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ORIGINAL ARTICLE

Retinoids produced by macrophages engulfing apoptotic cells contribute to the appearance of transglutaminase 2 in apoptotic thymocytes

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Abstract Transglutaminase 2 (TG2) has been known for a long time to be associated with the in vivo apoptosis program of various cell types including T cells. Though the expression of the enzyme was strongly induced in mouse thymocytes following apoptosis induction in vivo, no significant induction of TG2 could be detected, when thymocytes were induced to die by the same stimuli in vitro indicating that signals arriving from the tissue environment are required for the in vivo induction of the enzyme in apoptotic thymocytes. Previous studies have shown that one of these signals is transforming growth factor-β (TGF-β) which is released by macrophages engulfing apoptotic cells. Besides TGF-β, the TG2 promoter contains retinoic acid response elements as well. Here we show that in vitro retinoic acids, or TGF-β and retinoic acids together can significantly enhance the TG2 mRNA expression in dying thymocytes, and the apoptotic signal contributes to the TG2 induction. Inhibition of retinoic acid synthesis either by alcohol or retinaldehyde dehydrogenases significantly attenuates the in vivo induction of TG2 following apoptosis induction indicating that retinoids indeed might contribute in vivo to the apoptosis-related TG2 expression. What is more, the in vivo apoptosis induction in the thymus is accompanied by an enhanced retinoid-dependent transcriptional activity due to the enhanced retinoid synthesis by macrophages engulfing apoptotic cells. Our data reveal a new crosstalk between macrophages and apoptotic cells, in which apoptotic cell uptake-induced retinoid synthesis in macrophages enhances TG2 expression in the dying thymocytes.

Keywords Tissue transglutaminase · Macrophage · Thymocytes · Retinoid · Apoptosis

Abbreviations

9cRA	9-*cis* Retinoic acid
ATRA	All-*trans* retinoic acid
DEAB	4-Diethyl amino-benzaldehyde
DEX	Dexamethasone acetate
GAPDH	Glycerinaldehyde-3-phosphate dehydrogenase
LXR	Liver X receptor
PPAR	Peroxisome proliferator-activated receptor
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenases
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
TG2	Transglutaminase 2

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Introduction

Transglutaminases (Folk and Chung 1985) are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in

certain proteins. The reaction results in post-translational modification of proteins by establishing ε-(γ-glutamyl) lysine cross-linkages and/or covalent incorporation of polyamines and histamine into proteins. Transglutaminase 2 (TG2) is very unique in the transglutaminase family, because besides being a transglutaminase it also possesses GTPase, protein disulphide isomerase and protein kinase enzymatic activities. In addition, TG2 can also function in various biological settings as a protein/protein interaction partner (Fesus and Piacentini 2002).

TG2 has been known for a long time to be associated with the in vivo apoptosis program of various cell types including T cells (Fésüs and Szondy 2005). TG2 expression is induced in thymocytes dying in vivo following exposure to various apoptotic signals (Szondy et al. 1997), and TG2 also appears in the dying T lymphocytes of HIV-infected individuals (Amendola et al. 1996). The in vivo expression of TG2 in dying thymocytes is regulated at transcriptional level, since in mice, which carry the beta-galactosidase reporter gene under the control of a 3.8-kb fragment of the TG2 promoter, the beta-galactosidase expression showed strong correlation with the endogenous TG2 expression (Szegezdi et al. 2000). While, however, TG2 is strongly induced in dying thymocytes in vivo, no induction of TG2 was observed, when thymocytes were induced to die by the same stimuli in vitro (Szegezdi et al. 2000) indicating that signals arriving from the tissue environment contribute to the in vivo induction of the enzyme in apoptotic thymocytes.

Retinoic acids (RAs)—all-*trans* (ATRA)- and 9-*cis* (9cRA) retinoic acids—are vitamin A derivatives and act as ligands for the nuclear retinoic acid (RAR) and retinoid X (RXR) receptors. These receptors are ligand-dependent transcription factors which bind to specific hormone response element (RARE, RXRE) and transactivate specific target genes in the form of RAR/RXR heterodimers or RXR homodimers. ATRA and 9cRA are equipotent in activating RAR, while activation of RXR by ATRA is 50-fold less than by 9cRA. Though ATRA does not bind to RXR receptors, the latest observation is explained by the spontaneous conversion of ATRA to 9cRA within the cells. ATRA is produced in tissues by the oxidation of retinol. The first reaction, which converts retinol to retinaldehyde, is carried out by members of the alcohol dehydrogenase (ADH) family. Retinaldehyde is then efficiently oxidized into RA by three retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3) (Blomhoff and Blomhoff 2006).

The existence of retinoid (Nagy et al. 1996), and TGF-β response elements (Ritter and Davies 1998) in the TG2 promoter has been known for a long time. Macrophages engulfing apoptotic cells release TGF-β (Fadok et al. 1998), and we found that neutralization of TGF-β in vivo attenuates the expression of TG2 following apoptosis initiation in the thymus (Szondy et al. 2003) indicating that TGFβ might contribute to the induction of TG2 in dying thymocytes. However, neutralizing of TGF-β could not fully block the in vivo induction of TG2 expression suggesting that in addition to TGF-β, other signals must contribute to the thymic apoptosis-coupled TG2 expression. Since TG2 is a retinoid target gene (Nagy et al. 1996) and recently we found that retinoids are also actively produced in the thymus (Kiss et al. 2008), in the present study we decided to test, whether retinoids produced in the thymus could also contribute to the appearance of TG2 in the apoptotic thymocytes.

Materials and methods

Reagents

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except when indicated otherwise.

Experimental animals

The experiments were carried out with 1 or 2- to 4-month-old C57B6 mice. In some experiments RARE-hsp68-lacZ reporter transgenic line (Rossant et al. 1991) was used, which harbors a tetrameric repeat of the RARβ2 RARE linked to the hsp68 minimal promoter, which has been widely used as a RA-reporter transgene. Due to the hsp68 minimal promoter the lacZ has a background expression. The induced expression of the lacZ gene depends on the simultaneous presence of retinoid receptor ligands and the endogenous RARs. Mice were maintained in specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB) and tissue were obtained in accordance with the guidelines.

Inhibition of in vivo retinoid synthesis

To inhibit the endogenous retinoid synthesis in the thymus, 4-week-old mice were injected intraperitoneally with disulfiram (1.33 mg/g body weight on first day, 0.33 mg/g on the second and third day) or with 4-diethyl amino-benzaldehyde (DEAB) (0.2 mg/g body weight on first day and 0.1 mg/g on second and third day). Thymic apoptosis was induced with the apoptosis inducer added together with the third dose of the inhibitor.

Induction of thymic apoptosis in vivo

Four-week-old mice were injected intraperitoneally with 0.3 mg dexamethasone acetate—dissolved in DMSO/

physiological saline—or with 50 μg of anti-CD3 antibodies (eBioscience, Hatfield, UK) to induce thymic apoptosis. Controls received the same dose of DMSO/physiological saline. Thymuses were removed at the indicated time points. Thymic apoptosis was evaluated by measuring the change in the amount of thymic weight.

Western blot analysis

Collected thymuses were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of each samples were diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in 10% SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilion-P transfer membrane (Millipore, Budapest, Hungary) and were probed with rabbit polyclonal anti-TG2 antibody (SantaCruz, Heidelberg, Germany) or mouse anti-β-actin antibody. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate (Millipore, Budapest, Hungary).

Induction of thymocyte apoptosis in vitro

For TG2 expression experiments thymocytes (10^7 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% charcoal stripped FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2 for indicated time periods. Apoptosis was induced by addition of 0.1 μM dexamethasone acetate. In some experiments thymocytes were also exposed to 5 ng/ml recombinant human TGF-β1 (AbD Serotec, Kidlington, UK), 0.3 μM all-*trans* retinoic acid, 0.3 μM 9-*cis* retinoic acid, 0.3 μM AM580 (Tocris Bioscience, Eching, Germany) or 1 nM LG268 (from R. Heyman, Ligand Pharmaceuticals, La Jolla, CA).

Macrophage cell culture and treatment

Macrophages were harvested from 2- to 4-month-old C57B6 mice by peritoneal lavage with sterile physiological saline. Cells were plated onto 12-well plates (1.5×10^6 cells/well) after 3–4 h incubation the non-adherent cells were washed away, adherent macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2 for 2 days before use. Macrophages were treated with 1 μM GW3965 (Glaxo Smith Kline, Budapest, Hungary) a synthetic liver X receptor (LXR) agonist, 1 μM rosiglitazone (SPI-Bio, Montigny le Bretoneux, France), a peroxisome proliferator-activated receptor (PPAR)-γ agonist, 1 μM GW501516 (Alexis Biochemicals, Lörrach, Germany), a synthetic PPARδ agonist, for 4 h, or 0.1 μM dexamethasone acetate for 12 or 24 h.

In vitro phagocytosis

Thymocytes or NB4 cells (acute promyelocytic cell line) was used as apoptotic cells. To generate apoptotic thymocytes, thymus was collected from 4-week-old C57B6 mice, cells were isolated and cultured for 24 h (10^7 cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. In case of NB4 cells apoptosis was induced by 10 μM As_2O_3—treatment for 12 h. This method results in >80% apoptotic cells (as assessed by propidium iodide/AnnexinV-FITC staining). Apoptotic thymocytes were added to the peritoneal macrophages in 10:1 (apoptotic cells:macrophage) ratio and in 5:1 ratio in case of NB4 cells. After 6 h of coculture, apoptotic cells were washed away and macrophages were incubated for additional 6 or 18 h when RNA was isolated from the samples. In some experiments, macrophages were preincubated with 5 μg/ml actinomycin D for 30 min to block transcription, with 50 mM cytochalasin D or recombinant Annexin V (10 μg/10^5 cells) for 15 min prior to phagocytosis to block it.

Analysis of mRNA expression

Total RNA was isolated from control and treated thymuses, isolated thymocytes or macrophages by TRI reagent according to the manufacturer's guidelines. The concentration and purity of RNA were measured by means of NanoDrop spectrophotometer (Thermo Scientific, Schwerte, Germany). Total RNA (1 μg/samples) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) according to the manufacturer's instruction. QRT-PCR was carried out in triplicate using pre-designed FAM-MGB-labeled assays for TG2 and RALDHs (Applied Biosystems, Budapest, Hungary) on an ABI Prism 7900. To detect lacZ mRNA the following primers and FAM-TAMRA-labeled TaqMan probes (designed and ordered from Eurogentec, Seraing, Belgium) were used: forward, 5′-TGC-CGT-CTG-AAT-TTG-ACC-TGA-G-3′; reverse, 5′-CCG-CCA-CAT-ATC-CTG-ATC-TTC-C-3′; probe, FAM-ACT-CCA-ACG-CAG-CAC-CAT-CAC-CGC-TAMRA. ROX reference dye (Invitrogen, Budapest, Hungary) was used for normalization of fluorescent reporter signal. Relative mRNA levels were calculated using comparative C_T method and were normalized to cyclophilin or glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. ABI Prism SDS2.1 software was used for data analysis (Applied Biosystems, Budapest, Hungary).

Immunodetection of RALDH1 and 2 in macrophages of the thymus

Fluorescence light microscopic detection of macrophages (F4/80-immunopositive cells), RADLH1 or RADLH2 was performed in frozen thymus sections from mice exposed to dexamethasone for 24 h. Thymuses were frozen in embedding medium (Shandon Cryomatrix, Thermo Scientific, Schwerte, Germany) by liquid nitrogen. Tissue was sectioned at 7 μm (Leica Instruments, Nussloch, Germany), mounted on Superfrost Ultra Plus microscope slides (Menzel, Branschweig, Germany) stored at −20°C until use. Before IF staining, slides were kept at room temperature (RT) for 1 h, fixed in ice-cold acetone for 10 min and washed with PBS containing 0.1% Triton X. To minimize the non-specific staining by antibodies, sections were incubated with 2% BSA in PBS for 30 min at 37°C in a humid chamber. Sections were then incubated with goat polyclonal RALDH1 (Abcam, Budapest Hungary) or RALDH2 (SantaCruz, Heidelberg, Germany) antibodies in 1:150 dilution in 2% BSA-PBS in a humid chamber overnight at 4°C. After washings the samples were incubated with rat anti-mouse FITC-conjugated monoclonal F4/80 antibody (Hycult Biotech, Uden, Netherlands) in 1:250 dilution in 2% BSA-PBS in a humid chamber at RT for 1 h. Following washes sections were further incubated with secondary antibodies (anti-goat Nl557 conjugated IgG polyclonal antibody, 1:250, R&D systems, Minneapolis, MN) and mounted in DAPI aquamount. Appropriate negative control immunohistochemical reactions were performed. The region of fluorescent images were captured with a digital camera (Olympus DP50) connected to Nikon Eclipse 800 (Nikon, Tokyo, Japan). Acquired and presented images are representative of all the samples examined. For documentation, images were processed using Adobe PhotoShop software (Version 5.5, Adobe Systems Inc., CA, USA).

Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± SD; p values were calculated by using two-tailed Student's t test for two samples of unequal variance.

Results

Retinoic acids and TGF-β together effectively enhance the mRNA expression of TG2 in dexamethasone-exposed thymocytes dying in vitro

Since our previous studies indicated that TGF-β might be one of the signals that regulate TG2 expression in apoptotic thymocytes in vivo, we decided to test the possible effect of RAs on TG2 mRNA expression added alone or together with TGF-β. Both retinoids and TGF-β were applied in a concentration that was shown to be effective in our previous studies to affect apoptosis of thymocytes (Szondy et al. 1998, 2003). As shown in Fig. 1a, addition of TGF-β, ATRA or 9cRA alone slightly increased the mRNA expression of TG2 in the cultured thymocytes, while no increase was found on the endogenous mRNA expression of TG2 during the 8-h culture period. Among the compounds tested alone, 9cRA, which is also an apoptosis inducer in thymocytes (Szondy et al. 1998), was the most effective in inducing the expression of TG2.

As we reported previously (Szegezdi et al. 2000), exposure of thymocytes to dexamethasone acetate (DEX), a very efficient apoptosis inducer of thymocytes (Wyllie 1980) only slightly affected the endogenous TG2 expression. Further addition of ATRA was insufficient to promote DEX-induced TG2 expression (Fig. 1b). However, addition of either TGF-β or 9cRA alone or in combinations could significantly elevate the TG2 expression in thymocytes exposed to DEX (Fig. 1b, c). When, RAs were applied together with TGF-β to DEX-exposed thymocytes, a more significant induction of TG2 mRNA expression could be detected. In the case of 9cRA, this induction was more pronounced than the one observed with simultaneous addition of TGF-β and 9cRA in the absence of DEX suggesting that the apoptotic signal provided by the glucocorticoid hormone, which alone is insufficient to induce significant TG2 expression, when applied together with 9cRA and TGF-β, also contributes to the appearance of TG2.

9cRA appeared more effective in each experimental setting than ATRA. Since ATRA is an RAR agonist, while 9cRA is a ligand for both RAR and RXR receptors, the difference in their efficacy indicated that RXR receptors might participate in the phenomenon. Indeed, tested at 8 h of culture, the RXR agonist LG268, significantly induced the expression of TG2 in each experimental settings. However, it was more effective, when it was added together with AM580, an RARα agonist (Fig. 1d). Altogether these data indicate that in vitro retinoids and TGF-β together can significantly enhance TG2 expression in dying thymocytes, and RAR/RXR heterodimers ligated at both sides might mediate most effectively the effect of retinoids.

Inhibition of the in vivo retinoic acid synthesis attenuates apoptosis-induced TG2 expression in the mouse thymus

If retinoic acids are produced in vivo in the thymus and contribute to the TG2 expression of dying thymocytes, inhibition of RA synthesis prior to apoptosis induction

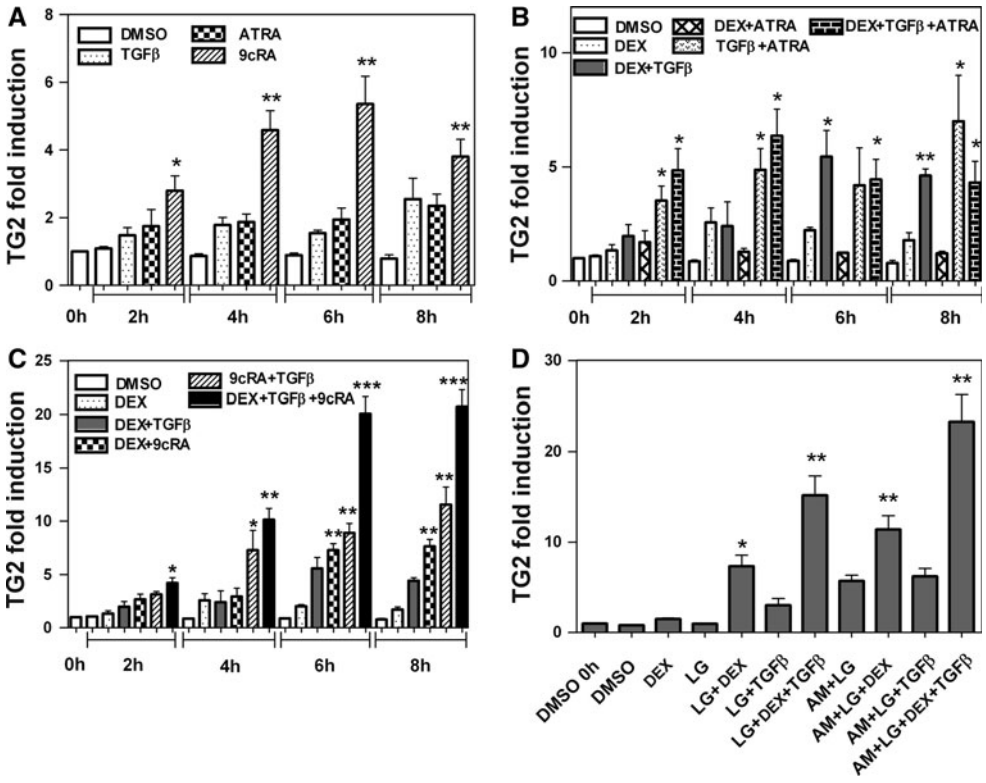

Fig. 1 Retinoids and TGF-β significantly enhance the TG2 mRNA expression in isolated mouse thymocytes induced to die by dexamethasone acetate. Thymocytes were exposed to the indicated compounds (DMSO, 0.5%; DEX, 0.1 μM; rTGF-β, 5 ng/ml; ATRA, 0.3 μM; 9cRA, 0.3 μM; LG268, 1 nM; AM580, 0.3 μM) for the indicated time period (**a**–**c**) or for 8 h (**d**). TG2 gene expression levels were measured by quantitative PCR using cyclophilin as a control. Results are expressed as mean ± SD of four independent experiments ($*p < 0.05$)

should prevent or attenuate TG2 induction. For inhibiting in vivo RA synthesis, mice were pretreated either with disulfiram, an alcohol dehydrogenase, or 4-diethyl aminobenzaldehyde (DEAB), an inhibitor of RALDHs for 2 days. Apoptosis was then induced by intraperitoneal injection of dexamethasone acetate. As seen in Fig. 2a–d, in line with our previous observations (Szondy et al. 1997) injection of DEX induced a significant increase in the TG2 expression on both mRNA and protein levels in the mouse thymus detected at 24 h. Pretreatment with either disulfiram or DEAB, significantly reduced the DEX-induced TG2 expression indicating that endogenously produced retinoids might contribute to its apoptosis-related in vivo appearance. However, the DEX-induced expression of TG2 was never fully inhibited indicating that other signals are also contributing to its in vivo appearance.

The in vivo apoptosis induction is coupled to enhanced retinoid-dependent transcription in the mouse thymus

To demonstrate that both disulfiram and DEAB at the concentration used did efficiently inhibit the in vivo retinoid synthesis, the effect of these compounds was tested on the thymic β-galactosidase mRNA expression of the RARE–lacZ transgenic mice (Rossant et al. 1991). In these mice lacZ expression demonstrates the in vivo retinoid-dependent transcriptional activity. To make sure that retinoid production and the consequent retinoid-dependent transcriptional activity was also inhibited by DEAB or disulfiram when the thymus was exposed to DEX, lacZ mRNA expression was determined also in DEX-exposed thymuses. To our surprise DEX treatment alone induced a significant increase in the reporter gene expression (Fig. 2e, f). This expression was nearly fully inhibited by DEAB (Fig. 2f), but only partially by disulfiram (Fig. 2e) indicating a full inhibition of retinoid-induced transcription by DEAB, but only partial inhibition by disulfiram. However, we could not further increase the dose of disulfiram, as higher doses of it were lethal for the mice.

The fact that DEX treatment induced a strong lacZ expression indicated that retinoids not only contribute to the TG2 induction in the mouse thymus, but retinoid-dependent transcription is enhanced following apoptosis induction. To check whether this response is related only to DEX treatment, or is apoptosis specific, we induced thymocytes to die also by injecting anti-CD3 antibodies, which trigger the T cell receptor-mediated cell death pathway. As seen in Fig. 3a, both apoptotic stimuli induced an enhanced retinoid transcriptional activity in the thymus detected by measuring lacZ mRNA expression, though the effect of DEX was much more pronounced. That is why we decided to check the expression of RALDH1 and 2, two RA synthesis-specific enzymes, which were found to be expressed by the mouse thymus (Kiss et al. 2008) following in vivo apoptosis induction. As shown in Fig. 3b, c, e, f, the mRNA expression of both enzymes increased in a

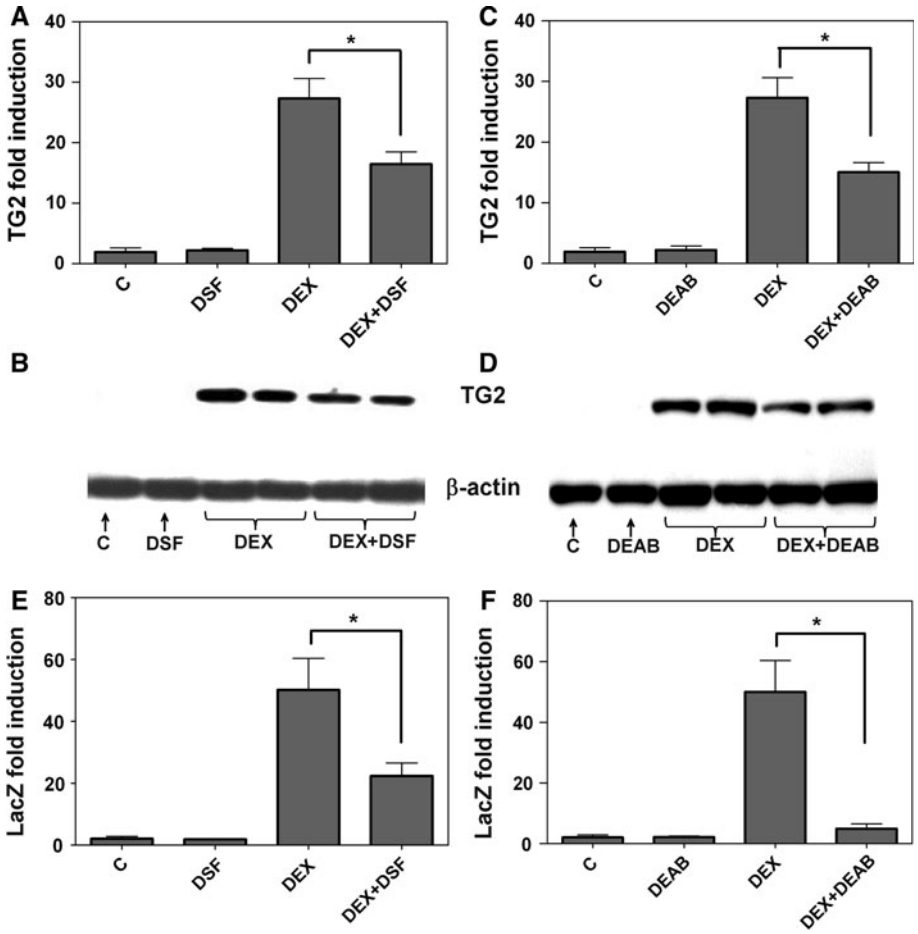

Fig. 2 In vivo inhibition of either alcohol dehydrogenases or RALDHs significantly attenuates the apoptosis-linked TG2 expression in the mouse thymus. After 2 days pretreatment with disulfiram, an alcohol dehydrogenase inhibitor (**a** and **b**), or DEAB, an inhibitor of RALDHs (**c** and **d**), in vivo thymocyte apoptosis was induced by intraperitoneal injection of 0.3 mg dexamethasone acetate in 4-week-old C57B6 mice. Thymuses were collected 24 h later. TG2 gene expression levels were measured by quantitative PCR using cyclophilin as a control, while TG2 protein levels were determined by Western blot using β-actin as loading control. The effect of the same treatments was also determined on the thymic LacZ expression of 4 weeks old *RARE-hsp68-lacZ* reporter mice using GAPDH as a reference gene (**e** and **f**). Results are expressed as mean ± SD of four independent experiments (*$p < 0.05$, **$p < 0.01$)

time-dependent manner following injection of either DEX or anti-CD3 antibodies. In line with the lacZ results, the expression of RALDH1 increased much more significantly following DEX, than anti-CD3 antibody injection, while less difference was found in the RALDH2 response. The different response might be partially related to the fact that DEX induced a more significant cell death in the thymus at each time point demonstrated by the remaining thymic weight (Fig. 3d, g).

Macrophages engulfing apoptotic cells express increased levels of RALDHs

Though previous studies indicated that thymic epithelial cells express RALDHs (Kiss et al. 2008), it seems unlikely that they could sense the amount of thymocyte apoptosis induced. However, it was recently indicated that macrophages engulfing apoptotic cells sense the amount of apoptosis via three lipid-sensing receptors, and respond to it by enhanced phagocytosis (Mukundan et al. 2009; A-Gonzalez et al. 2009; Roszer et al. 2011). That is why we decided to test whether the expression of RALDHs can be altered during phagocytosis of apoptotic cells in macrophages. For this purpose peritoneal macrophages were isolated, and exposed to apoptotic thymocytes or NB4 cells, which are larger and contain more lipids. As shown in Fig. 4a, phagocytosis of apoptotic thymocytes enhanced the mRNA expression of RALDH1, but did not alter that of RALDH2 in engulfing macrophages (Fig. 4b). The induction was not thymocyte specific, as a more pronounced induction of RALDH1 was observed, when the same macrophages engulfed apoptotic NB4 cells (Fig. 4c). What is more, uptake of apoptotic NB4 cells also triggered the expression of RALDH2 (Fig. 4d). Preincubation of macrophages for 30 min with actinomycin D, a transcription inhibitor, prevented the apoptotic cell-associated induction of RALDH mRNA expression, indicating that regulation occurs at the transcriptional level (Fig. 4e, f).

Cytochalasin D does inhibit the engulfment process, but it does not influence the recognition of apoptotic cells (Cvetanovic and Ucker 2004). Binding of phosphatidylserine on the surface of apoptotic cells plays a role in their recognition and subsequent uptake by macrophages, and this recognition can be inhibited by preincubation of apoptotic cells with recombinant annexin V, which binds to phosphatidylserine (Hoffmann et al. 2001). Both cytochalasin D and recombinant annexin V inhibited the induction of RALDH mRNA levels by apoptotic cells (Fig. 4e, f)

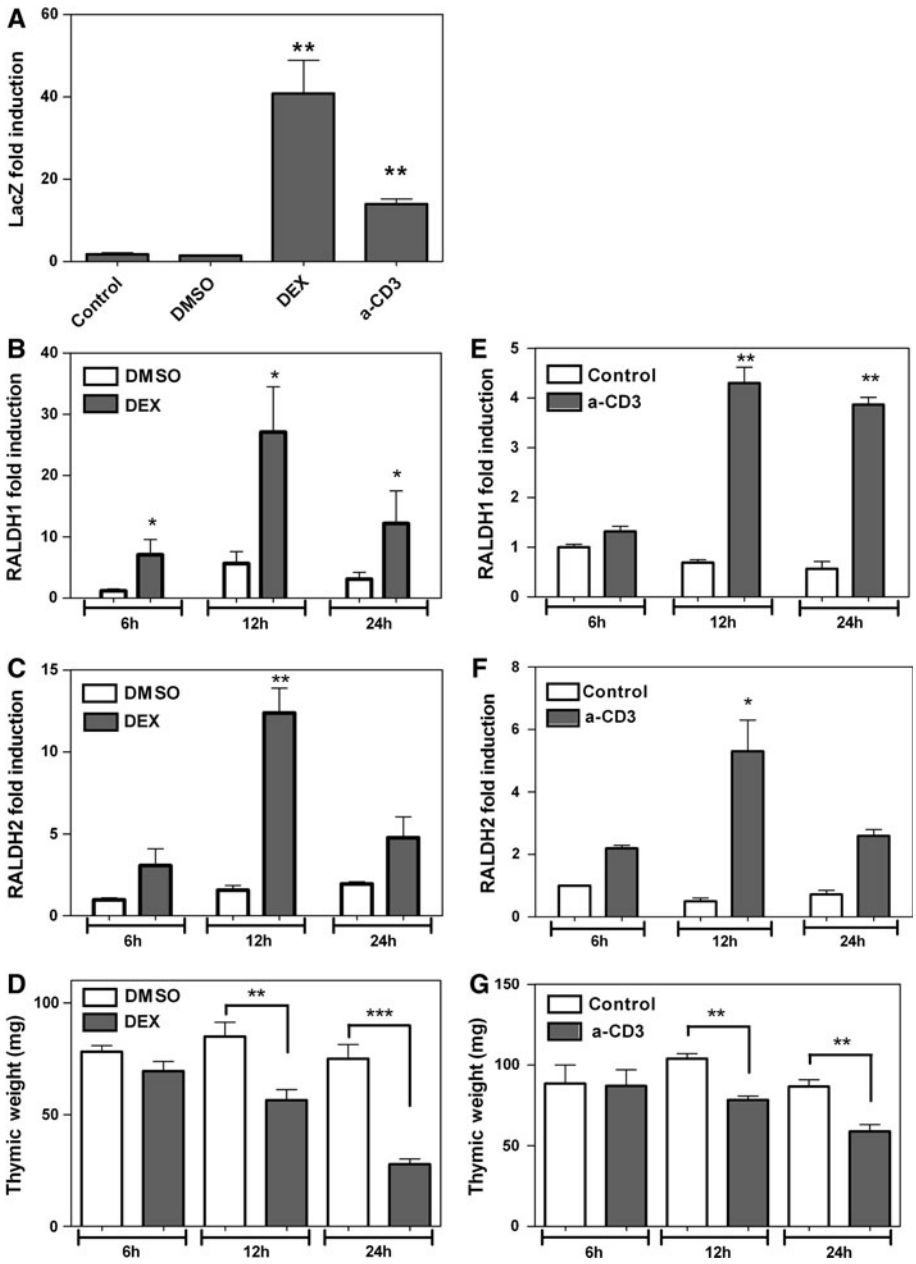

Fig. 3 In vivo apoptosis induction of thymocytes is accompanied by enhanced expression RALDH1 and 2, and by increased retinoid-dependent transcriptional activity in the mouse thymus. In vivo thymocyte apoptosis was induced by intraperitoneal injection of 0.3 mg dexamethasone acetate or by that of 50 μg anti-CD3 antibodies in *RARE-hsp68-lacZ* reporter mice. LacZ (**a**), RALDH1 (**b** and **d**), RALDH2 (**c** and **e**) relative gene expressions and thymic weight (**f** and **g**) were determined at 24 h or at the indicated time points. Results are expressed as mean ± SD of four independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

suggesting that it is the engulfment of apoptotic cells, rather than their recognition per se, that triggers RALDH expression.

Since the three lipid-sensing nuclear receptors (LXR, PPARγ and δ) have been implicated in the macrophage response to the engulfed apoptotic cells (Mukundan et al. 2009; A-Gonzalez et al. 2009; Roszer et al. 2011), we decided to test whether triggering of these receptors affect the expression of RALDH1 or 2. Tested at 4 h., all the three agonists, GW3965, an LXR agonist, rosiglitazone, a PPARγ agonist, and GW1516, a PPARδ agonist, promoted the mRNA expression of RALDH1 in peritoneal macrophages (Fig. 4g), and only GW1516 was ineffective in inducing RALDH2 expression. These observations indicate that all the three lipid-sensing receptors might mediate the effect of apoptotic cell engulfment on the RALDH expression.

RALDHs are expressed by macrophages in the thymus following in vivo apoptosis induction

To prove that RALDHs are indeed expressed by macrophages in the thymus following in vivo apoptosis induction, frozen thymic sections were co-stained for the macrophage marker F4/80 and RALDH1 or 2 at 24 h following apoptosis induction by DEX. As demonstrated in Fig. 5, both enzymes were co-stained with macrophages following apoptosis induction.

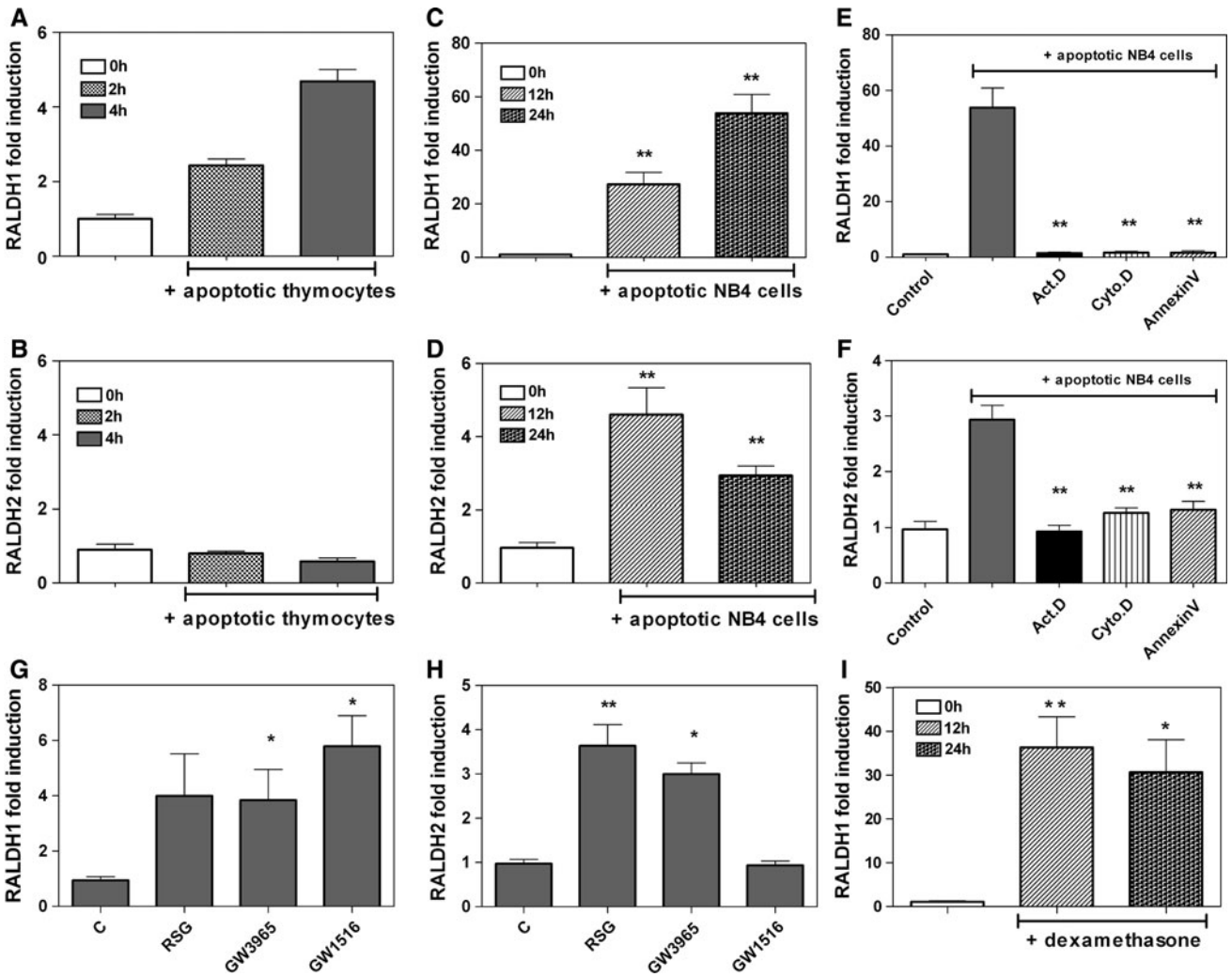

Fig. 4 Engulfment of apoptotic cells induces the expression of RALDHs in macrophages possibly via activation of three lipid-sensing receptors. Macrophages were exposed to apoptotic thymocytes (**a** and **b**) or to apoptotic NB4 cells (**c**–**f**) in the absence or presence of actinomycin D (5 μg/ml), cytochalasin D (50 mM) or recombinant Annexin V (10 μg/10^5 apoptotic cells); to 1 μM rosiglitazone, 1 μM GW3965 or 1 μM GW1516 (**g** and **h**) or to 0.1 μM dexamethasone acetate (**i**) for the indicated time period, for 4 h (**e**–**h**)) or for 24 h (I), and the relative expression levels of RALDH1 and 2 were determined were by quantitative PCR using cyclophilin as a control. Results are expressed as mean ± SD of four independent experiments (*$p < 0.05$, **$p < 0.01$)

Since injection of DEX induced much more RALDH1 expression in the thymus in vivo than injection of anti-CD3 antibodies (Fig. 3b), we also checked whether DEX affects RALDH1 expression in macrophages in vitro. As shown in Fig. 4i, DEX could efficiently enhance RALDH1 expression in macrophages, while had no effect on RALDH2 expression (data not shown).

Discussion

TG2 has long been associated with the in vivo apoptosis program of T cells. In vivo in thymocytes TG2 appears before caspase-mediated events, such as DNA breakdown, could be detected (Szondy et al. 1997) indicating that the enzyme might have a role in the initiation phase of apoptosis. Indeed our recent results indicate that inducible overexpression of the enzyme in Jurkat T cells alone can induce apoptosis. Though the cross-linking mutant of the enzyme was also efficient in apoptosis induction, the wild-type TG2 induced a more pronounced cell death indicating that both its cross-linking and other activities can contribute to cell death induction. However, thymocytes die in vitro efficiently in the absence of TG2 indicating that in these cells TG2 might contribute to, but is not necessary for the cell death induction. In fact, isolated thymocytes do not express high levels of TG2, and the TG2 levels do not increase significantly following in vitro apoptosis induction. That is why we proposed that signals arriving from the tissue environment must contribute to the strong TG2 induction

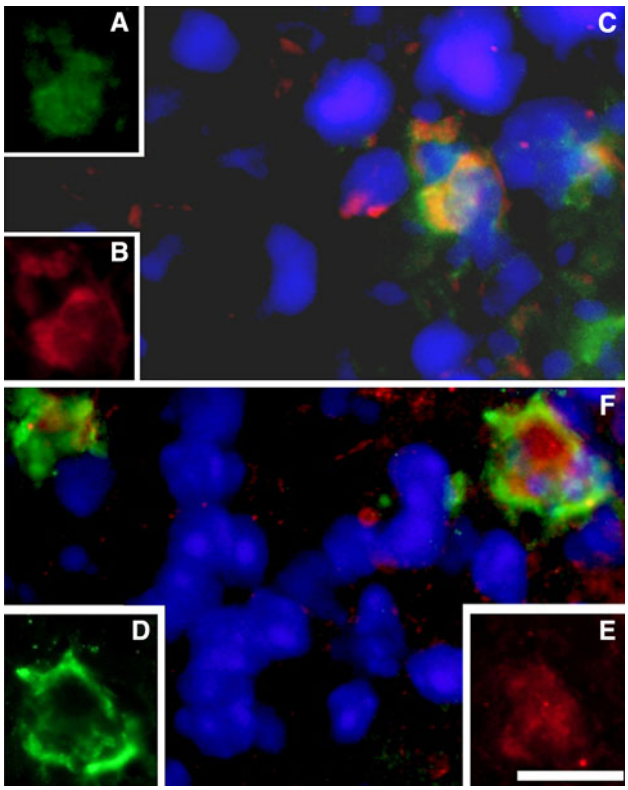

Fig. 5 Colocalisation of RALDH1 and 2 with macrophages in tissue sections of mouse thymus exposed to dexamethasone acetate for 24 h. **a** F4/80 staining of a macrophage (*green*) in the thymus at 24 h dexamethasone injection. **b** The endogenous *red* labeling of RADLH1. **c** Double-labeled macrophages (*green* F4/80, and *red* RADLH1) along with staining for nuclei (*blue* DAPI) to confirm both the cellular localization of the label in macrophages and the anatomical location of these cells in the section presented. **d** Macrophages (F4/80), **e** RADLH2 (*red labeling*), and **f** the dual-labeled correlation (*yellow*), along with DAPI (*blue*) staining, respectively. *Scale bar* 15 μm (color figure online)

in vivo (Szegezdi et al. 2000). Previous studies (Szondy et al. 2003) indicated that TGF-β released by macrophages might be one such a signal, while in the present paper we investigated the possible contribution of retinoids.

Here we have shown that in vitro retinoic acids, or TGF-β and retinoic acids together can significantly enhance the TG2 mRNA expression in dying thymocytes, and the apoptotic signal contributes to the TG2 induction. We have also demonstrated that inhibition of retinoic acid synthesis either by alcohol or retinaldehyde dehydrogenases significantly attenuates the in vivo induction of TG2 following apoptosis induction indicating that retinoids might indeed contribute in vivo to the enhanced TG2 expression. In fact, we found that retinoids not only contribute to TG2 expression by being constantly present in the thymic environment, but the in vivo apoptosis induction is accompanied by an enhanced retinoid-dependent transcriptional activity in the thymus indicating the possibility of enhanced retinoid synthesis. Indeed, in vivo apoptosis induction in the thymus was accompanied by enhanced RALDH1 and 2 expression. Though thymic cortical and medullary epithelial cells were shown to express RALDHs (Kiss et al. 2008), based on our data we propose that similar to TGF-β, retinoids might also be produced by macrophages engulfing apoptotic cells in a phagocytosis-dependent manner. Our data also indicate that the apoptotic cell-induced retinoid production might be mediated via the three lipid-sensing receptors already indicated in the engulfment-dependent regulation of phagocytosis. In vivo DEX treatment was more efficient than that of anti-CD3 in promoting retinoid-dependent transcriptional activity. This later event might be related to the fact that DEX is a more potent apoptosis inducer leading possibly to more phagocytosis, and in addition, it alone can induce retinoid production in macrophages by stimulating the expression of RALDH1 independently of the engulfment.

Our previous studies have already indicated a strong crosstalk between apoptotic cells and macrophages engulfing apoptotic cells in the context of TG2 function (Szondy et al. 2003). Our present data provide a further example for the existence of such a crosstalk demonstrating that uptake of apoptotic cells induces synthesis of retinoids in macrophages, which in turn contributes to the appearance of TG2 in apoptotic cells. Since retinoids also regulate apoptosis of thymocytes (Szondy et al. 1998), this crosstalk involves not only the feedback regulation of TG2 expression, but also that of apoptosis in the surrounding thymocytes.

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