dose dependent manner (Figure 1b). The concentration of dexamethasone, at which the effect of the retinoids was tested, was selected for 0.1  $\mu$ M, where about 45% of DNA degradation was observed when added alone (Figure 1a). Retinoids were able to induce about a 30%

further increase in the DNA fragmented at this time point, however, ATRA at physiological concentrations was ineffective. 9cRA, however, was very effective suggesting that RXR receptors stimulated selectively by 9cRA may participate in the phenomenon. Indeed increasing concentrations of LG268, an RXR agonist, also promoted dexamethasone-induced apoptosis (Figure 1b). However, addition of LG268 at 0.1 nM concentration, which alone had only slight effect on the GR-induced DNA fragmentation (Figure 1 c), effectively lowered the concentration of ATRA required to enhance GR-induced apoptosis of thymocytes implying that stimulation of both RAR and RXR receptors might play a role in the enhancement of GR-induced death.

To investigate which of the RARs is involved in the phenomenon, the effect of various RAR specific agonists was also tested. In agreement with the lack of RAR $\beta$  expression in mouse thymocytes<sup>25</sup>, the RAR $\beta$ -selective compound (CD2314) tested could not promote GR-induced apoptosis (data not shown). Though RAR $\gamma$  was shown to be expressed by mouse thymocytes, three RAR $\gamma$ -binding compounds (CD437, CD666 and CD2325) found previously to induce apoptosis in thymocytes<sup>25</sup> (Figure1c), were also ineffective. These data suggested that neither RAR $\beta$  nor RAR $\gamma$  are good candidates for mediating the effect of retinoids on GR-induced apoptosis.

Two RAR $\alpha$ -selective agonists, which alone have no effect on the background cell death rate (Figure 1c), however, effectively promoted GR-induced death of thymocytes (Figure 1b). The EC<sub>50</sub> values for apoptosis inhibition of the compounds were around 5 nM for CD2081 and Am580, respectively. These data suggest that ligation of RAR $\alpha$  may be responsible for the observed cell death promotion by RAs. To prove this further, the effect of retinoids was also tested in dexamethasone exposed thymocytes derived from RAR $\alpha$  knock out mice<sup>33</sup>. As shown in Figure 1d, while the RAR $\alpha$  agonists ATRA and AM580 were practically ineffective in enhancing GR-mediated death in these thymocytes, the biological activity of the RXR agonist LG268 and 9cRA remained unaffected. These data provide a direct proof that retinoids mediate

their apoptosis promoting effect via both RAR $\alpha$  and RXRs. Interestingly, when CD2503, an RAR $\alpha$  antagonist, was added to the culture, which alone did not affect spontaneous thymocyte death up to 10  $\mu$ M concentration (Figure 1c), also stimulated GR-induced thymocyte apoptosis with an EC value at around 5 nM (Figure 1b). CD2503 was acting also via RAR $\alpha$ , as it was ineffective in the RAR $\alpha$  knock out thymocytes (Figure 1d). Since CD2503, being an RAR $\alpha$  antagonist, cannot trigger the transcriptional activity of RAR $\alpha$ , this observation indicates that the effect of retinoids on the GR-induced death of thymocytes might not require the retinoid receptor's transcriptional activity.

Though DNA fragmentation is specific for the apoptotic form of cell death, possible DNA rearrangements during thymocyte differentiation might interfere with the assay. To prove that retinoids indeed enhance glucocorticoid induced death dying cells were simultaneously labeled with annexin V-FITC and propidium iodide. As seen in Figure 1e, 9cRA, AM580, CD2503 and LG268 all enhanced dexamethasone induced apoptosis. In addition, Am580 and LG268 promoted glucocorticoid-induced thymocyte death also under *in vivo* conditions resulting in enhanced cell death especially in the CD4CD8 double positive immature thymocyte population, which is known to be sensitive to glucocorticoids (Figure 1f)<sup>34</sup>.

**Retinoids do not promote glucocorticoid-induced PI-PLC phosphorylation.** Since activation of PI-PLC was reported to be non-genomic effect participating in dexamethasone-induced thymocyte cell death<sup>18</sup>, we decided to test whether retinoids could promote glucocorticoid-induced phosphorylation of PI-PLC. However, 9cRA, which was the most powerful natural RA in promoting glucocorticoid-induced apoptosis of thymocytes, was unable to enhance dexamethasone-induced phosphorylation of PI-PLC (Figure 1g), indicating that not PI-PLC is the main target of the retinoid action.



**Retinoids enhance glucocorticoid-induced expression of GILZ, a glucocorticoid target gene, during thymocyte apoptosis.** Increasing evidence suggests that in addition to transactivation of their own target genes, nuclear receptors are also capable of cross-talking with other transcription factors. The original observation was made in 1990, when it was shown that GR could inhibit, in a ligand-dependent manner, the ability of AP1 to transactivate its target gene promoters<sup>35</sup>. This transrepression was mutual and required an unknown state of the receptor, which could be induced by both receptor agonists and certain, but not all receptor antagonists. Since then it was also discovered that this crosstalk does not per se imply negative regulation of transcription, as several reports show that under certain conditions this cross-talk can lead to positive transcriptional effects<sup>36</sup>. As previous studies have shown that glucocorticoid-induced apoptosis is dependent on the transcriptional activity of GR<sup>4</sup>, we decided to investigate whether GR-induced transcriptional activity changes in the presence of various retinoids.

First we investigated whether addition of retinoids affect the amount of GR. However, no such effect was found indicating that retinoids do not affect the level of GR (Figure 2a).

Several genes have been reported to be upregulated during dexamethasone-induced death of thymocytes<sup>10</sup>. From these genes we selected GILZ<sup>6</sup> to test how its expression changes under the effect of various retinoids, since its promoter carries several glucocorticoid response elements, thus its transcription can be used a readout of the transcriptional activity of GR in the thymocytes<sup>37</sup>. As shown in Figure 2b, the expression of GILZ was not effected by retinoids alone, but its glucocorticoid-induced expression was further induced by the RAR $\alpha$  agonist and antagonist, and the RXR agonist tested in a dose dependent manner. These data indicate that retinoids are capable of enhancing glucocorticoid-induced gene expression, and ligation of RAR $\alpha$  or RXR alone is sufficient for the effect.

**RAR $\alpha$ /RXR heterodimers mediate the transactivating effects of retinoids.** To investigate further the transactivating phenomenon, the effect of retinoids was tested in an *in vitro* glucocorticoid reporter assay system using COS-1 cells. These cells express sufficient amount of GR to transactivate a GRE-luc construct in the presence of glucocorticoids, but lack detectable ATRA binding<sup>38</sup>. Figure 3A shows that following transient transfection of the

GRE-luc reporter plasmid the reporter enzyme is induced in COS-1 cells in a dose-dependent manner, but 10  $\mu$ M dexamethasone decreased the viability of these cells. Based on these results 0.1  $\mu$ M dexamethasone concentration was selected to test the effect of various retinoids on the glucocorticoid-induced transcription. Preliminary experiments have shown that none of the retinoids tested affected the basal luciferase expression (Supplementary figure 1). Neither did addition of retinoids affect the glucocorticoid-induced transactivation, in line with the lack of sufficient amount of functional RAR expression in these cells (Figure 3b-f).

To test the effect of various retinoid receptors involved, RAR $\alpha$  and RXR $\alpha$  were transfected alone or together (Figure 3g), and the dexamethasone-induced luciferase expression was tested in the presence of increasing concentrations of retinoids. As shown in Figure 3b-f, transfection of various retinoid receptors in the absence of retinoids did not affect the dexamethasone-induced luciferase expression. However, in the presence of retinoids we detected various transcription efficiencies. ATRA, the pan RAR agonist, at physiological concentrations, had no effect on the GR-induced transcription in the presence of RXR, and only slightly elevated the transcription in the presence of RAR $\alpha$  or RAR $\alpha$ /RXR $\alpha$  (Figure 3b). 9cRA, the pan RAR/RXR agonist, however, significantly elevated the GR-induced transcription in the presence of RXR $\alpha$ , but a most pronounced enhancement was observed when both receptors were present and activated by 9cRA (Figure 3c). These data suggested that ligation of RXR is capable of affecting GR-induced transcription, but a maximal enhancement is seen only, when both receptors are present and ligated by retinoic acids. To prove this statement further, we tested the effect of the synthetic retinoids. The RXR agonist LG268 significantly enhanced transcription in the presence of RXR alone, and to a similar degree in the presence of RAR $\alpha$ /RXR heterodimer (Figure 3d). AM580 (Figure 3e) and CD2503 (Figure 3f), similar to ATRA, had no effect in the presence of RXR, and only slightly elevated transcription, when only RAR $\alpha$  was expressed. In contrast to ATRA, however, when both retinoid receptors were expressed, these compounds significantly enhanced GR-induced transcription. When both Am580 and LG268 were added together in the presence of RAR $\alpha$ /RXR $\alpha$  heterodimers (Figure 3h), a more pronounced transcription was detected indicating that ligation of both sides of the receptor heterodimer results in a more

effective enhancement of GR-induced transcription. These data imply that RAR $\alpha$ /RXR heterodimers must mediate the effect of RAs on the glucocorticoid-induced transcription. Physiological concentration of ATRA, which bind only RARs, cannot induce the required conformation even if RXR is present. Simultaneous ligand binding of RXR is also required. The synthetic retinoid Am580 and CD2503, however, can induce the necessary conformation also in the absence of RXR binding. On the other side, RXR ligation alone is also sufficient to enhance GR-induced transcription.

Since CD2503 cannot transactivate RAR $\alpha$ , but is as effective as the RAR $\alpha$  agonists, we tested the possibility that DNA binding of the retinoid receptors is not required for promoting the transcriptional activity of the GR by using DNA binding mutants of the retinoid receptors (RAR $\alpha$ -LBD and RXR $\alpha$ -LBD) (Figure 3 g). As shown in Figure 3i, these receptors tested at one effective concentration of the retinoids were as effective as their wild-type variants.

**Glucocorticoid, RAR $\alpha$  and RXR receptors interact following ligand binding.** Since the cross-talks between various transcription factors are very often mediated via direct interaction which does not necessarily require DNA binding of the interacting partner, we investigated a possible interaction between the two nuclear receptors by immunoprecipitating the GR from thymocytes in the presence of various ligands, and searched for RAR $\alpha$  among the co-immunoprecipitated proteins. As shown in Figure 4a, equal amount of GR proteins were immunoprecipitated from thymocytes in the presence of various ligands. When, however, the co-immunoprecipitated proteins were investigated, co-immunoprecipitation of RAR $\alpha$  was not seen in the absence of dexamethasone, while addition of all the other ligands with the exception of physiological concentrations of ATRA were able to co-immunoprecipitate RAR $\alpha$  in the presence of dexamethasone. The fact that LG268, which is an RXR ligand, also induces interaction between RAR $\alpha$  and the ligated GR indicates that within the thymocytes LG268 must bind to RAR $\alpha$ /RXR heterodimers.

To prove that the interaction between the two nuclear receptors is direct and is not mediated for example by a protein present in the coactivator complex of the glucocorticoid receptor, the two mammalian hybrid technique was used. For this purpose 293 T fibroblast cells were transfected with the pmH100-TK-luc plasmid alone or together with the pCMX-Gal-L-hGR and/or the VP-hRAR $\alpha$ -LBD plasmids. As shown in Figure 4b, in cells transfected with pmH100-TK-luc alone, or together with pCMX-Gal-L-hGR or VP-hRAR $\alpha$ -LBD no significant luciferase activity could be detected. However, in pmH100-TK-luc/ pCMX-Gal-L-hGR transfected cells the presence of the VP-hRAR $\alpha$ -LBD was able to induce a similar degree of luciferase expression as dexamethasone in the absence of VP-hRAR $\alpha$ -LBD proving that the GR and RAR $\alpha$  can indeed directly interact. However, this interaction was not affected significantly by addition of retinoids or dexamethasone.

Since ligation of RXR $\alpha$  in the presence of RXR $\alpha$  alone could promote dexamethasone-induced transcription in the transient transfection assays (Figure 3d), using again the two mammalian hybrid technique we checked whether RXR $\alpha$  can also interact with GR. As shown in Figure 4c, in cells transfected with pmH100-TK-luc alone, or together with pCMX-Gal-L-hGR or VP-hRXR $\alpha$ -LBD no significant luciferase activity could be detected. However, in pmH100-TK-luc/ pCMX-Gal-L-hGR transfected cells the presence of the VP-hRXR $\alpha$ -LBD was able to induce a similar degree of luciferase expression as dexamethasone in the absence of VP-hRXR $\alpha$ -LBD proving that the GR and RXR $\alpha$  can indeed directly interact. This interaction was enhanced by addition of the RXR ligands. In the presence of the ligands of both GR and RXR receptors the interaction became more efficient detected by the enhanced luciferase activity. These data imply that strength of the interaction between GR and RXR $\alpha$  is enhanced by ligand binding of the RXR $\alpha$ , and might be also influenced by the ligand binding of the GR.

**Retinoids do not affect the Ser212 phosphorylation of the glucocorticoid receptor.** Although ligand binding is essential for initiating the transcriptional activity of the GR, the receptor is also subject of post-translational modification through phosphorylation<sup>39</sup>. Seven phosphorylation sites have been identified in the N terminal region of the mouse GR, from which Ser212 has been shown to be phosphorylated to a greater extent upon hormone exposure<sup>40</sup> and specifically on GRs located in the nucleus<sup>39</sup>. It has been also shown that phosphorylation can enhance the transcriptional activity of the GR in a promoter specific manner<sup>41</sup>. That is why we decided to test whether treatment of thymocytes with retinoids alter the dexamethasone-induced phosphorylation of Ser212 of the GR. Though addition of dexamethasone to thymocytes enhanced the phosphorylation of Ser212 on GR, retinoids had no effect on it indicating that ligated retinoid receptors must act via a different mechanism.

**Retinoids also promote glucocorticoid-induced apoptosis in malignant T cell lines.** To investigate whether the observed enhancing effect of retinoids on the GR-induced apoptosis is specific only for thymocytes, the effect of retinoids on GR-induced apoptosis was further tested using a murine (IP-12-7)<sup>42</sup> and two human (IG3<sup>43</sup> and CCRF-CEM<sup>44</sup>) glucocorticoid-sensitive T cell lines. As shown in Figure 6, 9cRA efficiently promoted the glucocorticoid-induced apoptosis of these T cell lines as well. In addition, in Figure 6d we also demonstrate that various retinoid specific ligands at selected concentrations affect GR-induced apoptosis of these T cell lines, similarly as they do in mouse thymocytes.

## **Discussion**

Retinoic acids, the derivatives of vitamin A, are widely known to affect various immune functions<sup>45</sup>. Here we show that retinoids can also stimulate glucocorticoid-induced apoptosis in immature thymocytes. Using various receptor specific retinoids or RAR $\alpha$  knock out thymocytes we have demonstrated that the effect is mediated via RAR $\alpha$  and RXR, and simultaneous ligation of both RAR $\alpha$  and RXRs by the natural RAs, or ligation of RXR $\alpha$  or

RARs alone by the synthetic retinoids are required for the phenomenon. Since an RAR $\alpha$  specific antagonist also enhanced glucocorticoid-induced apoptosis, we proposed that the enhancing capability of the retinoid receptor does not involve its transcriptional activity. As phosphorylation of PI-PLC, one of the known non-genomic effect of glucocorticoids reported to participate in dexamethasone-induced death of thymocytes<sup>18</sup>, was not affected by retinoids, we investigated other mechanisms to explain the observed phenomenon.

Because nuclear receptors are known to interact with various transcription factors and regulate their transcriptional activity in a ligand-dependent manner<sup>35,36</sup>, we tested the possibility that retinoid receptors interact with the GR to regulate its transcriptional activity. We found that retinoids can enhance glucocorticoid-induced transcription of GILZ in thymocytes and a GR-driven reporter construct in COS-1 cells in an RAR $\alpha$ /RXR-dependent manner. In the presence of the RAR $\alpha$ /RXR heterodimer all the investigated RAR $\alpha$  and RXR agonists and the RAR $\alpha$  antagonist could enhance the transcription added alone, while in the case of physiological concentrations of ATRA, ligation of RAR $\alpha$  alone was not sufficient. On the other hand, RXR agonists could also enhance GR-induced transcription if only RXR was expressed. These data pointed for a strong role of RXR in regulating GR-induced transcription, but also a contribution from the RAR $\alpha$  side in the heterodimer.

GR and RXR $\alpha$  or RAR $\alpha$  directly interacted in a two mammalian hybrid assay in the absence of ligands, and only the RXR ligand enhanced this basal interaction especially in the presence of the GR ligand. On the other hand RAR $\alpha$  could be co-immunoprecipitated with GR from thymocytes, but only if thymocytes were exposed simultaneously to dexamethasone and to those retinoids which were effective in enhancing glucocorticoid-induced thymocyte cell death, including the RXR agonist LG268. Based on these data we propose that in cells ligated RAR $\alpha$ /RXR heterodimers and ligated GR interacting with each other result in the enhanced transcriptional activity of the GR. Under physiological conditions, when the receptors are expressed at physiological levels one role of the GR ligand in mediating the GR/RAR $\alpha$ /RXR interaction is to promote the nuclear translocation of GR into the nucleus, where the GR and retinoid receptors can physically interact. In the two mammalian hybrid assay overexpressed GR might saturate the levels of proteins that keep it in the cytosol

resulting in nuclear translocation of the GR and interaction with retinoid receptors even in the absence of the dexamethasone, as in other experiments we found overexpressed GR-GFP proteins in the nucleus even in the absence of the GR ligand (Brázda P. unpublished observations). Since in the transient transfection assays ligated RAR $\alpha$  alone only slightly affected GR-mediated transcription, while ligated RXR $\alpha$  was fully effective, and the RXR ligand enhanced the GR/RXR $\alpha$  interaction, while the RAR $\alpha$  ligand had no effect on the GR/RAR $\alpha$  interaction, we propose that the RXR side of the RAR $\alpha$ /RXR heterodimer regulates the transcriptional activity of the GR. The conformation of RXR required for the interaction with GR can be stabilized by both the RAR $\alpha$  and the RXR ligands acting on the heterodimer. Since ATRA, and the RAR $\alpha$  synthetic ligands differ in their ability to regulate RAR $\alpha$ /RXR-mediated transcription and to induce interaction with the GR, we propose that they all stabilize a different conformation of RAR $\alpha$ , some of which promote or inhibit transcription, while others promote the interaction. The mechanism through which this interaction promotes GR-mediated transcription does not require the DNA binding of the retinoid receptors and does not alter the phosphorylation status of Ser212, known to regulate the transcriptional activity of GR. All together our data reveal a novel signal crosstalk between the GR and RAR signaling pathways demonstrating that RARs similarly to GRs<sup>35,36</sup>, can also enhance the transcriptional activity of other transcription factors with which they interact.

Thymic epithelial cells play a central role in guiding the development of immature thymocytes. Both glucocorticoids<sup>31</sup> and retinoids<sup>32</sup> were shown to be produced by thymic epithelial cells. Since they both can stimulate the death of neglected thymocytes either alone<sup>2,23-25</sup> or, as it is shown here, by interacting with each other, we propose that under *in vivo* conditions the production of the two molecules will provide an excellent environment for the fast removal of the neglected cells, the TCR of which is unable to interact with self MHC.

Retinoids are already widely used in the treatment of cutaneous T-cell lymphoma and certain B cell malignancies<sup>46</sup>. Our data, which demonstrate that retinoids can also promote glucocorticoid-induced apoptosis of T cell lines, indicate that retinoids could also be used in

the treatment of glucocorticoid sensitive T cell malignancies to enhance the therapeutical efficacy of glucocorticoids.

## **Materials and methods**

**Retinoids and plasmids.** All the retinoids used in this study were from the Galderma Research & Development (Sophia Antipolis, France), with the exception of ATRA and 9cRA, which were from Sigma–Aldrich (Budapest Hungary), Am580, which was purchased from Tocris Bioscience (Ellisville, MO) and LG00268 (LG268), which was from a gift from R. Heyman (Ligand Pharmaceuticals). These retinoids were characterized in our previous papers<sup>25,27,28</sup>. All the plasmids used in these studies were a kind gift from Ron Evans (San Diego, California) and were described previously<sup>47</sup>.

**Mice.** 4 weeks old male NMRI mice purchased from LATI (Gödöllő, Hungary) were used. For the induction of *in vivo* thymic apoptosis mice were treated i.p. with 0.5 mg dexamethasone-acetate (Sigma-Aldrich) alone or either with 50 µg Am580 or with 50 µg LG268 dissolved in a mixture of 0.1 ml ethanol and 0.4 ml physiological saline. Control animals were injected with the same amount of vehicle. Study protocols were approved by the Animal Care Committee of the University of Debrecen.

**Characterization of thymocyte subpopulations.** Thymocytes were isolated after 24 hr of various *in vivo* treatments. Cells were washed twice and resuspended in ice-cold PBS containing 0.1% (w/v) sodium azide before staining with PE-labeled anti-CD4 and FITC-conjugated anti-CD8 (BD Biosciences Pharmingen, Erembodegen, Belgium). The cells were incubated with agitation for 30 min at 4°C, washed twice with ice cold PBS supplemented with 1% BSA and 0.1% sodium azide, and resuspended in PBS containing 0.1% sodium



azide. Unstained thymocytes treated similarly served as autofluorescence controls. Dual fluorescence was analyzed on a Becton Dickinson FACScan (BD Biosciences) with excitation at 488 nm.

**Thymocyte culture, cell lines and apoptosis detection.** Thymocyte suspensions were prepared from thymus glands of 4 week-old NMRI or RAR $\alpha$  knock out<sup>33</sup> mice by mincing the glands in RPMI 1640 media (Sigma-Aldrich, Budapest, Hungary) supplemented with 10% charcoal-treated FCS, 2 mM glutamine, and 100 IU penicillin/100  $\mu$ g streptomycin/ml. Thymocytes were washed three times and diluted to a final concentration of  $5 \times 10^6$  cells/ml before incubation at 37°C in a humidified incubator under an atmosphere of 5% CO<sub>2</sub> /95% air. Cell death was measured by trypan blue uptake. A total of 95-98% of cells routinely excluded trypan blue after the isolation procedures. The IP-12-7 CD4<sup>+</sup> T cell hybridoma was developed from BALB/c mouse pre-immunized with a synthetic peptide 317-329 H1 (covering the C-terminal of the HA1 subunit of the human influenza virus A/PR/34/8) and subsequently infected with the a/PR/8/34 human influenza virus<sup>42</sup>. The Kit225 IG3 cell line is an IL-2-independent subclone of the Kit225 human T leukemic cells with helper/inducer phenotype<sup>43</sup>. CCRF-CEM cells derived from the peripheral blood buffy coat of a child (CEM) with acute lymphoblastic leukemia who had originally presented with lymphosarcoma<sup>44</sup> was a kind gift from Edit Buzas (Budapest). Thymocytes and the T cell lines were treated with dexamethasone acetate (0.1  $\mu$ M) and various retinoids for 6hrs in the presence of 10% charcoal-treated FCS (Sigma-Aldrich, Budapest, Hungary). At each culture the final concentration of DMSO used as the dissolvent for retinoids was 0.5%. At the end of culture the percentage of DNA degraded in the thymocyte cultures was determined by diphenylamine reagent, as it was described previously<sup>23,25,28</sup>. In the case of the T cell lines the percentage of cells containing degraded DNA (sub G<sub>0</sub>-G<sub>1</sub> cells) was determined by flow cytometry analysis

in ethanol-fixed cells following RNAaseA and propidium iodide treatment. For further confirmation of apoptosis induction, Annexin V binding was performed on thymocytes treated at various ways using a standard kit from BD Pharmingen (San Diego, CA) to measure apoptosis. After rinsing cells twice with PBS, cells were resuspended in 100  $\mu$ l of 1x binding buffer in a flow cytometry tube, to which 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l of propidium iodide were added and mixed well. After a 15-min incubation at room temperature in the dark, 400  $\mu$ l of 1x binding buffer was added and flow cytometry was performed within 15 min. 293 T fibroblast cells used for the mammalian two-hybrid system and COS-1 cells used for the reporter assay were grown in DMEM medium supplemented with 10% charcoal treated FCS and antibiotics.

**PI-PLC immunoprecipitation and Western blot.** Thymocytes were treated with vehicle alone, dexamethasone-acetate (1  $\mu$ M) alone or together with 9-cRA (0.3  $\mu$ M) for 30 min. After treatment, cells were harvested, and whole cell lysates were prepared in RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin. Phosphotyrosine-containing proteins were immunoprecipitated with agarose-conjugated 4G10 antibodies (Upstate Biotechnology, Waltham, MA). PI-PLC in the 4G10 immunoprecipitates was measured by Western blot. Membranes were incubated with the monoclonal anti-PI-PLC antibody (Upstate Biotechnology, Waltham, MA). Antigen-antibody complexes were detected by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL).

**Q-PCR for detecting changes in the mRNA expression of GILZ .** Total RNA was isolated with RNeasy® Mini Kit (Quiagen, Hilden, Germany) from treated cells. Transcript

quantitation was performed via quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman probes. Every sample was assayed in triplicates. The RT reaction was performed at 42°C for 30 min and 72°C for 5 min using 100 ng total RNA, specific reverse primer (Bio-Science, Pécs, Hungary) and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). Real-time monitoring was carried out using an ABI Prism 7900 performing 40 cycles of 94°C for 12 sec and 60°C for 1 min. Values of transcripts in unknown samples were calculated from standard curve derived from transcript specific oligonucleotides. Transcript levels were normalized to the level of cyclophilin (Bio-Science, Pécs, Hungary). The following primers were used: mGILZ reverse: 5'-CTT CAG TGG ACA GAT CAG GGA G-3'; mGILZ forward: 5'-AGA CCA GCC TCA CAA TGC G-3'; mCyc reverse: 5'-TCT GCT GTC TTT GGA ACT TTG TC-3'; mCyc forward: 5'-CGA TGA CGA GCC CTT GG-3'.

**Reporter gene assays following transient transfections.** COS-1 cells were transfected at 60-80% confluency with the pCMX-GRE-luc and pCMX- $\beta$ -GAL plasmids alone, or together with pCMX-FL-hRAR $\alpha$  and/or pCMX-FL-hRXR $\alpha$  plasmids using polyethylene-imine (Promega, Madison, Wisconsin). Transfection was carried out in Dulbecco's modified essential medium containing 10% charcoal-stripped bovine calf serum (Sigma-Aldrich, Budapest, Hungary). After 6 hrs the medium was changed to Dulbecco's modified essential medium containing the indicated ligands or vehicle. Cells were lysed and assayed for reporter expression 48 hrs after transfection. Luciferase activity of each sample was normalized to the galactosidase activity as described above. Each transfection was carried out in triplicates.

**Western blot analysis of RAR $\alpha$  or RXR receptor expression in COS1 cells following transient transfections.** At 48 hours following transfection cells were harvested and boiled in

2x sample buffer and loaded onto SDS PAGE gels. PVDF membranes were probed for anti-mouse RAR $\alpha$  or RXR $\alpha$  (SantaCruz, California) and  $\beta$ -actin antibodies (Sigma).

**Co-immunoprecipitation.** To demonstrate possible direct interaction between the GR and the RAR $\alpha$ , thymocytes were exposed to dexamethasone-acetate (1  $\mu$ M) and to various retinoids alone or together for 1 hr in an RPMI medium containing 10% charcoal treated FCS. Thymocytes ( $4 \times 10^7$ ) were then lysed, and the GR was immunoprecipitated with agarose-conjugated anti-mouse GR antibody (SantaCruz, California) according to the manufacturer's instructions. Following 12% SDS electrophoresis immunoprecipitated proteins were analysed with Western blot technique using anti-mouse GR and anti-mouse RAR $\alpha$  antibodies (SantaCruz, California). In some experiments the immunoprecipitated GR was analysed for Ser211 phosphorylation by a site specific antibody from Abcam.

**Mammalian two hybrid system.** To demonstrate direct interaction between GR and RAR $\alpha$  or RXR $\alpha$  a mammalian two hybrid system was used. For this purpose 293 T fibroblast cells were transfected with pCMX-Gal-L-hGR and pMH100-TK-luc plasmids alone or either with VP-hRAR $\alpha$ -LBD or VP-hRXR $\alpha$ -LBD together. pCMX- $\beta$ -galactosidase plasmid was used to normalize transfection efficiency, VDR<sup>-1</sup> plasmid, which codes a reverse sequence for the vitamin D receptor, was used to equalize the total amount of plasmid DNA (1  $\mu$ g/  $10^5$  cells/well) transfected. Luciferase activities were detected 48 hrs later by using the kit from Promega (Madison, Wisconsin), while  $\beta$ -galactosidase activities were detected by the kit from Fermentas (St. Leon-Rot, Germany) according to the manufacturer's directions. In this assay luciferase enzyme will be induced only if a, either glucocorticoid acts on a pmH100-TK-luc and pCMX-Gal-L-hGR transfected cell, because following ligand binding the transfected GR bound to the luciferase promoter with its Gal fusion DNA binding domain will drive the

transactivation, or b, in the absence of glucocorticoids the VP-hRAR $\alpha$ -LBD or VP-hRXR $\alpha$ -LBD plasmid coded RAR $\alpha$  or RXR $\alpha$  fusion proteins that carry a strong transactivation domain of Herpes simplex virus origin (VP), but have no DNA binding ability interact with the pCMX-Gal-L-hGR coded GR fusion protein and drive the transactivation. This later luciferase activities demonstrate the interaction between the glucocorticoid and two retinoid receptors.

## **Acknowledgement**

This work was supported by Hungarian National Research Fund (OTKA T049445, K 77587 and NI 67877) Ministry of Welfare T (115/2006), EU (MRTN-CT-2006-036032 and 2006-035624, LSHB-CT-2007-037730) and TÁMOP 4.2.1./B-09/1/KONV-2010-0007 project. The latest project is implemented through the New Hungary Development Plan, co-financed by the European Social Fund. We thank the Galderma Research & Development for providing the retinoid receptor ligands. The technical assistance of Edit Komóczi and Zsolt Hartmann is gratefully acknowledged.

## References

1. Tuckermann JP, Kleiman A, McPherson KG and Reichardt HM, Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. *Crit Rev Clin Lab Sci* 2005; 42: 71-104
2. Wyllie A, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980; 284: 555-556
3. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K *et al.* The nuclear receptor superfamily: the second decade. *Cell* 1995; 83: 835-839
4. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R *et al.* DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 1995; 93: 531-541
5. Cannarile L, Zollo O, D'Adamio F, Ayroldi E, Marchetti C, Tabilio A *et al.* Cloning, chromosomal assignment and tissue distribution of human GILZ, a glucocorticoid hormone-induced gene. *Cell Death Differ* 2001; 8: 201-203
6. Delfino DV, Agostini M, Spinicelli S, Vito P and Riccardi C, Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. *Blood* 2004; 104: 4134-4141
7. Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F and Distelhorst CW, Dexamethasone-induced gene 2 (dig2) is a novel prosurvival stress gene induced rapidly by diverse apoptotic signals. *J Biol Chem.* 2003; 278: 27053-27058
8. Malone MH, Wang Z and Distelhorst CW, The glucocorticoid-induced gene ttag8 encodes a pro-apoptotic G protein-coupled receptor whose activation promotes glucocorticoid-induced apoptosis. *J Biol Chem* 2004; 279: 52850-52859

9. Wang Z, Malone MH, He H, McColl KS and Distelhorst CW, Microarray analysis uncovers the induction of the proapoptotic BH3 only protein Bim in multiple models of glucocorticoid-induced apoptosis. *J Biol Chem* 2003; 278: 23861-23867
10. Woodward MJ, de Boer J, Heidorn S, Hubank M, Kioussis D, Williams O and Brady HJ, Tnfrsf8 is an essential gene for the regulation of glucocorticoid-mediated apoptosis of thymocytes. *Cell Death Differ* 2010; 17: 316-323
11. Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ, Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 1993; 75: 229-240
12. Rathmell JC, Lindsten T, Zong WX, Cinalli RM and Thompson CB, Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nat Immunol* 2002; 3: 932-939
13. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F *et al.* Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; 286: 1735-1738
14. Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ *et al.* p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003; 302: 1036-1038
15. Ma A, Pena JC, Chang B, Margosian E, Davidson L, Alt FW *et al.* Bclx regulates the survival of double positive thymocytes. *Proc Natl Acad Sci USA*. 1995; 92: 4763-4767
16. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS *et al.* Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* 1998; 94: 339-352

17. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R *et al.* Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 1998; 94: 739-750
18. Cifone MG, Migliatorati G, Parroni R, Marchetti C, Millimaggi D, Santoni A *et al.* Dexamethasone-induced thymocyte apoptosis: apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. *Blood* 1999; 93: 2282-2296
19. Marchetti C, Di Marco B, Cifone MG and Riccardi C, Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. *Blood* 2003; 101:585-593
20. Lépine S, Lakatos B, Courageot M, Le Stunff H, Sulpice J and Giraud F, Sphingosine Contributes to Glucocorticoid-Induced Apoptosis of Thymocytes Independently of the Mitochondrial Pathway. *J Immunol* 2004; 173: 3783-3790
21. Salmena L, Lemmers B, Hakem A, Matysiak-Zablocki E, Murakami K, Au PY *et al.* Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev.* 2003; 17: 883-895
22. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM *et al.* 9-cis retinoic acid is a high affinity ligand for the retinoic acid X receptor. *Cell* 1992; 68: 397-406
23. Fésüs L, Szondy Z and Uray I, Probing the molecular program of apoptosis by cancer chemopreventive agents. *J Cell Biochem* 1995; Suppl.22: 151-161
24. Xue Y, Chomez P, Castanos-Velez E, Biberfeld P, Perlmann T and Jondal M, Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression. *Eur J Immunol* 1997; 27: 2048-2056



25. Szondy Z, Reichert U, Bernardon JM, Michel S, Tóth R, Ancian P *et al.* Induction of apoptosis by retinoids and RAR gamma selective compounds in mouse thymocytes through a novel apoptosis pathway. *Mol Pharmacol* 1997; 51: 972-982
26. Iwata M, Mukai M, Nakai Y and Iseki R, Retinoic acids inhibit activation-induced apoptosis in T cell hybridomas and thymocytes. *J Immunol* 1992; 149: 3302-3308
27. Szondy Z, Reichert U and Fésüs L, Apoptosis regulation of T lymphocytes by retinoic acids: a novel mode of interplay between RAR and RXR receptors in regulating T lymphocyte death. *Cell Death Diff* 1998, 5: 4-10
28. Szondy Z, Reichert U, Bernardon JM, Michel S, Tóth R, Karászi E *et al.* Inhibition of activation-induced apoptosis of thymocytes by all-trans and 9-cis retinoic acids is mediated via retinoic acid receptor alpha. *Biochem J* 1998; 331: 767-774
29. Szegezdi E, Kiss I, Simon A, Blaskó B, Reichert U, Michel S *et al.* Ligand of RAR $\alpha$  regulates negative selection of thymocytes by inhibiting both DNA binding of Nur77 and the synthesis of Bim. *J Immunol* 2003; 170: 3014-3022
30. Zhang XK, Lehmann J, Hoffmann B, Dawson ML, Cameron J, Graupner G *et al.* Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* 1992; 358: 587-591
31. Vacchio MS, Papadopoulos V and Ashwell JD, Steroid production in the thymus: implications for thymocyte selection. *J Exp Med* 1994; 179:1835-1846
32. Kiss I, Rühl R, Szegezdi E, Fritzsche B, Tóth B, Pongrácz J *et al.* Retinoid receptor activating ligands are produced within the mouse thymus during postnatal development. *Eur J Immunol* 2008; 38:147-155
33. Chapellier B, Mark M, Garnier JM, LeMeur M, Chambon P and Ghyselinck NB, A conditional floxed (loxP-flanked) allele for the retinoic acid receptor alpha (RARalpha) gene. *Genesis* 2002 ; 32:87-90

34. Cohen JJ, Glucocorticoid-induced apoptosis in the thymus. *Semin Immunol* 1992; 4:863-869
35. Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J *et al.* Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* 1990; 62: 1205-1215
36. Shemshedini L, Knauth R, Corsi-Sassone P, Pornon A and Gronemeyer H, Cell specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. *EMBO J* 1991; 10: 3839-3849
37. Muzikar KA, Nickols NG and Dervan PB, Repression of DNA-binding dependent glucocorticoid receptor-mediated gene expression. *Proc Natl Acad Sci USA* 2009; 106:15598-15603
38. Crettaz, M, Baron A, Siegenthaler G and Hunziker W, Ligand specificities of recombinant retinoic acid receptors RAR alpha and RAR beta. *Biochem J* 1990; 272: 391–397
39. Wang Z, Frederick J and Garabedian MJ, Deciphering the phosphorylation "code" of the glucocorticoid receptor in vivo. *J Biol Chem* 2002; 277:26573-26580
40. Bodwell, JE, Orti E, Coull JM, Pappin DJC, Smith LI and Swift FJ, Identification of phosphorylated sites in the mouse glucocorticoid receptor. *J Biol Chem* 1991; 266:7549-7555
41. Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M and Cidlowski JA, Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *J Biol Chem* 1997; 272:9287–9293

42. Rajnavölgyi E, Nagy Z, Kurucz L, Gogolák P, Tóth G, Váradi G *et al.* T cell recognition of the posttranslationally cleaved intersubunit region of influenza virus haemagglutinin. *Mol Immunol* 1994; 31: 1403-1414
43. Eicher DM and Waldmann TA, IL-2R alpha on one cell can present IL-2 to IL2R beta/gamma (c) to another cell to augment IL-2 signaling. *J Immunol* 1998; 161: 5430-5437
44. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA and McCarthy RE, Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukaemia. *Cancer* 1965; 18:522-529
45. Mora JR, Iwata M and von Adrian UH, Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol* 2008; 8: 685-698
46. Zhang C and Duvic M, Retinoids: therapeutic applications and mechanisms of action in cutaneous T-cell lymphoma. *Dermatol Ther* 2003; 16: 322-330
47. Chen JD and Evans RM, A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 1995; 377:454-457

## LEGENDS TO THE FIGURES

**Figure 1** Retinoids promote glucocorticoid-induced apoptosis of thymocytes via RAR $\alpha$ /RXR.

(a) Dexamethasone-acetate induces DNA fragmentation in mouse thymocytes in a dose dependent manner detected at 6h following addition. (b) The RAR $\alpha$  agonists ATRA, 9cRA, Am580 and CD2081, the RXR agonist LG268, a combination of ATRA with LG268 (0.1 nM), and an RAR $\alpha$  antagonist CD2503, all promote dexamethasone (0.1  $\mu$ M) -induced DNA fragmentation of mouse thymocytes. The amount of DNA degradation in the presence of glucocorticoid alone was  $45 \pm 4$  %. (c) The RAR $\gamma$  agonists CD437, CD666 and CD2325 alone induce DNA fragmentation, while compounds acting on RXR or RAR $\alpha$  do not alter the basal DNA fragmentation ( $8 \pm 3$  %) of thymocytes detected at 6h following addition of the retinoids. (d) The RXR agonists 9cRA and LG268 can, but the RAR $\alpha$  agonists are unable to promote significantly the dexamethasone acetate (0.1  $\mu$ M) -induced DNA fragmentation of RAR $\alpha$  knock out mouse thymocytes. The amount of DNA degradation in the presence of glucocorticoid alone was  $45 \pm 4$  %. Data represent mean $\pm$ S.D. of three determinations. (e) 9cRA, AM580, CD2503 and LG268 all promote glucocorticoid-induced apoptosis of thymocytes *in vitro* detected by propidium iodide/annexin V labeling. (f) Injection of both Am580 (50 $\mu$ g) and LG268 (50 $\mu$ g) significantly enhances dexamethasone (0.2mg)-induced CD4<sup>+</sup>CD8<sup>+</sup> thymocyte apoptosis *in vivo* determined at 24hrs following treatment. Data (one representative experiment out of three) show number of surviving thymocytes and the distribution of various thymocyte cell populations following *in vivo* treatments. (g) Glucocorticoid-induced PI-PLC activation is not enhanced by retinoids in mouse thymocytes. Tyrosine phosphorylated proteins were immunoprecipitated (IP) from cell lysates with agarose conjugated 4G-10 antibodies, and PI-PLC in the immunoprecipitate was assessed by Western blot with anti-PI-PLC antibodies. The results are representative of one of three independent experiments.

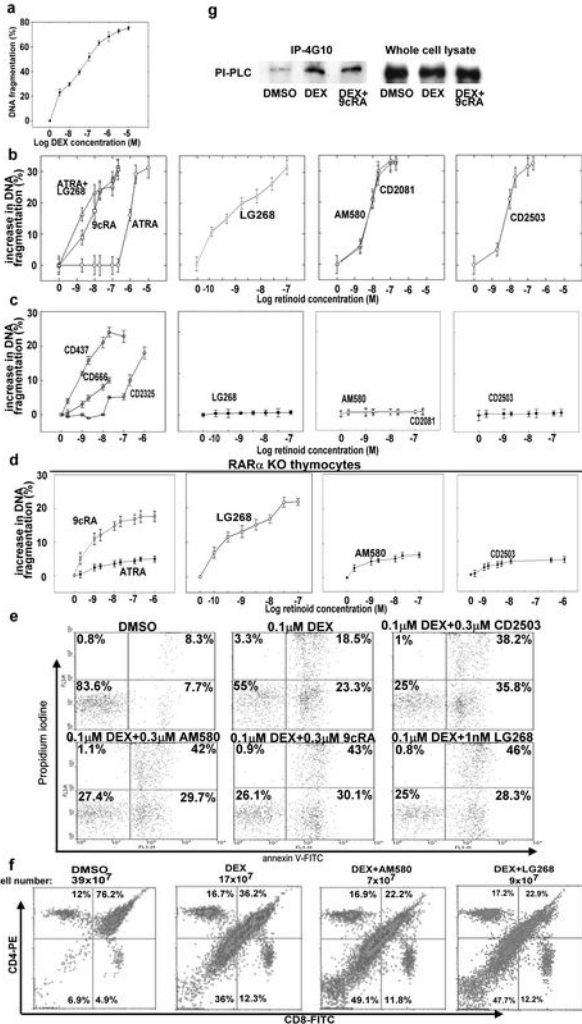
**Figure 2** Retinoids enhance glucocorticoid-induced expression of GILZ in mouse thymocytes. **(a)** Retinoids do not affect the basal levels of glucocorticoid receptors. Isolated thymocytes ( $10^7$  cells/ml) were exposed to 0.1  $\mu$ M dexamethasone acetate alone or together with 0.3  $\mu$ M ATRA, 9cRA, Am580 or CD2503 for 2 hours. Levels of the glucocorticoid receptors were determined by immunoblot analysis.  $\beta$ -actin was used as loading control. **(b)** Retinoids enhance the glucocorticoid-induced expression of GILZ in a dose dependent manner. Isolated mouse thymocytes were exposed to 0.1  $\mu$ M dexamethasone acetate and the indicated concentrations of retinoids. mRNA levels of GILZ were determined 2 hours later. Data represent mean $\pm$ S.D. of three determinations. \*Significantly different from glucocorticoid-treated control determined by student paired *t* test ( $p < 0.05$ ).

**Figure 3** RAR $\alpha$ /RXR heterodimers mediate the transactivating effects of retinoids. **(a)** Dexamethasone-acetate induces the expression of the pCMX-GRE-luc reporter construct in a dose dependent manner in COS1 cells transfected transiently. Effect of increasing concentrations of ATRA **(b)**, 9cRA **(c)**, LG268 **(d)**, AM580 **(e)**, CD2503 **(f)** on the dexamethasone (0.1  $\mu$ M)-induced expression of the pCMX-GRE-luc reporter in the presence of the indicated full length retinoid receptors. **(g)** Western blot analysis of retinoid receptor expression before and after transient transfections of COS-1 cells. In comparison the endogenous level of retinoid receptors in the IG3 T cell line<sup>43</sup> is also shown. **(h)** Effect of the combination of AM580 and LG268 on the dexamethasone (0.1  $\mu$ M)-induced expression of the pCMX-GRE-luc reporter in the presence of the full length RAR $\alpha$ /RXR $\alpha$  receptors. **(i)** Effect of the indicated concentrations of 9cRA, LG268 and AM580 on the dexamethasone (0.1  $\mu$ M)-induced expression of the pCMX-GRE-luc reporter in the presence of retinoid receptors not capable of DNA binding. Data represent mean $\pm$ S.D. of three independent experiments. \*Significantly different from glucocorticoid-treated control determined by student paired *t* test ( $p < 0.05$ ).

**Figure 4** The ligated retinoid receptors directly interact with the ligated GR. (a) RAR $\alpha$  coimmunoprecipitates with GR only when the ligand of both receptors is present. GR was immunoprecipitated from cell lysates with agarose conjugated anti-GR antibodies, and GR and RAR $\alpha$  in the immunoprecipitate was assessed by Western blot with anti-GR and anti-RAR $\alpha$  antibodies. The dose of glucocorticoid was 0.1  $\mu$ M, that of retinoids 0.3  $\mu$ M, except for LG268, which was 100 nM. The results are representative of one of three independent experiments. (b) Two-hybrid mammalian system reveals a direct interaction between GR and RAR $\alpha$ . (c) Two-hybrid mammalian system reveals a direct interaction between GR and RXR $\alpha$ , which is influenced by the presence of the receptor ligands.

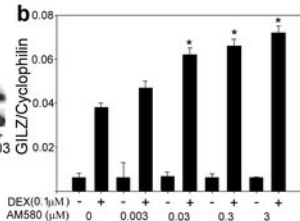
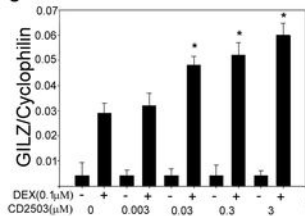
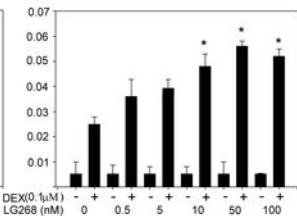
**Figure 5** Glucocorticoid-induced Ser211 phosphorylation of GR is not affected by retinoids. Thymocytes were exposed to 0.1  $\mu$ M dexamethasone acetate alone or with 0.3  $\mu$ M Am580, 0.3 $\mu$ M CD2503 or 50 nM LG268 for 1 hr. GR was immunoprecipitated from cell lysates with agarose conjugated anti-GR antibodies, and GR and its phosphorylated Ser211 in the immunoprecipitate was assessed by Western blot by using specific antibodies.

**Figure 6** Retinoids enhance glucocorticoid-induced apoptosis of various T cell lines as well. 9cRA enhances dexamethasone-acetate (0.1  $\mu$ M)-induced apoptosis of (a) IP-12-7 T hybridoma (b) IG3 and (c) CCRF-CEM T cells in a dose dependent manner determined at 6h following treatment. (d) RAR $\alpha$  and RXR $\alpha$  ligands also enhance dexamethasone acetate (0.1  $\mu$ M)-induced apoptosis in CCRF-CEM T cells. Data represent mean $\pm$ S.D. of three determinations. \*Significantly different from glucocorticoid-treated control determined by student paired *t* test ( $p < 0.05$ ).

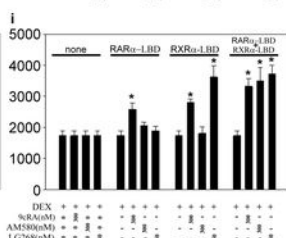
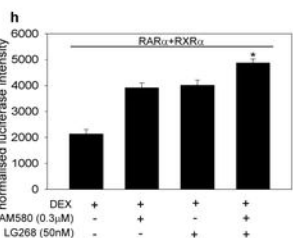
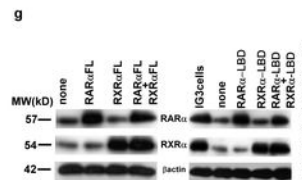
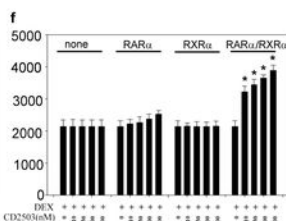
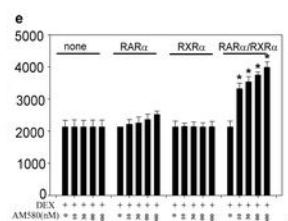
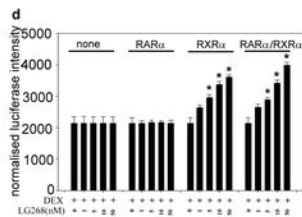
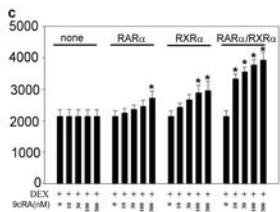
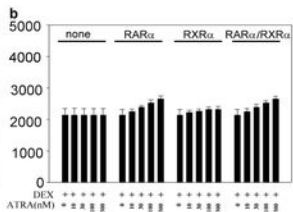
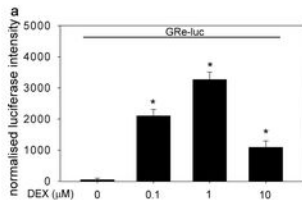




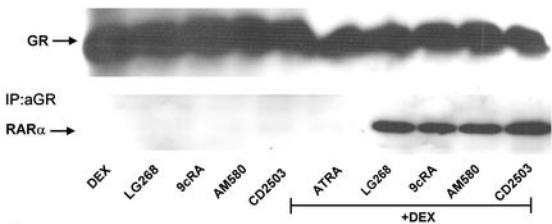
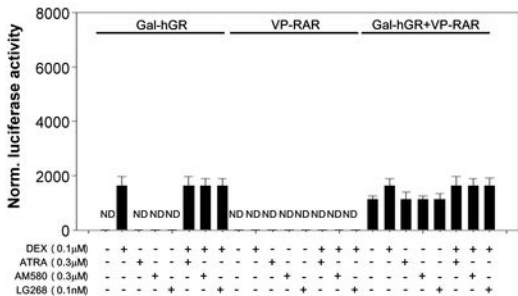
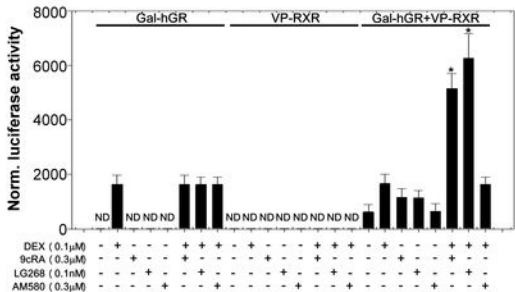


**a****b****c****d**







**a****b****c**



S211 ↓

GR ↓

DMSO

DEX

DEX  
+  
LG268

DEX  
+  
AM580

DEX  
+  
CD2503

