

Mitochondrial translocation of the glucocorticoid receptor in double-positive thymocytes correlates with their sensitivity to glucocorticoid-induced apoptosis

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Abstract

Glucocorticoid receptor (GR) signaling plays an important role in the selection and apoptosis of thymocytes. Besides nuclear translocation, mitochondrial translocation of the ligand-bound GR in lymphoid cells was also shown, which might determine glucocorticoid (GC)-induced apoptosis sensitivity. In the present work, we followed the ligand-induced GR trafficking in CD4+CD8+ double-positive (DP) thymocytes. Using confocal microscopy, we found that upon short-term *in vitro* GC analog [dexamethasone (DX)] treatment, the GR translocates into the mitochondria but not into the nucleus in DP cells. We also analyzed the GR redistribution in cytosolic, nuclear and mitochondrial fractions of unseparated thymocytes by western blot and confirmed that in DX-treated cells a significant fraction of the GR translocates into the mitochondria. DX reduced the mitochondrial membrane potential of DP cells within 30 min, measured by flow cytometry, which refers to a direct modulatory activity of mitochondrial GR translocation. The abundant mitochondrial GR found in DP cells well correlates with their high GC-induced apoptosis sensitivity.

Introduction

Glucocorticoids (GCs) are key regulators of T lymphocyte apoptosis (1) and are commonly used as immunosuppressive drugs in the treatment of many diseases, like hematological malignancies (2) and autoimmune diseases (3). Classically, they act via nuclear receptors: upon ligand binding, the receptor translocates to the nucleus and interacts with respective DNA sequences, called glucocorticoid-responsive elements (GRE), regulating the synthesis of several proteins, including apoptosis-related proteins and cytokines (4). Recently, a number of reports underlined that GCs can act through rapid non-genomic mechanisms, too, for example, by the modulation of ion-exchange processes and the alteration of the physical properties of the plasma membrane (5, 6). Moreover, the GC–glucocorticoid receptor (GR) complex interacts with several cytoplasmic proteins modulating their phosphorylation status independent of the nuclear GR (7–9).

Besides the 'classical' cytoplasmic and nuclear expression, lately, GR has been described in other cell compart-

ments, too. Sionov *et al.* (10, 11) demonstrated the presence of the GR in the mitochondria of several lymphoid cell types and reported that the cell lineage-specific GC-induced apoptosis sensitivity is determined rather by the mitochondrial and not by the nuclear translocation of the GC–GR complex. Moreover, membrane GR is suspected to predict the GC resistance in lympho-haemopoietic cells (11, 12).

In the thymic cortex, GCs act as regulators of the differentiation and selection of T cell precursors (1). GCs are among the important soluble factors eliciting key signals in maturing thymocytes together with cytokines (13), chemokines (14) and other soluble factors, for example Wnt molecules (15). Thymocyte–thymic epithelial cell and other stromal cell interactions are also critical during thymocyte development (16). Thymic stromal cells are known to produce GCs locally (17), and more recently, it has also been demonstrated that thymocytes can secrete GCs, too, in an age-dependent manner (18); therefore, thymocytes are located in a GC-rich microenvironment (19).

As thymocytes differentiate in a complex stromal network of the thymus, they proceed through well-defined developmental stages, characterized by their cell surface marker CD4 and CD8 expression: CD4-CD8-DN cells express a pre-TcR; then in the next stage of maturation, CD4+CD8+ DP cells undergo positive and negative selection steps, testing their ability to bind to self MHC molecules and to recognize self-structures; and finally, CD4 or CD8 single-positive (SP) cells with the features of the naive mature T cells emigrate from the thymus (20).

GCs have been shown to control selection steps of double-positive (DP) cells (21) by modifying their TCR signal (22, 23). Paradoxically, DP cells, the most GC-sensitive thymocyte subpopulation, express the lowest level of GR both at protein and mRNA level when compared with thymocytes at other developmental stages (19, 24, 25). These findings have suggested that alternative (non-nuclear) GR-signaling pathways might exist in thymocytes (24). In a preliminary work, in a TCR transgenic mouse model, we have shown that thymocytes surviving during T cell selection up-regulate their mitochondrial anti-apoptotic Bcl-2 protein, suggesting that mitochondria were directly involved in regulating thymocyte apoptosis (23).

In our present work, we show that upon *in vitro* exposure to GC the GR translocates to the mitochondria in DP thymocytes within 30 min, having a direct effect on the mitochondrial function as shown by changes in the mitochondrial membrane potential. Quantitative confocal microscopic analysis confirmed the ligand-induced mitochondrial GR translocation. Additionally, we performed subcellular fractionation and western blotting of thymocytes in order to examine the subcellular distribution of the GR after rapid GC exposure. We found that some GR is already present in the mitochondria of untreated DP cells. In the presence of GC agonist, a dominant mitochondrial GR translocation occurred but the nuclear GR trafficking was absent in DP thymocytes. We conclude that the sensitivity of DP thymocytes to GC-induced apoptosis correlates with rapid mitochondrial GR translocation upon ligand binding, which could initiate apoptotic pathways.

Methods

Mice

Three- to four-week-old BALB/c mice were kept under conventional conditions and provided with pelleted rodent chow and acidified water *ad libitum*. All animal experiments were carried out in accordance with the regulations set out by the university's committee on animal experimentations (#BA 02/2000-2/2006).

Cell lines

Sp2/0-Ag14 (mouse myeloma) and RBL2H3 (rat basophilic leukemia) cells were cultured in humidified atmosphere, containing 5% CO₂ at 37°C, in DMEM (Sigma) supplemented with 5 and 10% FCS (Gibco, Gaithersburg, MD, USA), respectively.

mAbs

The following mAbs from BD Pharmingen (San Jose, CA, USA) were used for flow cytometry: anti-CD4-FITC (clone#

YTS-191.1) and anti-CD8-cychrome (CyC) (clone# Ly-2), and for confocal microscopy: anti-CD4-Pacific Blue (clone# RM4-5), anti-CD8-Alexa fluor 647 (clone# 53-6.7) and anti-GR-FITC (5E4-B1) (26).

Antibodies for western blot were the following: mouse monoclonal anti-Cytochrome C (BD Pharmingen), mouse monoclonal anti-GR (5E4-B1), mouse monoclonal anti-β-actin (Sigma) and rabbit polyclonal anti-Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase conjugated anti-mouse or anti-rabbit IgG antibody were used as secondary antibodies.

Short-term *in vitro* GC treatment of isolated thymocytes and cell lines

After sacrifice, thymi were removed and homogenized mechanically in RPMI (Sigma) followed by filtration through nylon mesh. Cell viability was determined by trypan-blue exclusion using a hemocytometer.

One million thymocytes, RBL2H3 or Sp2 cells were treated *in vitro* with 1 μM dexamethasone (DX, Sigma; dissolved in DMSO until use in 10 mM stock) in serum-free media for 5, 10 or 30 min. Control samples were kept under the same conditions for the same time in the presence of the solvent alone. The treatment was stopped by adding ice-cold PBS containing 0.1% NaN₃.

Flow cytometric analysis of mitochondrial function with mitotracker chloromethyl-X-rosamine

Chloromethyl-X-rosamine (CMX-Ros; Invitrogen) is a lipophilic reagent that diffuses across the cell membrane and accumulates in intact mitochondria due to normal mitochondrial membrane potential (27). Briefly, 10 μl CMX-Ros stock solution (1 μg ml⁻¹ in DMSO) was added to 10⁶ cells in 1 ml of serum-free RPMI, following the manufacturer's instructions and then cells were incubated for 30 min at 37°C parallel with 1 μM DX treatment. Cell surface labeling with anti-CD4-FITC and anti-CD8-CyC was performed on ice in binding buffer (PBS containing 0.1% BSA and 0.1% NaN₃) followed immediately by flow cytometric analysis.

Flow cytometric data acquisition and analysis

Samples were measured and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest software. First, thymocytes were gated according to their size and granularity on forward and side scatter dot plots. Thymocyte subpopulations according to their cell surface CD4/CD8 expression were analyzed separately for CMX-Ros intensity that was detected in the FL2 channel (ex.: 579 nm, em.: 599 nm). Fluorescent histogram plots were used to compare the mean fluorescence intensity (MFI) values of the different samples.

Labeling of cells for confocal laser scanning microscopy

CMX-Ros loaded ± DX-treated CD4-Pacific Blue and CD8-Alexa Fluor 647-labeled thymocytes, RBL2H3 or Sp2 cells were fixed in 4% PFA and washed in permeabilization buffer (PBS containing 0.1% BSA, 0.1% NaN₃ and 0.1% saponin). The intracellular labeling of the cells was performed in

saponin buffer with $1 \mu\text{g ml}^{-1}$ anti-GR-FITC antibody (26). Briefly, cells were incubated on ice for 30 min and then washed twice in saponin buffer and finally once with PBS and cytospined onto slides. For nuclear staining, RBL2H3-cells and Sp2-cells were labeled with DAPI (Invitrogen) for 5 min. The excess fluid was carefully aspirated and the slides were covered using 50% glycerol-PBS.

Confocal microscopic image acquisition and analysis

Visualization and analysis of the samples were carried out using an Olympus Fluoview 300 confocal microscope with an Olympus Fluoview FV1000S-IX81 image acquisition software system. Data were collected in four separate channels, including differential interference contrast (DIC), UV for CD4, FITC for GR, red for mitochondria and far red for CD8. Sequential scanning and Kalman setting were used for image acquisition to avoid cross-talk between the channels and to eliminate noise, respectively. Signals were collected from cells in 3–3 frames and CMX-Ros-GR morphological association was analyzed.

Images were overlaid in the following alignment: CD4-Pacific Blue with CD8-Alexa fluor 647 or CMX-Ros with GR-FITC. Cell contours were controlled using DIC images. CMX-Ros-GR images were analyzed further with the ImageJ software (<http://rsb.info.nih.gov/ij>) using the co-localization plug-in. Based on the analysis of pixel fluorescence intensities, ranging from 0 to 255, specific staining was distinguished from background by using a threshold value of 50 as described elsewhere (28, 29). Then, co-localized pixels between CMX-Ros and GR were counted. One hundred DP cells per sample were analyzed altogether using this approach.

Subcellular fractionation

Mitochondria Isolation Kit (Pierce) was used to obtain cytoplasmic, mitochondrial and nuclear fraction from thymocytes, according to manufacturer's instructions, with minor modifications according to Stasik *et al.* (30). Briefly, isolated solvent control and DX-treated thymocytes were washed in cold PBS and lysed. After centrifugation at $800 \times g$ for 10 min, the nuclear pellet was washed $3 \times$ in 0.5% NP-40 in Tris-buffered saline, centrifuged at $1000 \times g$ for 5 min, suspended in SDS sample buffer (125 mM Tris, 4% SDS, 10% glycerol, 0.006% Bromo-phenol-blue and 10% mercaptoethanol) and boiled. The post-nuclear supernatant was centrifuged first at $3000 \times g$ for 15 min and then at $12\,000 \times g$ for 5 min. The pellet containing mitochondria was dissolved in SDS sample buffer and the clear supernatant was used as a cytosolic fraction.

Western blotting

Cell fractions were boiled and subjected to SDS-PAGE on a 10 or 15% gel. The gels were blotted overnight to nitrocellulose membranes using a Trans-Blot cell blotting equipment (both from Bio-Rad, Hercules, CA, USA). After transfer, nitrocellulose membranes (Bio-Rad) were soaked in blocking buffer (2% BSA, 10 mM Tris, 100 mM sodium chloride and 0.1% Tween 20, pH 7.4) and then incubated with mouse monoclonal anti-GR antibody (5E4-B1) (26). Mouse mono-

clonal anti- β -actin (Sigma), mouse monoclonal anti-Cytochrome C (BD) and rabbit polyclonal anti-Histone H1 (Santa Cruz Biotechnology) antibodies were used as controls for the protein content and purity of the fractions. Blots were then probed with secondary anti-mouse IgG or anti-rabbit IgG HRP-labeled antibodies in 1:1000 dilutions. For washing, the blots washing buffer was used (10 mM Tris, 100 mM sodium chloride and 0.1% Tween 20, pH 7.4). Western blot visualization was performed by enhanced chemiluminescence as described in the manufacturer's instructions (SuperSignal West Femto Chemiluminescent substrate, Pierce, Rockford, IL, USA). Luminescent light signals were detected with Kodak Image Station 2000R blot documentary system.

Analysis of blots

Densitometry of blots was done with the Quantityone software (Bio-Rad). Relative densities of GR blots were normalized to the relative densities of β -actin, cytochrome C and Histone H1 blots from the same samples to determine the relative expression of GR in the subcellular fractions.

Statistical analysis

Data are presented as mean \pm SD. The effect of various treatments between groups was tested for statistical significance using Student's *t*-test. $P < 0.05$ denoted statistical significance.

Results

Different GR staining pattern of DP and CD4 SP cells

In previous works, we (19, 24) and others (25) have shown that DP thymocytes have the lowest GR expression among the thymocyte subpopulations both in BALB/c and also in a TCR transgenic mouse model (23). Low GR expression of DP cells was verified both on protein level, with flow cytometry (24, 25), and on the mRNA level, with quantitative PCR (19). However, the subcellular localization of the GR in thymocytes remained to be elucidated. Therefore, we performed a morphological comparison of the GR expression in the four main thymocyte subpopulations.

Figure 1 shows confocal microscopic images of CD4 (blue), CD8 (red) and GR (green)-labeled subpopulations of thymocytes. Note: the dominance of the DP and CD4 SP thymocytes is typical in 3- to 4-week-old BALB/c mice (31). On Fig. 1, the DIC (Fig. 1A and D), merged images of the CD4 and CD8 double-cell surface staining (blue and far red, respectively) (Fig. 1B and E) and intracellular GR (FITC green) (Fig. 1C and F) labeling is shown. The weak GR staining in DP cells and strong GR staining in CD4 SP cells (Fig. 1E and F) is in line with previous flow cytometric quantifications (24). There was also a marked difference in the GR staining pattern of the DP and the CD4 SP subpopulations: in DP cells, weak and granular GR staining was localized mostly to the outer rim of the cells (Fig. 1E and F); on the other hand, CD4 SP cells exhibited a more homogenous cytoplasmic and also some nuclear GR staining (Fig. 1E and F).

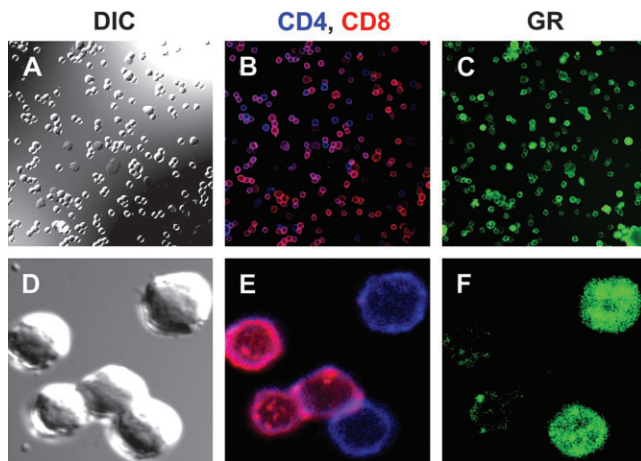


Fig. 1. DP and CD4 SP thymocytes show different GR staining pattern. Representative confocal microscopic images of the major BALB/c thymocyte subpopulations, DIC (A and D), CD4 (blue channel) and CD8 (far red channel) overlaid (B and E) and intracellular GR (green channel) (C and F) images are shown. DP thymocytes (magenta) (B and E) show weak and dominantly granular GR staining (C and F), CD4 SP cells (blue) (B and E), on the other hand, show a more intensive and rather homogenous cytoplasmic and some nuclear GR staining (C and F). The panels in this figure show representative images of at least three separate experiments.

After ligand induction, mitochondrial localization of the GR increased in DP cells

Among the major thymocyte subpopulations, DP cells are the most sensitive to GC-induced apoptosis (24). Since Sionov *et al.* (10, 11) have proposed that GC-induced apoptosis sensitivity correlates with the ligand-induced mitochondrial rather than nuclear translocation of the GR, we studied whether the mitochondrial GR translocation was present in DP cells. Moreover, the granular staining pattern specific for DP cells (Fig. 1E and F) also raised the possibility of the mitochondrial localization of the GR. To test this hypothesis, the mitochondrial dye CMX-Ros and GR co-localization was analyzed in double negative (DN), DP, CD4 SP and CD8 SP cells (Fig. 2), and a comparative analysis was done on DP cells before and after short-time GC treatments (Figs 3 and 4).

CMX-Ros-GR overlaid images of the four major thymocyte subpopulations are shown on Fig. 2. Some mitochondrial GR staining (CMX-Ros-GR co-localization) was observed in all studied cell types (Fig. 2). Upon 30 min DX treatment, the association between the GR and the mitochondria increased in DP cells, but no nuclear translocation was observed (Fig. 3A and B). To quantify the ligand-induced mitochondrial GR translocation, we compared the number of co-localized pixels (both GR and CMX-Ros signals present) in individual DP cells before and after the DX treatment (Fig. 4). There was a nearly 4-fold increase in the number of mitochondrial GR pixels after 30 min DX treatment (350 ± 109 versus 87 ± 36 in the control) (Fig. 4).

As morphological controls for the ligand-induced nuclear or mitochondrial GR translocations (based on our previous unpublished observation), in an additional experiment, we studied two cell lines of lympho-haemopoietic origin, Sp2/0-

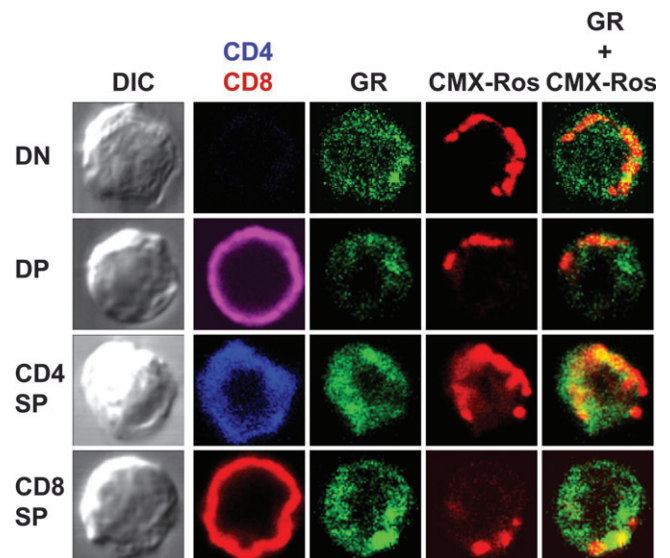


Fig. 2. GR and mitochondrial staining of thymocytes. Confocal microscope images of GR-CMX-Ros co-localization in the four major thymocyte subpopulations according to their CD4 (blue) and CD8 (far red) cell surface expression. Representative images show DN, DP, CD4 SP and CD8 SP thymocytes from at least three separate experiments. Co-localization of the GR with the mitochondria (GR-CMX-Ros merge) is indicated by yellow areas.

Ag mouse myeloma and RBL2H3 rat mast cells, respectively (supplementary Figure 1, available at *International Immunology Online*). In RBL2H3-cells, some nuclear translocation of the GR appeared already after 10 min of DX exposure (data not shown) that completed after 30 min (supplementary Figure 1, available at *International Immunology Online*). In contrast, in Sp2-cells, mainly mitochondrial localization of GR was observed and no nuclear GR translocation appeared after 30 min DX treatment (supplementary Figure 1, available at *International Immunology Online*).

DP cells possessed the lowest level of mitochondrial membrane potential among thymocytes that was further decreased by short-time in vitro DX exposure

Mitochondria are important signal integrating and cellular target organelles for apoptosis (32). Since we have found ligand-induced mitochondrial translocation of the GR in DP cells (Figs 3 and 4), we analyzed the functional consequences of DX exposure on mitochondria by flow cytometry. Besides labeling intact mitochondria, CMX-Ros can also be used for monitoring the mitochondrial membrane potential (ψ_m) (27). In our previous work, we studied the mitochondrial function of thymocytes after *in vivo* DX exposure (23).

In the present study, *in vitro* CMX-Ros-loaded cells were DX treated and cell surface labeled with CD4 and CD8 for flow cytometric analysis. CMX-Ros MFI was detected in FL2-H and analyzed in all thymocyte subpopulations after different treatment times. In control (solvent-treated) samples, the DP cells had significantly lower ($P < 0.05$) CMX-Ros MFI when compared with the other thymocyte subpopulations [80 ± 19 in DP cells versus 136 ± 33 in DN cells, 112 ± 8 in CD4 SP and 196 ± 32 in CD8 SP cells, respectively (Fig. 5A and B)].

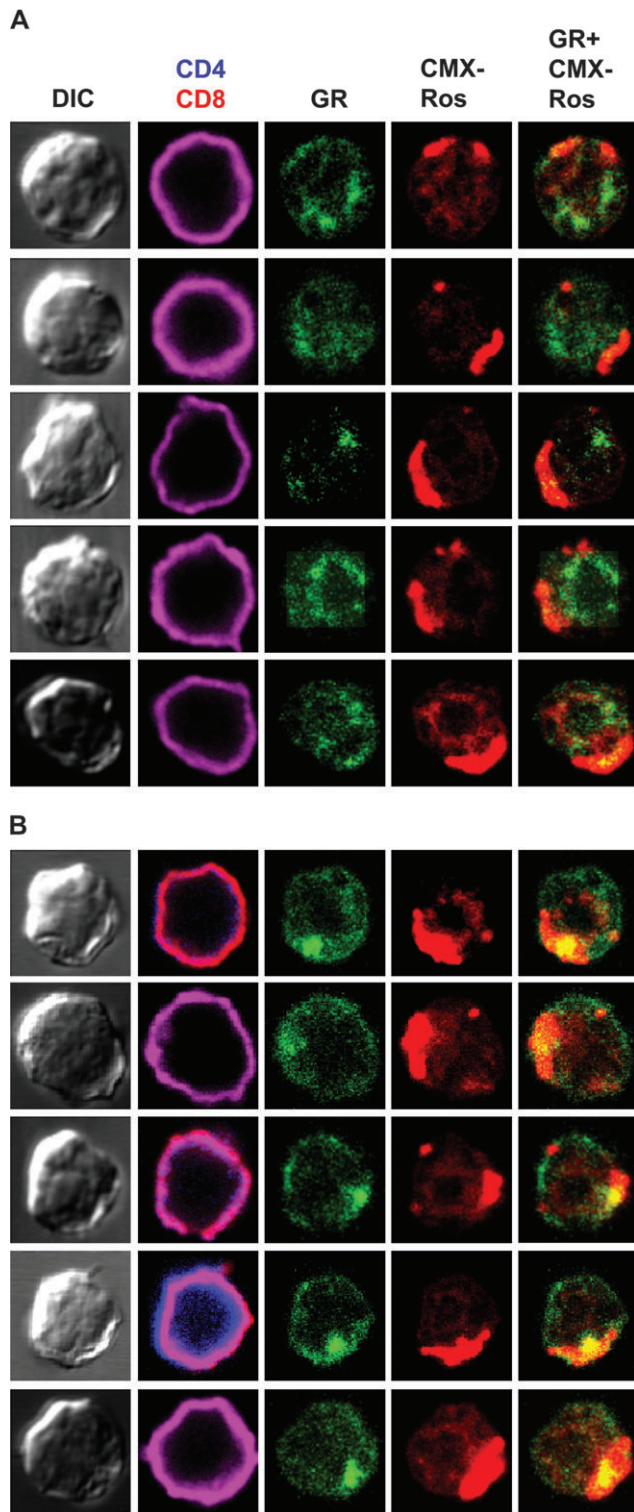


Fig. 3. Ligand-induced mitochondrial translocation of the GR in DP thymocytes after 30 min *in vitro* DX treatment. Representative confocal microscopic DIC, CD4 (blue channel) and CD8 (far red channel) double labeled, mitochondria (CMX-Ros, red channel), intracellular GR (green channel) and finally GR–CMX-Ros merged images show 5–5 control (A) and *in vitro* DX-treated (B) DP cells. Colocalization of the GR with the mitochondria (GR–CMX-Ros merge) is indicated by yellow areas. The panels in this figure show representative images of at least three separate experiments.

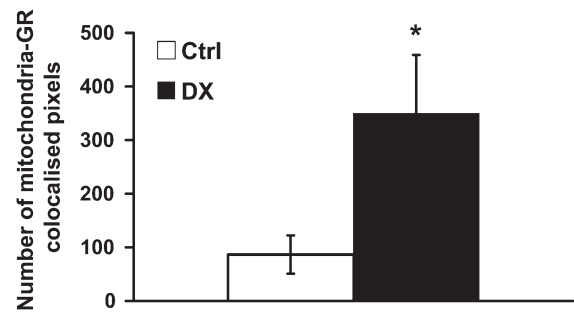


Fig. 4. Quantification of the changes in the GR–CMX-Ros colocalization in DP thymocytes after *in vitro* DX treatment. Bars represent the number of co-localized pixels (GR with CMX-Ros) showing the mitochondrial translocation. The mean \pm SD was calculated from the data of 100 DP cells per treatment, respectively. Significantly higher ($P < 0.05$) values in DX-treated cells versus controls are indicated by asterisk.

The CMX-Ros MFI decreased significantly ($P < 0.05$) in DP cells after 30 min of DX exposure from 80 ± 19 to 45 ± 15 when compared with solvent-treated control (Fig. 5C and 5D) but remained unchanged upon shorter (5 or 10 min) DX exposure (79 ± 16 and 79 ± 15 , respectively).

Subcellular fractionation of thymocytes confirmed the ligand-induced mitochondrial translocation of the GR

Finally, in order to confirm the short-term DX-induced GR trafficking to the mitochondria observed with confocal microscopy, we performed subcellular fractionation and isolated cytoplasmic, nuclear and mitochondrial fractions from unseparated thymocytes. Note: although here thymocytes were not separated based on their cell surface phenotype, 70–80% of the cells are in the DP stage in 3- to 4-weeks-old BALB/c mice (31); therefore, results from our western blot experiments most likely give an impression about the DP cells. In solvent-treated samples, GR was found in all fractions, including the cytoplasm, the nucleus and in trace amounts in the mitochondria (Fig. 6A). Upon DX treatment, the level of the GR decreased in the cytoplasm and rapidly translocated into the nucleus and the mitochondria (Fig. 6A). Densitometric quantification of the western blots confirmed that DX treatment resulted in a significant ($P < 0.05$) decrease of GR expression (relative GR expression: 0.42 ± 0.25) in the cytoplasm, but the mitochondrial and nuclear GR levels increased significantly ($P < 0.05$) at the same time (relative GR expression: 2.58 ± 1.35 and 2.32 ± 0.21 , respectively) (Fig. 6B). The presence of the GR in the nuclear fractions in the western blots is most likely the result of the presence of SP and DN (non-DP) cells in the unseparated thymocyte samples. The strong nuclear signals might be due to the 2–3 times higher GR expression of the non-DP cells (19, 24, 25).

Discussion

The molecular mechanisms and importance of GC-exerted signaling pathways during thymocyte apoptosis and differentiation are still not fully understood (33). The seemingly contradictory phenomenon that in DP thymocytes, the high GC-induced apoptosis sensitivity is coupled with low GR

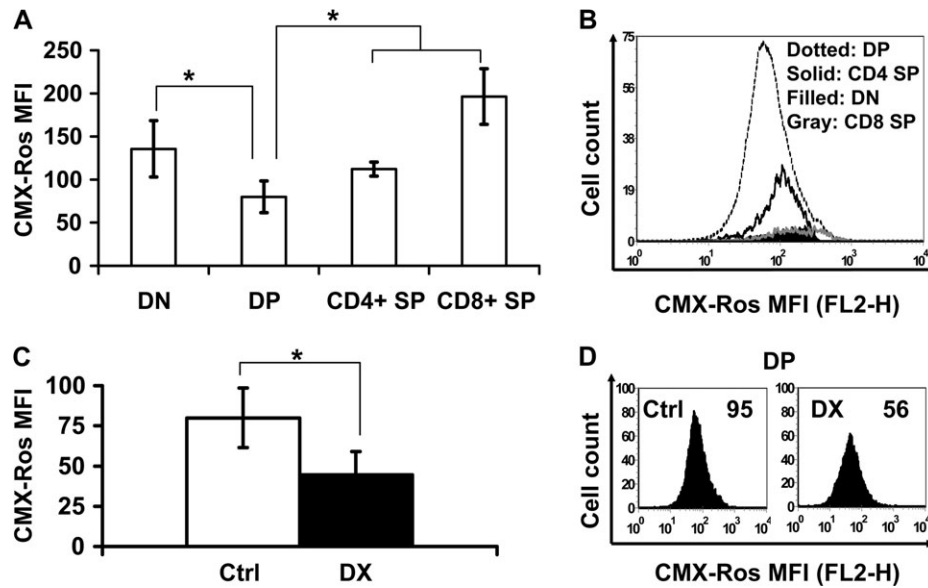


Fig. 5. Mitochondrial membrane potential in thymocyte subpopulations (A and B), and its changes in DP cells upon 30 min of DX treatment (C and D). (A) Bars indicate mean \pm SD of CMX-Ros MFI (calculated from the data of three animals) in the different thymocyte subpopulations. Significant ($P < 0.05$) differences are indicated by asterisk. (B) The overlaid fluorescent histogram plot shows the CMX-Ros MFI of DN, DP, CD4 SP and CD8 SP thymocyte subpopulations. (C) CMX-Ros MFI changes upon *in vitro* DX treatment in DP thymocytes. Bars represent mean \pm SD of CMX-Ros MFI. Significant ($P < 0.05$) differences are indicated by asterisk. (D) Corresponding fluorescent histogram plots show CMX-Ros MFI of control and DX-treated DP cells.

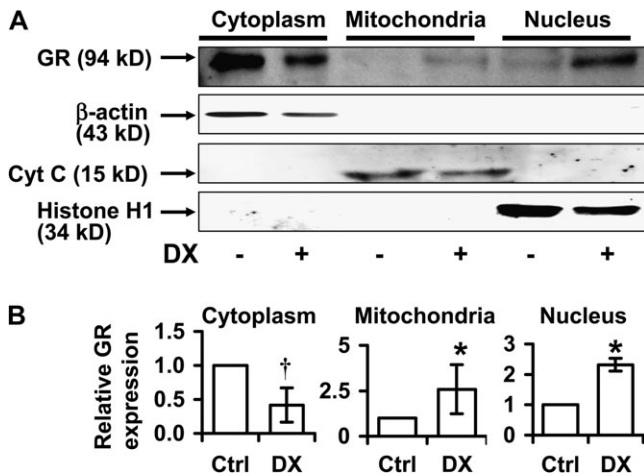


Fig. 6. Redistribution of the GR in the cytoplasmic, mitochondrial and nuclear fractions of unseparated thymocytes. (A) GR was detected in thymocyte subcellular fractions probed with anti-GR mAb (5E4-B1) by western blotting. Blots were also probed with anti- β -actin, anti-Cytochrome C (Cyt C) and anti-Histone H1 antibodies to confirm the purity of the cytoplasmic, mitochondrial and nuclear fractions, respectively. Upon DX treatment, mitochondrial and nuclear GR increase was coupled by cytoplasmic GR decrease. (B) Diagrams show the relative GR expression in the cytoplasm (normalized to β -actin), the mitochondria (normalized to Cytochrome C) and the nucleus (normalized to Histone H1). Bars represent the mean of relative densities compared with the controls \pm SD. Significantly ($P < 0.05$) higher (asterisk) and lower (dagger) values compared with the controls are indicated. The figure shows representative blots (A) and densitometry data of at least three independent experiments (B). Blots were detected using the Kodak Image Station blot documentation system.

expression (24, 25) have directed the attention toward alternative GC actions. Sionov *et al.* demonstrated, for the first time, in different haemopoietic cell lines that, after ligand binding parallel to nuclear translocation, GR can also translocate to the mitochondria (10, 11). In our present work, we show that in BALB/c DP thymocytes, there is a significant association between the GR and the mitochondria. Moreover, a ligand-dependent rapid mitochondrial GR translocation could be verified, after short-time *in vitro* GC-analog treatment. Therefore, we hypothesize that there is a connection between mitochondrial GR translocation and GC sensitivity of DP cells.

Mitochondria-directed mechanisms (including hydrogen peroxide production) and mitochondrial proteins are possible key players in GC-induced thymocyte apoptosis (1, 34). In a TcR-transgenic mouse model, we have already demonstrated that the anti-apoptotic mitochondrial protein, Bcl-2, was up-regulated in thymocytes undergoing positive selection and coupled with significant changes in the mitochondrial membrane potential (23). These results already suggested that *in vivo* GC-induced apoptotic signals could target the mitochondria in DP cells (23). The morphological association between mitochondria and GR, described in the present work, verified the possibility of alternative GR signaling at this developmental stage.

Mitochondria are target organelles of apoptosis since the apoptotic enzyme cascade ends in the disintegration of the mitochondria and cytoplasmic release of Cytochrome C (35). Therefore, the presence of the GR in the mitochondria of DP thymocytes is of special importance in the regulation of apoptosis by GCs. Here, we found that the GR was

present to some extent in the mitochondria of all major murine thymocyte populations. The ligand-induced mitochondrial GR translocation was dominant in DP cells that are known to be the most sensitive to GC-induced apoptosis *in vivo* (22–24). Interestingly, no nuclear translocation of the GR was observed after short-term DX treatment in DP cells. Sionov *et al.* (10, 11) showed that the translocation of the GR into the mitochondrion, but not nucleus could be the main regulator of GC-induced apoptosis sensitivity in lympho-haemopoietic cell lines. Therefore, the ligand-induced mitochondrial GR translocation found in DP cells could dictate their high sensitivity to GC-induced apoptosis.

Interestingly, the mitochondrial GR was also found in ligand (GC)-free experimental condition. This could be caused either by *in vivo* GC exposure, prior to extraction, in thymocytes that are located in the GC-secreting microenvironment of the thymus (19) or mitochondrial GR translocation could occur in a ligand-independent manner, too (10).

In our present work, it was also found that DP cells possess the lowest mitochondrial membrane potential among thymocyte subpopulations, which could also confer to an increased sensitivity to GC-induced apoptosis (21). Short-time GC treatment induced significant but not complete loss of mitochondrial membrane potential in DP cells, most likely due to the rapid non-genomic modulatory effect of glucocorticoids on the mitochondrial function, similar to what was shown by others previously in high-dose GC-treated and conA-stimulated rat thymocytes (36, 37). These results raise the possibility that the mechanism of non-genomic action of GCs is universal in GC-sensitive cells.

It was already reported that the mitochondrial genome contains similar sequences to GRE (38, 39); therefore, it is possible that GR binds to mitochondrial DNA and influences mitochondrial gene transcription similar to what was demonstrated in other experimental systems (40). However, the present results seem to exclude the gene transcription-dependent mechanisms due to their rapid (within 30 min) nature. Besides, steroid hormone receptors, like the GR, transcription factors (41), for example RelB and NFAT (30), or nuclear orphan receptors, like Nur77 (42, 43), which is an important regulator during thymocyte negative selection (44), can also translocate to the mitochondria in thymocytes (42, 43). Recently, it has been also found that incomplete TCR chains can also target the mitochondria and trigger apoptosis (45). It is tempting to speculate that the GR interacts with the above mentioned transcription factors, as in the nucleus (4), or other apoptosis-related proteins in the mitochondria, similar to the cross-talk between Nur77 with Bcl-2 (43).

Other rapid effects of GCs, already elucidated, also involve interactions with important signaling proteins, including TCR signaling associated molecules, like the ZAP-70 in Jurkat T cells (9, 46) and Lck and Fyn in primary T cells (47, 48). Based on these findings, together with our results, presented here, mitochondria may serve as important signal-integrator organelles during the selection processes of the thymocytes regulated by GR signaling.

In conclusion, here, we provide new data about the GR intracellular distribution in BALB/c thymocyte subpopulations and evidence of an alternative GR-mediated signal targeting the mitochondria and regulating mitochondrial function in im-

mature DP thymocytes. However, the fine details of the molecular mechanisms how mitochondrial GR exert their effects during thymocyte apoptosis need further intensive studies.

Supplementary data

Supplementary Figure 1 is available at *International Immunology* Online.

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Conflict of Interest: None.

Abbreviations

CMX-Ros	chloromethyl-X-Rosamine
CyC	cychrome
DIC	differential interference contrast
DN	double negative
DP	double positive
DX	dexamethasone
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
MFI	mean fluorescent intensity
SP	single positive

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