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

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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 shown. 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 by retinoic acid receptor (RAR) alpha/retinoid X receptor (RXR) heterodimers, and occurs when both RARα and RXR are ligated by retinoic acids. We show that the ligated RARα/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 Ser232, 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.

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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.[1] Glucocorticoid-induced apoptosis of thymocytes is one of the first recognized forms of programmed cell death.[2] For this effect, glucocorticoids passively diffuse into the cell and bind to the glucocorticoid receptor (GR), a member of the nuclear receptor super-family.[3] Subsequently, the hormone–receptor complex trans-locates into the nucleus, where it modulates gene expression either by direct binding to its cognate response element or by interaction with other transcription factors. In the case of thymocyte apoptosis, gene activation is essential to the process, as 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.[4] During the past decades, many of the genes involved have been identified.[5–10] Increasing evidence suggests that the effector phase of glucocorticoid-induced apoptosis is mediated by the mitochondrial pathway involving Bcl-2 family members.[11–15] 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.[16,17]

In addition to the genomic effects, glucocorticoids induce a rapid Src-dependent phosphorylation of the phosphatidyl-inositol-specific phospholipase C (PI-PLC),[18,19] 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.[18,20,21]

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

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have a physiological role in regulating thymocyte differentiation and apoptosis.

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

Results

Ligation of both RARα 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 6 h following addition, detected by determining the amount of DNA degradation (Figure 1a). As it was reported previously,[23,26] 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 μM, in which 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 1c), 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 RARβ expression in mouse thymocytes,[25] the RARβ-selective compound (CD2314) tested could not promote GR-induced apoptosis (data not shown). Although RARγ was shown to be expressed by mouse thymocytes, three RARγ-binding compounds (CD437, CD666 and CD2325) found previously to induce apoptosis in thymocytes[25] (Figure 1c) were also ineffective.

These data suggested that neither RARβ nor RARγ are good candidates for mediating the effect of retinoids on GR-induced apoptosis.

Two RARα-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$_{50}$ values for apoptosis inhibition of the compounds were around 5 nM for CD2081 and Am580, respectively. These data suggest that ligation of RARα 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 RARα knockout mice.[33] As shown in Figure 1d, while the RARα 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 by both RARα and RXRs. Interestingly, when CD2503, an RARα antagonist, was added to the culture, which alone did not affect spontaneous thymocyte death up to 10 μM concentration (Figure 1c), also stimulated GR-induced thymocyte apoptosis with an EC value at around 5 nM (Figure 1b). CD2503 was acting also via RARα, as it was ineffective in the RARα knockout thymocytes (Figure 1d). As CD2503, being an RARα antagonist, cannot trigger the transcriptional activity of RARα, this observation indicates that the effect of retinoids on the GR-induced death of thymocytes might not require the retinoid receptor's transcriptional activity.

Although 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).[34]
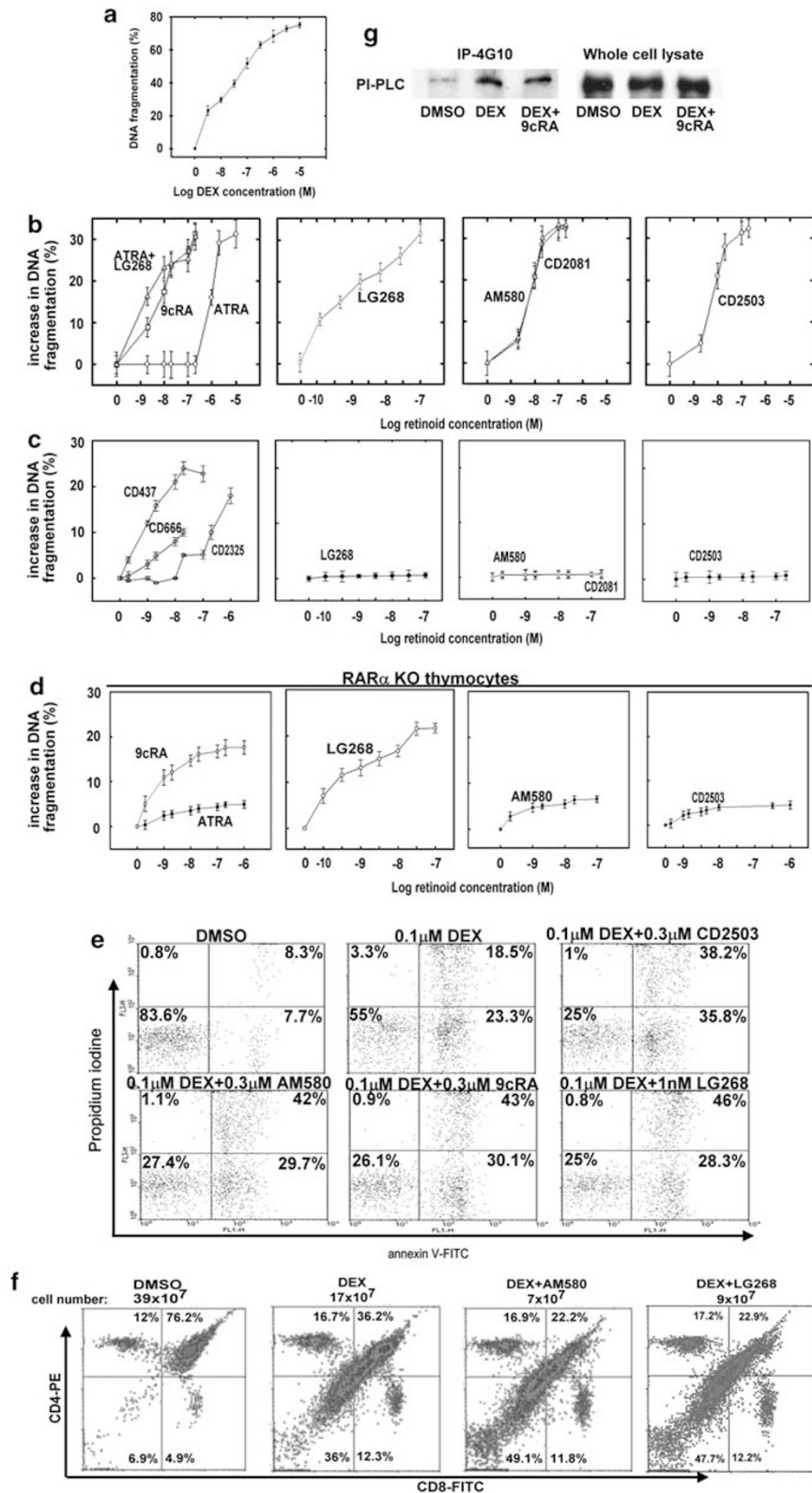
Retinoids do not promote glucocorticoid-induced PI-PLC phosphorylation. As activation of PI-PLC was reported to be non-genomic effect participating in

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Figure 1 Retinoids promote glucocorticoid-induced apoptosis of thymocytes by RARα/RXR. (**a**) Dexamethasone acetate induces DNA fragmentation in mouse thymocytes in a dose-dependent manner detected at 6 h following addition. (**b**) The RARα agonists ATRA, 9cRA, Am580 and CD2081, the RXR agonist LG268, a combination of ATRA with LG268 (0.1 nM), and an RARα antagonist CD2503 all promote dexamethasone (0.1 μM)-induced DNA fragmentation of mouse thymocytes. The amount of DNA degradation in the presence of glucocorticoid alone was 45 ± 4%. (**c**) The RARγ agonists CD437, CD666 and CD2325 alone induce DNA fragmentation, whereas compounds acting on RXR or RARα do not alter the basal DNA fragmentation (8 ± 3%) of thymocytes detected at 6 h following addition of the retinoids. (**d**) The RXR agonists 9cRA and LG268 can, but the RARα agonists are unable to promote significantly the dexamethasone acetate (0.1 μM)-induced DNA fragmentation of RARα knockout mouse thymocytes. The amount of DNA degradation in the presence of glucocorticoid alone was 45 ± 4%. Data represent mean ± 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 μg) and LG268 (50 μg) significantly enhances dexamethasone (0.2 mg)-induced CD4$^+$CD8$^+$ thymocyte apoptosis *in vivo* determined at 24 h following treatment. Data (one representative experiment out of three) show the 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

dexamethasone-induced thymocyte cell death,[18] 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
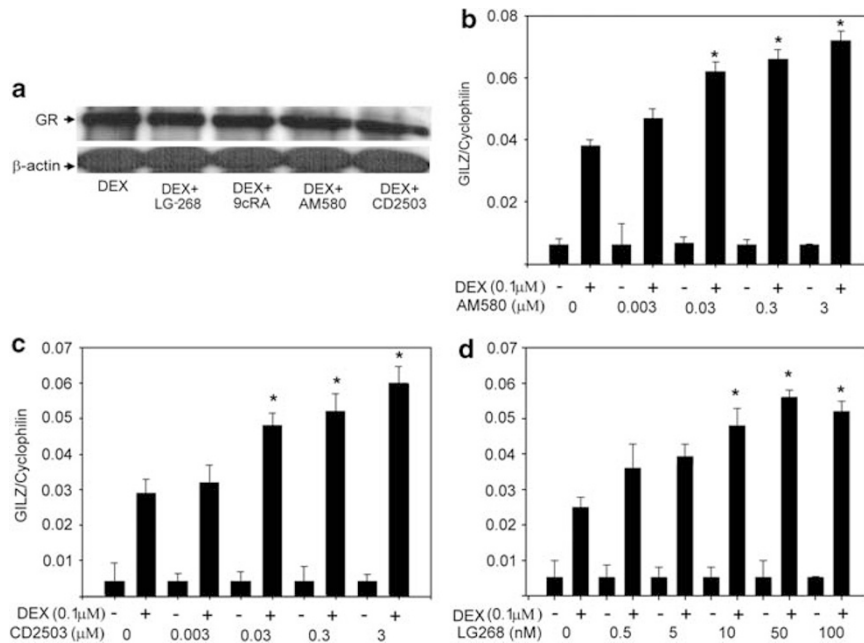
Figure 2 Retinoids enhance glucocorticoid-induced expression of GILZ in mouse thymocytes. (**a**) Retinoids do not affect the basal levels of glucocorticoid receptors. Isolated thymocytes (10^7 cells per ml) were exposed to 0.1 μM dexamethasone acetate alone or together with 0.3 μM ATRA, 9cRA, Am580, or CD2503 for 2 h. Levels of the glucocorticoid receptors were determined by immunoblot analysis. β-Actin was used as loading control. (**b–d**) Retinoids enhance the glucocorticoid-induced expression of GILZ in a dose-dependent manner. Isolated mouse thymocytes were exposed to 0.1 μM dexamethasone acetate and the indicated concentrations of retinoids. mRNA levels of GILZ were determined 2 h later. Data represent mean ± S.D. of three determinations. *Significantly different from glucocorticoid-treated control determined by Student's paired t-test ($P < 0.05$)

(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.[35] 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 cross-talk 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.[36] As previous studies have shown that glucocorticoid-induced apoptosis is dependent on the transcriptional activity of GR,[4] 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.[10] From these genes, we selected GILZ[6] to test how its expression changes under the effect of various retinoids, as its promoter carries several glucocorticoid response elements; thus, its transcription can be used as a read out of the transcriptional activity of GR in the thymocytes.[37] 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 RARα 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 RARα or RXR alone is sufficient for the effect.

RARα/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.[38] 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 μM dexamethasone decreased the viability of these cells. On the basis of these results, 0.1 μM 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, which is in line with the lack of sufficient amount of functional RAR expression in these cells (Figure 3b–f).
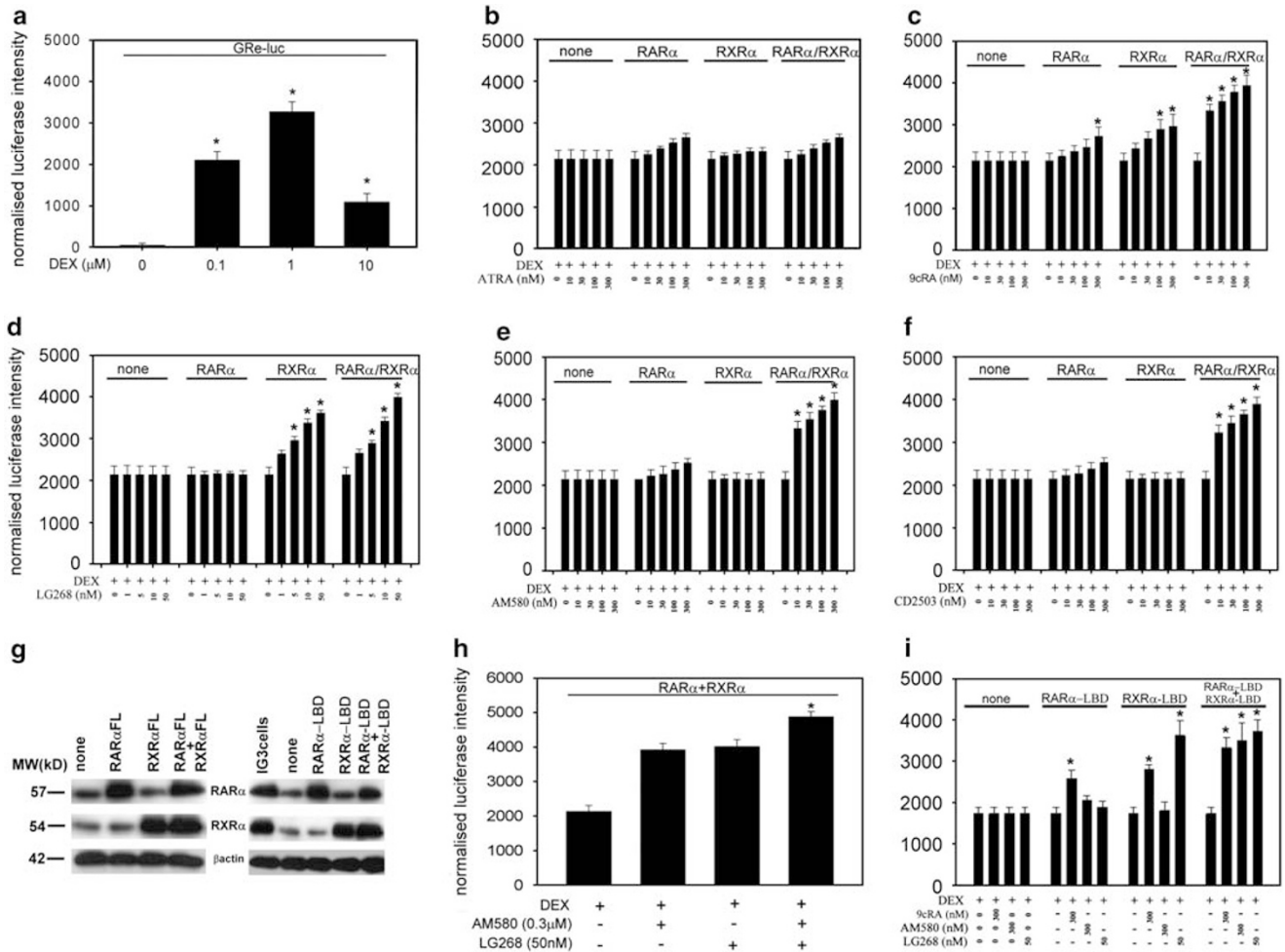
Figure 3 RARα/RXR heterodimers mediate the transactivating effects of retinoids. (**a**) Dexamethasone acetate induces the expression of the pCMX-GRE-luc reporter construct in a dose-dependent manner in COS1 cells transfected transiently. Effect of increasing concentrations of ATRA (**b**), 9cRA (**c**), LG268 (**d**), AM580 (**e**) and CD2503 (**f**) on the dexamethasone (0.1 μM)-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 IG3T cell line[43] is also shown. (**h**) Effect of the combination of AM580 and LG268 on the dexamethasone (0.1 μM)-induced expression of the pCMX-GRE-luc reporter in the presence of the full-length RARα/RXRα receptors. (**i**) Effect of the indicated concentrations of 9cRA, LG268 and AM580 on the dexamethasone (0.1 μM)-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's paired *t*-test ($P < 0.05$)

To test the effect of various retinoid receptors involved, RARα and RXRα 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 RARα or RARα/RXRα (Figure 3b). 9cRA, the pan-RAR/RXR agonist, however, significantly elevated the GR-induced transcription in the presence of RXRα, 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 RARα/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 RARα 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 RARα/RXRα heterodimers (Figure 3h), a more pronounced transcription was detected, indicating that ligation of both sides of the receptor hetero-dimer results in a more effective enhancement of GR-induced

transcription. These data imply that RARα/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.

As CD2503 cannot transactivate RARα, but is as effective as the RARα 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 (RARα-LBD and RXRα-LBD) (Figure 3g). As shown in Figure 3i, these receptors tested at one effective concentration of the retinoids were as effective as their wild-type variants.

Glucocorticoid, RARα and RXR receptors interact following ligand binding. As the cross-talks between various transcriptional factors are very often mediated by direct interaction, which does not necessarily require DNA binding of the interacting partner, we investigated a possible interaction between the two nuclear receptors by immuno-precipitating the GR from thymocytes in the presence of various ligands, and searched for RARα 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 investi-gated, co-immunoprecipitation of RARα was not seen in the absence of dexamethasone, whereas the addition of all the other ligands, with the exception of physiological concentrations of ATRA, was able to co-immunoprecipitate RARα in the presence of dexamethasone. The fact that LG268, which is an RXR ligand, also induces interaction between RARα and the ligated GR indicates that within the thymocytes LG268 must bind to RARα/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 GR, the mammalian two-hybrid technique was used. For this purpose, 293T fibroblast cells were transfected with the pmH100-TK-luc plasmid alone or together with the pCMX-Gal-L-hGR and/or the VP-hRARα-LBD plasmids. As shown in Figure 4b, in cells transfected with pmH100-TK-luc alone, or together with pCMX-Gal-L-hGR or VP-hRARα-LBD, no significant luciferase activity could be detected. However, in pmH100-TK-luc/pCMX-Gal-L-hGR-transfected cells, the presence of the VP-hRARα-LBD was able to induce a similar degree of luciferase expression as dexamethasone in the absence of VP-hRARα-LBD, proving that the GR and RARα can indeed interact directly. However, this interaction was not affected significantly by the addition of retinoids or dexa-methasone.

As ligation of RXRα in the presence of RXRα alone could promote dexamethasone-induced transcription in the transient transfection assays (Figure 3d), using again the mammalian two-hybrid technique, we checked whether
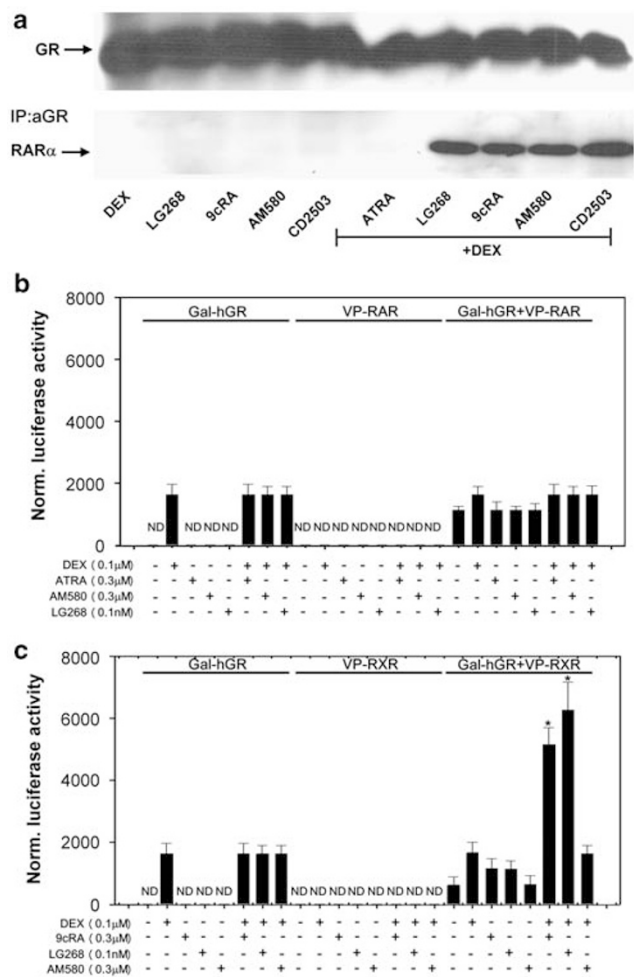

Figure 4 The ligated retinoid receptors directly interact with the ligated GR. (**a**) RARα co-immunoprecipitates with GR only when the ligand of both receptors is present. GR was immunoprecipitated from cell lysates with agarose-conjugated anti-GR antibodies, and GR and RARα in the immunoprecipitate was assessed by western blot with anti-GR and anti-RARα antibodies. The dose of glucocorticoid was 0.1 μM, that of retinoids 0.3 μM, except for LG268, which was 100 nM. The results are representative of one of three independent experiments. (**b**) Mammalian two-hybrid system reveals a direct interaction between GR and RARα. (**c**) Mammalian ttwo-hybrid mammalian system reveals a direct interaction between GR and RXRα, which is influenced by the presence of the receptor ligands

RXRα 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-hRXRα-LBD, no significant luci-ferase activity could be detected. However, in pmH100-TK-luc/pCMX-Gal-L-hGR-transfected cells, the presence of the VP-hRXRα-LBD was able to induce a similar degree of luciferase expression as dexamethasone in the absence of VP-hRXRα-LBD, proving that the GR and RXRα can indeed interact directly. This interaction was enhanced by the 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 the strength of the interaction between GR and RXRα is enhanced by ligand binding of

the RXRα, and might be also influenced by the ligand binding of the GR.

Retinoids do not affect the Ser232 phosphorylation of the GR. Although ligand binding is essential for initiating the transcriptional activity of the GR, the receptor is also subject of post-translational modification through phosphorylation.[39] Seven phosphorylation sites have been identified in the N-terminal region of the mouse GR, from which Ser232 (Ser211 in humans) has been shown to be phosphorylated to a greater extent upon hormone exposure[40] and specifically on GRs located in the nucleus.[39] It has also been shown that phosphorylation can enhance the transcriptional activity of the GR in a promoter-specific manner.[41] That is why we decided to test whether treatment of thymocytes with retinoids alter the dexamethasone-induced phosphorylation of Ser232 of the GR. Although the addition of dexamethasone to thymocytes enhanced the phosphorylation of Ser232 on GR, retinoids had no effect on it indicating that ligated
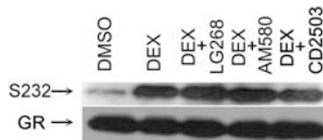

Figure 5 Glucocorticoid-induced Ser232 phosphorylation of GR is not affected by retinoids. Thymocytes were exposed to 0.1 μM dexamethasone acetate alone or with 0.3 μM Am580, 0.3 μM CD2503 or 50 nM LG268 for 1 h. GR was immunoprecipitated from cell lysates with agarose-conjugated anti-GR antibodies, and GR and its phosphorylated Ser232 in the immunoprecipitate was assessed by western blot by using specific antibodies (Cell Signaling Antibody No. 4161)

retinoid receptors must act by a different mechanism (Figure 5).

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)[42] and two human (IG3[43] and CCRF-CEM[44]) 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 show 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.[45] Here we show that retinoids can also stimulate glucocorticoid-induced apoptosis in immature thymocytes. Using various receptor-specific retinoids or RARα knockout thymocytes, we have shown that the effect is mediated via RARα and RXR, and simultaneous ligation of both RARα and RXRs by the natural RAs, or ligation of RXRα or RARs alone by the synthetic retinoids are required for the phenomenon. As an RARα-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
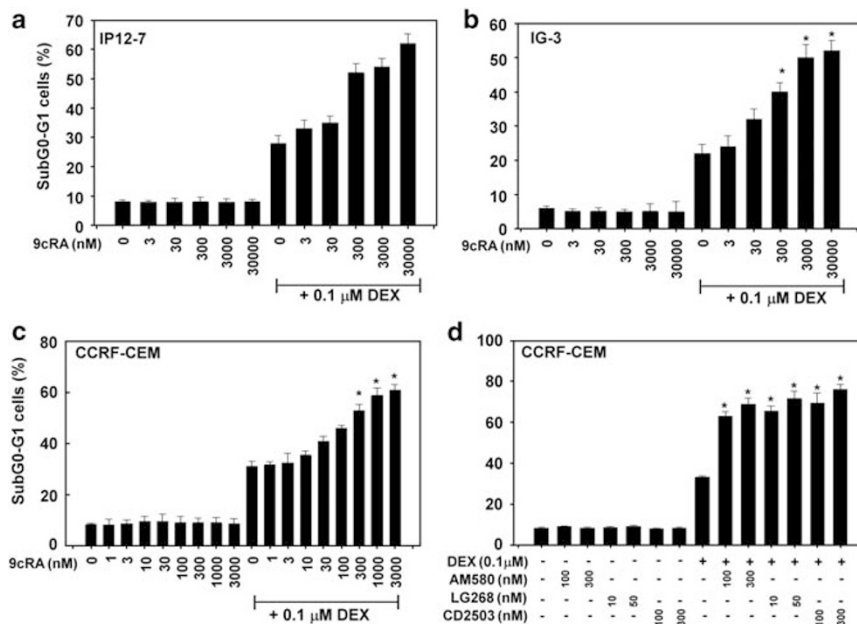

Figure 6 Retinoids enhance glucocorticoid-induced apoptosis of various T-cell lines as well. 9cRA enhances dexamethasone acetate (0.1 μM)-induced apoptosis of (**a**) IP-12-7T hybridoma, (**b**) IG3 and (**c**) CCRF-CEM T cells in a dose-dependent manner determined at 6 h following treatment. (**d**) RARα and RXRα ligands also enhance dexamethasone acetate (0.1 μM)-induced apoptosis in CCRF-CEM T cells. Data represent mean ± S.D. of three determinations. *Significantly different from glucocorticoid-treated control determined by Student's paired *t*-test ($P < 0.05$)

dexamethasone-induced death of thymocytes,[18] was not affected by retinoids, we investigated other mechanisms to explain the observed phenomenon.

As nuclear receptors are known to interact with various transcription factors and regulate their transcriptional activity in a ligand-dependent manner,[35,36] 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 RARα/RXR-dependent manner. In the presence of the RARα/RXR heterodimers, all the investigated RARα and RXR agonists and the RARα antagonist could enhance the transcription added alone, whereas in the case of physiological concentrations of ATRA, ligation of RARα 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 RARα side in the heterodimer.

GR and RXRα or RARα directly interacted in a mammalian two-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, RARα could be co-immunoprecipitated with GR from thymocytes, but only if thymocytes were exposed simultaneously to dexamethasone and to those retinoids that were effective in enhancing glucocorticoid-induced thymocyte cell death, including the RXR agonist LG268. On the basis of these data, we propose that in cells ligated RARα/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/RARα/RXR interaction is to promote the nuclear translocation of GR into the nucleus, in which the GR and retinoid receptors can physically interact. In the mammalian two-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 (P Brázda, unpublished observations). As in the transient transfection assays ligated RARα alone only slightly affected GR-mediated transcription, whereas ligated RXRα was fully effective, and the RXR ligand enhanced the GR/RXRα interaction, whereas the RARα ligand had no effect on the GR/RARα interaction, we propose that the RXR side of the RARα/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 RARα and the RXR ligands acting on the heterodimer. As ATRA and the RARα synthetic ligands differ in their ability to regulate RARα/RXR-mediated transcription and to induce interaction with the GR, we propose that they all stabilize a different conformation of RARα, some of which promote or inhibit transcription, whereas 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

Ser232, known to regulate the transcriptional activity of GR. Altogether, our data reveal a novel signal cross-talk between the GR and RAR signaling pathways showing that RARs similar to GRs[35,36] 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[31] and retinoids[32] were shown to be produced by thymic epithelial cells. As they both can stimulate the death of neglected thymocytes either alone[2,23–25] 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.[46] Our data, which show 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, USA) and LG00268 (LG268), which was a gift from R Heyman (Ligand Pharmaceuticals, San Diego, CA, USA). These retinoids were characterized in our previous papers.[25,27,28] All the plasmids used in these studies were a kind gift from Ron Evans (San Diego, CA, USA) and were described previously.[47]

Mice. Male NMRI mice (4 weeks old) purchased from LATI (Gödöllő, Hungary) were used. For the induction of *in vivo* thymic apoptosis, mice were treated intraperitoneally 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 h 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, San Jose, CA, USA) 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 RARα knockout[33] mice by mincing the glands in RPMI 1640 media (Sigma-Aldrich) supplemented with 10% charcoal-treated FCS, 2 mM glutamine and 100 IU penicillin/100 μg streptomycin per ml. Thymocytes were washed three times and diluted to a final concentration of 5×10^6 cells per ml before incubation at 37°C in a humidified incubator under an atmosphere of 5% CO_2 /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$^+$ 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.[42] The Kit225 IG3 cell line is an IL-2-independent subclone of the Kit225 human T leukemic cells

with helper/inducer phenotype.[43] CCRF-CEM cells derived from the peripheral blood buffy coat of a child (CEM) with acute lymphoblastic leukemia who had originally presented with lymphosarcoma[44] was a kind gift from Edit Buzas (Budapest, Hungary). Thymocytes and the T-cell lines were treated with dexamethasone acetate (0.1 μM) and various retinoids for 6 h in the presence of 10% charcoal-treated FCS (Sigma-Aldrich). 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.[23,25,28] In the case of the T-cell lines, the percentage of cells containing degraded DNA (sub-G_0–G_1 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 in various ways using a standard kit from BD Pharmingen (San Diego, CA, USA) to measure apoptosis. After rinsing cells twice with PBS, cells were resuspended in 100 μl of 1 $\times$ binding buffer in a flow cytometry tube, to which 5 μl of Annexin V-FITC and 5 μl of propidium iodide were added and mixed well. After a 15-min incubation at room temperature in the dark, 400 μl of 1 $\times$ binding buffer was added and flow cytometry was performed within 15 min. The 293T 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 μM) alone or together with 9-cRA (0.3 μM) 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, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 2 μg/ml aprotinin. Phosphotyrosine-containing proteins were immunoprecipitated with agarose-conjugated 4G10 antibodies (Upstate Biotechnology, Waltham, MA, USA). PI-PLC in the 4G10 immunoprecipitates was measured by western blot. Membranes were incubated with the monoclonal anti-PI-PLC antibody (Upstate Biotechnology). Antigen–antibody complexes were detected by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL, USA).

Q-PCR for detecting changes in the mRNA expression of GILZ. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) from treated cells. Transcript quantitation was performed via quantitative real-time RT 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, CA, USA). Real-time monitoring was carried out using an ABI Prism 7900 performing 40 cycles of 94°C for 12 s 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). The following primers were used: mGILZ reverse, 5′-CTTCAGTGGACAGATCAGGGAG-3′; mGILZ forward, 5′-AGACCAGCCTCACAATGCG-3′; mCyc reverse, 5′-TCTGCTGTCTTTGGAACTTTGTC-3′; and mCyc forward, 5′-CGATGACGAGCCCTTGG-3′.

Reporter gene assays following transient transfections. COS-1 cells were transfected at 60–80% confluency with the pCMX-GRE-luc and pCMX-β-Gal plasmids alone, or together with pCMX-FL-hRARα and/or pCMX-FL-hRXRα plasmids using polyethylene-imine (Promega, Madison, WI, USA). Transfection was carried out in Dulbecco's modified essential medium containing 10% charcoal-stripped bovine calf serum (Sigma-Aldrich). After 6 h, 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 h 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 RARα or RXR receptor expression in COS1 cells following transient transfections. At 48 h following transfection, cells were harvested and boiled in 2 $\times$ sample buffer and loaded onto SDS-PAGE gels. PVDF membranes were probed for anti-mouse RARα or RXRα (Santa Cruz) and β-actin antibodies (Sigma).

Co-immunoprecipitation. To show the possible direct interaction between the GR and the RARα, thymocytes were exposed to dexamethasone acetate (1 μM) and to various retinoids alone or together for 1 h in an RPMI medium containing 10% charcoal-treated FCS. Thymocytes (4×10^7) were then lysed, and the GR was immunoprecipitated with agarose-conjugated anti-mouse GR antibody (Santa Cruz) according to the manufacturer's instructions. Following 12% SDS electrophoresis, immunoprecipitated proteins were analyzed with western blot technique using anti-mouse GR and anti-mouse RARα antibodies (Santa Cruz). In some experiments, the immunoprecipitated GR was analyzed for Ser211 phosphorylation by a site-specific antibody from Abcam (Cambridge, MA, USA).

Mammalian two-hybrid system. To show direct interaction between GR and RARα or RXRα, a mammalian two-hybrid system was used. For this purpose, 293T fibroblast cells were transfected with pCMX-Gal-L-hGR and pMH100-TK-luc plasmids alone or either with VP-hRARα-LBD or VP-hRXRα-LBD together. pCMX-β-galactosidase plasmid was used to normalize transfection efficiency, VDR^{-1} plasmid, which codes a reverse sequence for the vitamin D receptor, was used to equalize the total amount of plasmid DNA (1 μg per 10^5 cells per well) transfected. Luciferase activities were detected 48 h later by using the kit from Promega (Madison, WI, USA), whereas β-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-hRARα-LBD or VP-hRXRα-LBD plasmid-coded RARα or RXRα 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 latter luciferase activities show the interaction between the glucocorticoid and two retinoid receptors.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)