International Immunology, Vol. 21, No. 10, pp. 1113–1123
doi:10.1093/intimm/dxp079

Nitric oxide cooperates with glucocorticoids in thymic epithelial cell-mediated apoptosis of double positive thymocytes

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Keywords: cytokines, death by neglect, inducible nitric oxide synthase, maturing T cells, synergy

Abstract

T cell development in the thymus is controlled by thymic epithelial cells (TE). While it is accepted that TE interact with maturing T cells, the mechanisms by which they trigger 'death by neglect' of double-positive (DP) thymocytes are poorly understood. We and others have demonstrated a role for TE-derived glucocorticoids (GCs) in this process. We have studied TE-induced apoptosis using an *in vitro* system based on co-culturing a thymic epithelial cell line (TEC) with DP thymic lymphoma cells or thymocytes (DP thymic cells). Here, we demonstrate that nitric oxide ($NO^{\cdot}$) is also involved in this death process. The inducible nitric oxide synthase (iNOS) inhibitors N^G-methyl-L-arginine and 1,4-PBIT attenuated TEC-induced apoptosis of DP thymic cells. Co-cultivation of TEC with DP thymic cells increased the expression of iNOS in TEC. A concomitant increase in $NO^{\cdot}$ was detected by staining with DAF-FM diacetate. Moreover, the iNOS-regulating cytokines IL-1α, IL-1β and IFNγ were up-regulated upon interaction of TEC with DP thymic cells. Neutralizing IL-1R or IFNγ reduced TEC-induced apoptosis of DP thymic cells. Cardinally, $NO^{\cdot}$ synergizes with GCs in eliciting apoptosis of DP thymic cells. Our data indicate that a cross-talk between DP thymic cells and TEC is required for proper induction of iNOS-up-regulating cytokines with a subsequent increase in iNOS expression and $NO^{\cdot}$ production in TEC. $NO^{\cdot}$, in turn, cooperates with GCs in promoting death by neglect. We suggest that $NO^{\cdot}$ together with GCs fine-tune the T cell selection process.

Introduction

Pre-T cells entering the thymus via its cortex acquire CD4 and CD8 and are therefore termed double-positive (DP) thymocytes. At this stage of their development such cells begin to express a TCR, which is generated by stochastic recombination of *V*, *D* and *J* genes. As a result, a large repertoire of pre-T cells appears in the thymus, most of which express TCR with low or no affinity to self-MHC. These cells are useless in the context of host immunity and are therefore excluded via apoptotic death in a process known as 'death by neglect'. Death by neglect requires interaction between DP thymocytes and thymic epithelial cells (TE), whose nature is still poorly understood.

We have studied TE-induced apoptosis using an *in vitro* experimental system that is based on co-culturing a thymic epithelial cell line (TEC) with DP thymic lymphoma cells (PD1.6) or thymocytes. PD1.6 cells are a retrovirus-immortalized cell line derived from DP thymocytes. In a previous study, we have shown that TEC resemble TE with regard to their effect on thymocyte apoptosis (1). PD1.6 cells undergo TCR- and MHC-independent apoptosis when co-cultured with TEC, thus recapitulating death by neglect (2). This system has the advantage of comprising only two of the major cell types in the thymus and is, thus, not hampered by contamination of other thymus-derived cells such as macrophages and dendritic cells, which are involved in negative selection. We have shown that this apoptotic process is mediated, in part, by glucocorticoids (GCs) (3) and is dependent on adequate glucocorticoid receptor (GR) expression in the responding DP thymic cells (4). DP thymocytes are the most sensitive thymic sub-population to GC-induced apoptosis (5).

So far, GC has been considered to be the major player in death by neglect (3, 6) based on the following observations: TE express all of the enzymes required for GC

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Received 7 December 2008, *accepted* 17 July 2009

Transmitting editor: R. Medzhitov

Advance Access publication 19 August 2009

synthesis (7, 8) and inhibition of GC synthesis attenuates TEC-induced apoptosis of DP thymic cells (3). Further studies showed correlation between GR expression level in DP thymic cells and their sensitivity to both TEC and GC-induced apoptosis (4). Elimination of GR strongly reduced the ability of the cells to undergo apoptosis in response to GC or TEC (4). Thus, GC seems to play a major role in death by neglect.

Although GR-deficient mice display normal T cell development (9), mice in which the GR level is conditionally up-regulated or down-regulated show altered T cell composition (10, 11). Also, transgenic rats overexpressing a mutant GR with increased ligand affinity in the thymus display perturbed T cell repertoire and altered adaptive immune responses (12). Thus, GCs affect T cell development in the thymus. An indication for the role of GC comes from the observation that GCs antagonize TCR-induced apoptosis and vice versa, TCR signaling inhibits GC-induced apoptosis (13). The level of TCR molecules on maturing DP thymocytes is initially low and increases gradually, achieving nearly full expression as the cells become committed to the SP phenotype (14). Hence, the strength of TCR signaling and the antagonistic effect of GC on cell viability vary upon thymocyte maturation. Accordingly, the intensity of TCR signaling together with GC signaling tune the apoptotic threshold of DP thymocytes.

Recent evidence suggests that in addition to GC, other signals are involved in TEC-induced apoptosis of DP thymic cells. First, we found that the level of corticosterone (Cort) secreted by TEC is below the threshold of effective concentration required to induce apoptosis (4). Second, a GR-deficient thymoma, which is fully resistant to GC-induced apoptosis, displays moderate apoptotic response when co-cultured with TEC (4).

A potential co-inducer of TEC-induced apoptosis is nitric oxide (NO·, formula N = O·). NO· is a highly diffusible free radical with diverse activities in multiple biological systems. The complexity of its biological effects is a consequence of its interactions with other molecules such as reactive oxygen species (ROS), metal ions and proteins (15). NO· reacts with superoxide (O_2^-·) to form peroxynitrite ($ONOO^-$), a potent inducer of cell death. Mouse thymocytes readily apoptose when exposed to various NO· donors such as *S*-nitroso-*N*-acetyl penicillamine (16–18) and *S*-nitrosoglutathione (GSNO) (17, 19). The T cell subset most sensitive to NO·-induced apoptosis is DP thymocytes (16–18). Peroxynitrite was also shown to cause apoptosis of human (20), mouse (21) and rat (22, 23) thymocytes.

NO· is produced when NO· synthases (iNOS, nNOS, eNOS) catalyze the oxidation of L-arginine to citrulline. Inducible nitric oxide synthase (iNOS or NOS2) is regulated primarily at the transcriptional level and can be induced by cytokines (24). iNOS mRNA and protein have been detected in mouse thymic stroma (16) and in human TE (20). Furthermore, nitrotyrosine, generated by the interaction of NO· with tyrosine, was detected in the cortex and corticomedullary junction of the human thymus (20) and in the corticomedullary junction of the mouse thymus (21). These data indicate that NO· is produced in the thymus.

The cortical localization of iNOS and the sensitivity of DP thymic cells to NO·-induced apoptosis suggest a role for this radical in death by neglect. Here, we demonstrate that NO· is indeed involved in TEC-induced apoptosis of DP thymic cells. Moreover, we observed that NO· and GC synergize in inducing apoptosis of DP thymic cells, indicating that these two mediators cooperate in executing death by neglect. Our findings explain the high sensitivity of DP thymic cells to TEC-induced apoptosis under conditions where GC is present at subtoxic concentrations. Furthermore, our data suggest a cross-talk between DP thymic cells and TEC, in which the cytokines IL-1α, IL-1β and IFNγ elevate iNOS and NO· levels in TEC, which in turn enhance the apoptotic effect of GC on DP thymic cells.

Methods

Reagents

N^G-methyl-L-arginine (L-NMMA), 1,4-PBIT, RU38486 (RU486), Cort, dexamethasone (Dex) and GSNO were purchased from Sigma (Rehovot, Israel). LPS was bought from Difco (Lawrence, KS, USA).

Cells

Thymocytes were obtained from thymi of BALB/c, C57BL/6 and B10A 7- to12-weeks-old mice, held under specific pathogen-free conditions at the animal facilities of our institute. Whole thymus was mechanically disintegrated using the flat end of a sterile 5 ml syringe and the supernatant containing thymocytes was collected. This method yields 94% Thy 1.2-positive cells (thymocytes) as determined by flow cytometry. The thymocytes were centrifuged and re-suspended in RPMI supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10 mM HEPES pH 7.4, 1 mM sodium pyruvate, non-essential amino acids, antibiotics and 50 μM β-mercaptoethanol. Unless specified, total thymic lymphocytes were used, of which >75% are $CD4^+8^+$. TEC, an SV40-transformed TEC of a (C57BL/6xBalb/C) F1 mouse, was kindly provided by Dr A. Kruisbeek, National Cancer institute, Bethesda, MD, USA (25). $CD4^+8^+$ PD1.6 thymic lymphoma cells were cloned in our laboratory from a thymic lymphoma generated in a C57BL/6 mouse by intra-thymic inoculation of radiation leukemia virus (RadLV) (26). RAW 264.7 murine macrophage cells were kindly provided by Dr R. Gallily, The Hebrew University of Jerusalem, Israel. The cell lines were grown in DMEM supplemented with the same ingredients as RPMI. All cells were kept in a humidified incubator containing 5% CO_2. In co-cultivation experiments, 12-well plates (containing 2 ml medium) or six-well plates (containing 5 ml medium) were used. In six-well plates, 6×10^5 to 9×10^5 TEC were seeded the day before the experiment to form a monolayer and were co-cultured on the following day with 4×10^5 to 5×10^5 PD1.6 cells in fresh DMEM medium or with 5×10^6 to 10×10^6 thymocytes in fresh RPMI medium. In 12-well plates, 3×10^5 to 4×10^5 TEC were seeded the day before and were co-cultured with 1.5×10^5 to 2×10^5 PD1.6 or with 2×10^6 to 4×10^6 thymocytes. The cells were co-cultured overnight (o.n.), i.e. for 18–24 h. RAW 264.7 cells treated with 1 μg ml^{-1} LPS for 24–48 h served as a positive control for iNOS and cytokine production.

Cell cycle analysis

Detection of apoptosis is based on loss of DNA fragments, as assessed by DNA staining by propidium iodide (PI) and flow cytometry using the FL3 channel. Sub-diploid cells are regarded as apoptotic. Cells were fixed in ice-cold methanol and kept at −20°C for at least 1 h. Following re-hydration in PBS, the cells were treated with RNase (5 μg ml^{-1}) and PI (1 μg ml^{-1}). Cell cycle analysis was performed using FACS-Calibur and Cellquest Software (Becton Dickinson, Istanbul, Turkey). To distinguish between DP thymic cells and TEC, DP thymic cells were pre-labeled with 1.25 μM carboxyfluorescein succinimidyl ester (CFSE) for 8 min in PBS prior to co-cultivation with TEC. Gating was done on CFSE-positive DP thymic cells using the FL1 channel.

PI uptake

Cells were collected, centrifuged and re-suspended in PBS prior to addition of PI (1 μg ml^{-1}) and analyzed by flow cytometry using the FL3 channel. PI-positive cells (PI+) are considered dead cells (these cells also showed a lower FSC and a higher SSC). In co-culture experiments, DP thymic cells were labeled with CFSE as described above.

Determination of thymocyte survival after co-cultivation with TEC

Thymocytes were stained with CFSE as described above prior to the experiment and gating was done on CFSE-positive cells. The amount of live thymocytes remaining after co-cultivation with TEC was determined on flow cytometry by collecting 30 000 PI-negative thymocytes. Thymocytes incubated alone were considered 100% survival. The relative survivals in percentages were calculated.

Analysis of activated caspase-3

DP thymic cells were fixed in ice-cold methanol and kept at −20°C for at least 1 h. Following re-hydration in PBS, the cells were blocked in PBS containing 2% BSA. The cells were then incubated with antibodies specific for active caspase 3 (Cell Signaling, Danvers, MA, USA), followed by incubation with $F(ab)_2$ fragment of Cy5-conjugated donkey anti-Rabbit IgG (Jackson Laboratories, Bar Harbor, ME, USA). Percentage of Cy5-positive cells was analyzed by flow cytometry using the FL4 channel. Thymocytes were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 (SouthernBiotech, Birmingham, AL, USA) prior to incubation with active caspase 3 antibody. Gating was done on $CD4^+CD8^+$ cells.

Reverse transcription–PCR

After o.n. incubation of DP thymic cells with TEC, the non-adherent DP thymic cells were separated from the adherent TEC. The TEC monolayer was washed several times prior to collection to remove thymic cells that have adhered to TEC. mRNA was isolated using the Ultraspec™ RNA isolation kit (BioTecx Laboratories, Houston, TX, USA) and reverse transcribed into complementary DNA (cDNA) using Revertaid™ first strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada). The cDNA was amplified

Table 1. Primer sequences and PCR programs

Primer name	5′ primer sequence	3′ primer sequence	Length of expected fragment (bp)	PCR program							
				Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
iNOS	CCCCGCTATGGCCGCTTTGATGTG	AGGACTGTGGCTCTGACCCGTGAA	735	95°C, 5 min	94°C, 1 min	60°C, 1 min	72°C, 2 min	Repeat step 2–4, 30 times	72°C, 10 min	—
IL-1α	ATGGCCAAAGTTCCTGACTTGTTT	CCTTCAGCAACACGGGCTGGTC	624	95°C, 5 min	94°C, 1 min	75°C, 1 min	60°C, 1 min	72°C, 2 min	Repeat step 2–5, 34 times	72°C, 10 min
IL-1β	CATCAGCACCTCACAAGCAGA	CAATTCATCCCCCACACGTT	410	95°C, 5 min	94°C, 30 s	60°C, 30 s	72°C, 30 s	Repeat Step 2–4, 32 times	72°C, 5 min	—
IFNα	CTCTCCTGCCTGAAGGACAGA	TTCTGCTCTGACCACTCTCCA	362	95°C, 5 min	94°C, 1 min	75°C, 1 min	60°C, 1 min	72°C, 2 min	Repeat step 2–5, 34 times	72°C, 10 min
IFNγ	TACTGCCACGGCACAGTCATTGAA	GCAGCGACTCCTTTCCGCTTCCT	401	95°C, 5 min	94°C, 1 min	60°C, 1 min	72°C, 2 min	Repeat step 2–4, 34 times	72°C, 10 min	—
GM-CSF	AGGATGTGGCTGCAGAATTTACTTTTC	TCATTTTTGGACTGGTTTTTTGCATTC	428	95°C, 5 min	94°C, 1 min	58°C, 1 min	72°C, 2 min	Repeat step 2–4, 37 times	72°C, 10 min	—
GAPDH	GGAGCCAAACGGGTCATCATCTC	GAGGGGCCATCCACAGTCTTCT	233	95°C, 5 min	94°C, 30 s	60°C, 30 s	72°C, 30 s	Repeat step 2–4, 30 times	72°C, 5 min	—

by PCR using specific primers (Sigma Genosys) to the gene products iNOS, GAPDH, IL-1α, IL-1β, IFNγ, IFNα, granulocyte macrophage colony-stimulating factor (GM-CSF) and Thy-1, according to the PCR programs specified in Table 1.

Western blot analysis

After o.n. incubation of DP thymic cells with TEC, the non-adherent DP thymic cells were separated from the adherent TEC. One million cells were lysed in 50 μl protein sample buffer (10% glycerol, 62.4 mM Tris–HCl pH 6.8, 3% SDS, 5% β-mercaptoethanol and 1 mg ml^{-1} bromophenolblue). Fifteen microliters of cell extracts were separated on SDS–PAGE and transferred to nitrocellulose. Western blot analysis was performed using antibodies to iNOS (Calbiochem, Gibbstown, NJ, USA) and α-tubulin (Sigma), followed by either HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (Jackson Laboratories).

NO· detection

After o.n. co-cultivation, the non-adherent DP thymic cells were separated from the adherent TEC, and each cell culture was loaded with 1–3 μM of the intracellular NO· probe DAF-FM diacetate (Molecular Probes, Carlsbad, CA, USA) for 1 h and then co-cultured for another 4 h. The cells were either analyzed by flow cytometry using the FL1 channel or visualized under confocal microscopy.

Statistical analysis

Statistical analysis was preformed using the Student's *t*-test. Significant differences were defined as P value <0.05. Statistical bars shown in the graphs correspond to mean ± SD.

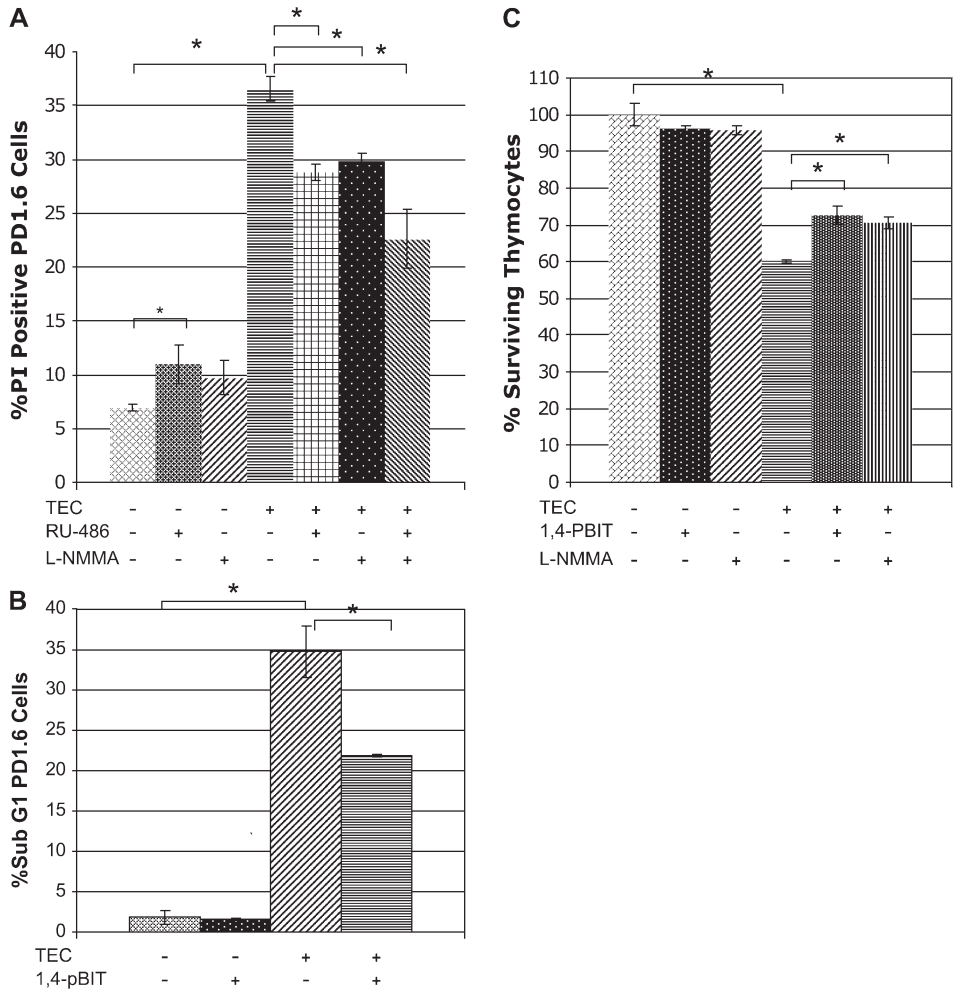

Fig. 1. NO· synthase inhibitors decrease TEC-induced apoptosis of DP thymic cells. (A) PD1.6 cells were harvested after an o.n. incubation with or without TEC, in the presence or absence of 5 μM L-NMMA (iNOS inhibitor) and/or 5 μM RU486 (GR antagonist). Percentage of PI-positive cells was measured by PI-uptake assay. A representative of four separate experiments is shown. (B) PD1.6 cells were harvested after an o.n. incubation with or without TEC, in the presence or absence of 10 μM 1,4-PBIT (an iNOS-specific inhibitor). Cell cycle analysis was done on each sample. A representative of two separate experiments is shown. (C) Thymocytes were harvested after an o.n. incubation with or without TEC, in the presence or absence of 100 μM L-NMMA and 5 μM 1,4-PBIT. Percentage of surviving thymocytes was calculated as described in 'Methods' following PI-uptake assay. A representative of three separate experiments is shown. Statistical bars shown in the graphs correspond to mean ± SD. Significant differences were defined as P value <0.05 and are marked by an asterisk (*).

Results

iNOS inhibitors attenuate TEC-induced apoptosis of DP thymic cells

In order to study the role of NO· in TEC-induced apoptosis of DP thymic cells, two iNOS inhibitors were added to TEC–thymocyte or TEC–PD1.6 co-cultures. Both the broad NO· synthase inhibitor L-NMMA and the specific iNOS inhibitor 1,4-PBIT decreased TEC-induced apoptosis of DP thymic cells by ~25% (Fig. 1A) to 40% (Fig. 1B). Since GC, and possibly other factors, also contributes to this cell death, a total abolishment of TEC-induced apoptosis was not expected. To assess the combined involvement of GC and NO· in the apoptotic process, the GR antagonist RU38486 (RU486) was added to the co-culture together with L-NMMA. As shown in Fig. 1(A), RU486 alone diminished TEC-induced apoptosis of PD1.6 (Fig. 1A). Combined treatment with RU486 and L-NMMA resulted in an additional reduction of TEC-induced apoptosis. These data indicate that GC and NO· cooperate in mediating TEC-induced apoptosis of DP thymic cells. Both L-NMMA and 1,4-PBIT increased the survival of thymocytes co-cultured with TEC (Fig. 1C), demonstrating the involvement of NO· in TEC-induced apoptosis of thymocytes.

DP thymic cells induce iNOS expression in TEC

To trace the cellular source of NO·, we looked for iNOS expression in TEC and DP thymic cells before and after co-cultivation. We took advantage of the fact that TEC are adherent cells, whereas thymocytes and PD1.6 thymic cells grow in suspension. Thus, the two cell populations in the co-culture can be easily separated. The TEC monolayer was washed several times prior to collection to ensure enriched TEC population. Flow cytometry analysis of CFSE-labeled TEC after cultivation with unlabeled thymocytes showed 88–94% purity (data not shown). Reverse transcription–PCR of isolated cell populations show that basal iNOS mRNA expression was detected in TEC, but not in PD1.6 (Fig. 2A and B) or thymocytes (Fig. 2B). Interestingly, the iNOS mRNA level in TEC was significantly elevated after incubation with PD1.6 (Fig. 2A and B) and even more so after incubation with thymocytes (Fig. 2B). This suggests that interaction of TEC with DP thymic cells up-regulates iNOS expression in the former. A corresponding increase in iNOS protein level was detected in TEC after incubation with PD1.6 (Fig. 2C) and with thymocytes (Fig. 2D). RAW 264.7 macrophage cells stimulated with 1 μg ml^{-1} LPS served as a positive control. As iNOS is active once expressed and catalyzes NO· synthesis until substrate depletion (27), we assumed a correlation between its expression and the amount of NO· produced.

NO· is detected in DP thymic cells after interaction with TEC

To track the production of NO·, we applied the NO· probe DAF-FM diacetate, which diffuses into the cells and becomes fluorescent upon NO· binding. NO· was detected in PD1.6 after interaction with TEC both by flow cytometry (Fig. 3A) and by confocal microscopy (Fig. 3B), indicating that NO· diffused into these cells. NO· could also be detected in thymocytes co-cultured with TEC (Fig. 3C and D). Green patches were seen in TEC as well, indicating NO· production in these cells (Fig. 3B and D). The amount of NO· detected in PD1.6 after co-cultivation with TEC is

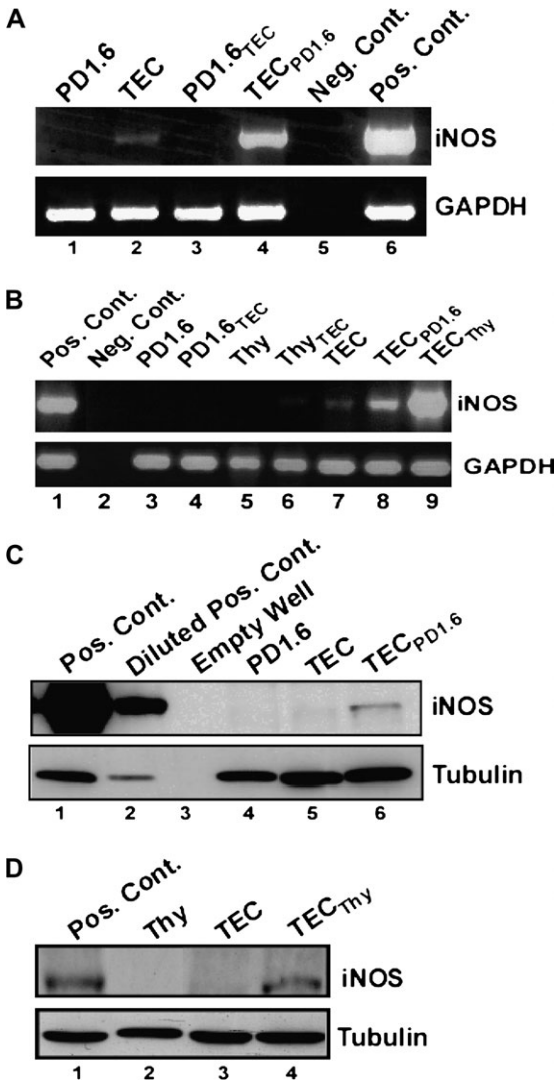

Fig. 2. DP thymic cells induced iNOS mRNA and protein expression in TEC. TEC and DP thymic cells were incubated alone or together o.n.. As TEC are adherent cells and DP thymic cells grow in suspension, we harvested these cells separately after co-cultivation. PD1.6 after incubation with TEC were labeled '$PD1.6_{TEC}$' and vice versa, TEC after incubation with PD1.6 were labeled '$TEC_{PD1.6}$'. This goes also for thymocytes, which were labeled 'Thy'. RAW 264.7 macrophages treated with 1 μg ml^{-1} LPS for 24 h served as a positive control. (A) Reverse transcription–PCR analysis of iNOS mRNA expression. GAPDH house keeping gene served as a control. A representative of two separate experiments is shown. (B) Reverse transcription–PCR analysis of iNOS mRNA expression. GAPDH served as a control. A representative of three separate experiments is shown. (C) Western blot analysis of iNOS protein expression. 1:10-diluted positive control is presented to clearly show the location of iNOS. α-Tubulin served as a marker for equal loading. A representative of five separate experiments is shown. (D) Western blot analysis of iNOS protein expression. α-Tubulin served as a marker for equal loading. A representative of two separate experiments is shown.

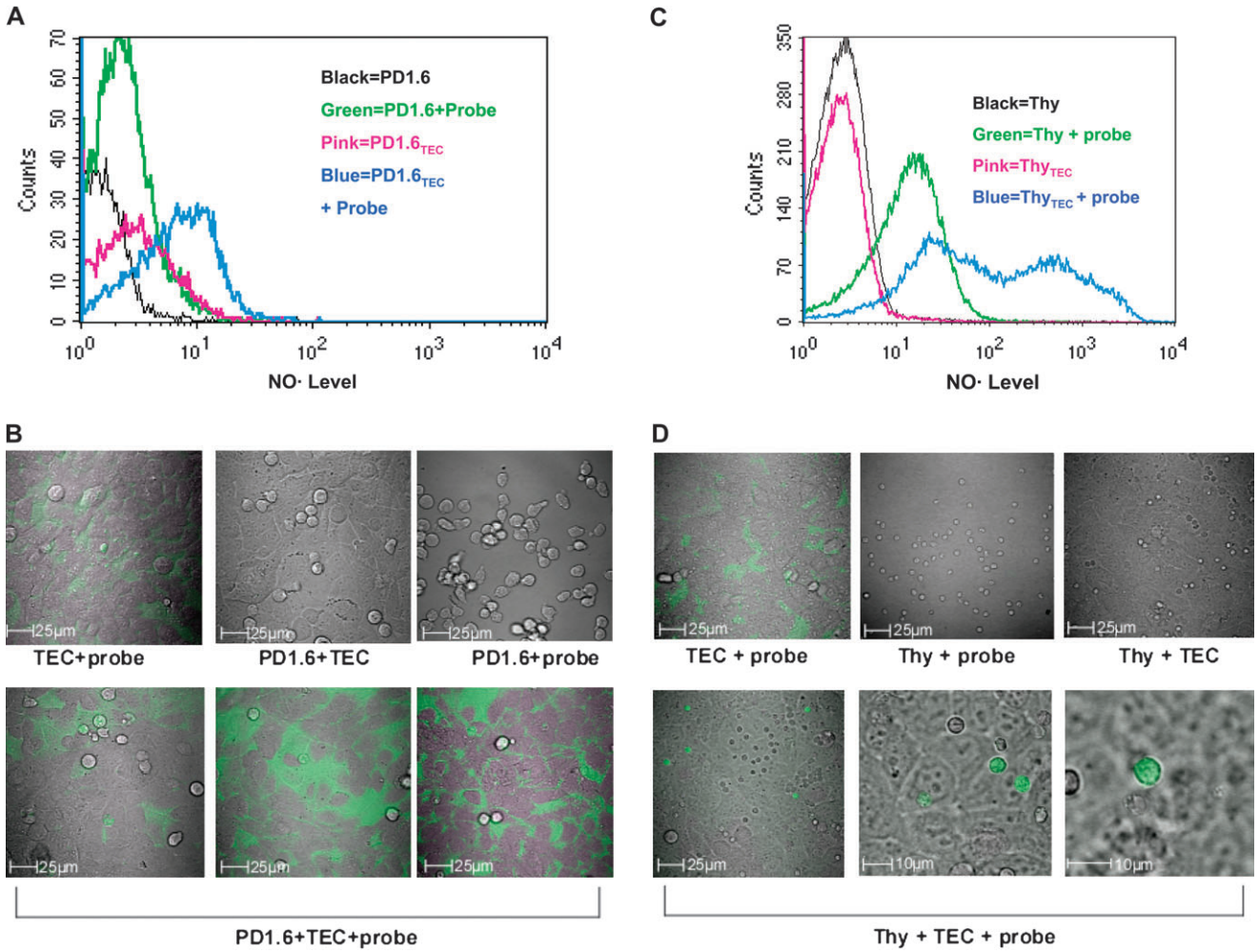

Fig. 3. NO· is detected in DP thymic cells after interaction with TEC. NO· was detected by flow cytometry (A and C) and by confocal microscopy (B and D). After an o.n. incubation, the cells were loaded with the NO· probe DAF-FM diacetate (1–3 μM) and co-cultured for another 4 h before analysis. (A) NO· detection in PD1.6 alone or after co-cultivation with TEC (labeled 'PD1.6$_{TEC}$') by flow cytometry. A representative of three separate experiments is shown. (B) NO· detection in PD1.6–TEC co-cultures by confocal microscopy. A representative of three separate experiments is shown. (C) NO· detection in thymocytes alone or after co-cultivation with TEC (labeled 'Thy$_{TEC}$') by flow cytometry. A representative of four separate experiments is shown. (D) NO· detection in thymocytes co-cultured with TEC by confocal microscope. A representative of three separate experiments is shown.

lower than that detected in thymocytes (Fig. 3A versus Fig. 3C), which correlates with the more efficient induction of iNOS by the latter (Fig. 2B). Altogether, our data show that NO· is produced in TEC when co-cultured with DP thymic cells.

DP thymic cells co-cultured with TEC produce iNOS-regulating cytokines

Since iNOS expression can be induced by pro-inflammatory cytokines (24), we studied the expression of such cytokines in DP thymic cells and TEC (Fig. 4). Co-cultivation of DP thymic cells with TEC led to elevation of IL-1α and IL-1β mRNA level in the co-cultures (Fig. 4). It therefore appears that IL-1α and IL-1β contribute to iNOS induction in TEC. Since IFNα was detected at a basal level in all cell types and was only up-regulated in PD1.6 after co-cultivation with TEC (Fig 4), it seems unlikely to play a major role in iNOS induction. Strong expression of IFNγ was detected in TEC–thymocyte co-cultures, but not in TEC–PD1.6 co-cultures (Fig. 4). This observation correlates with a more efficient induction of iNOS in TEC by thymocytes than by PD1.6 (Fig. 4) and suggests that IFNγ is a central inducer of iNOS expression. Further studies show that GM-CSF is highly expressed in TEC at a basal level and does not correlate with iNOS induction (Fig. 4). As for tumour necrosis factor-α, it could not be detected in DP thymic cells–TEC co-cultures (data not shown). Hence, these cytokines do not seem to contribute to iNOS up-regulation.

In conclusion, our data show that IL-1α, IL-1β and IFNγ are strongly up-regulated in co-cultures of DP thymic cells and TEC and are likely to be involved in the subsequent induction of iNOS expression in the latter.

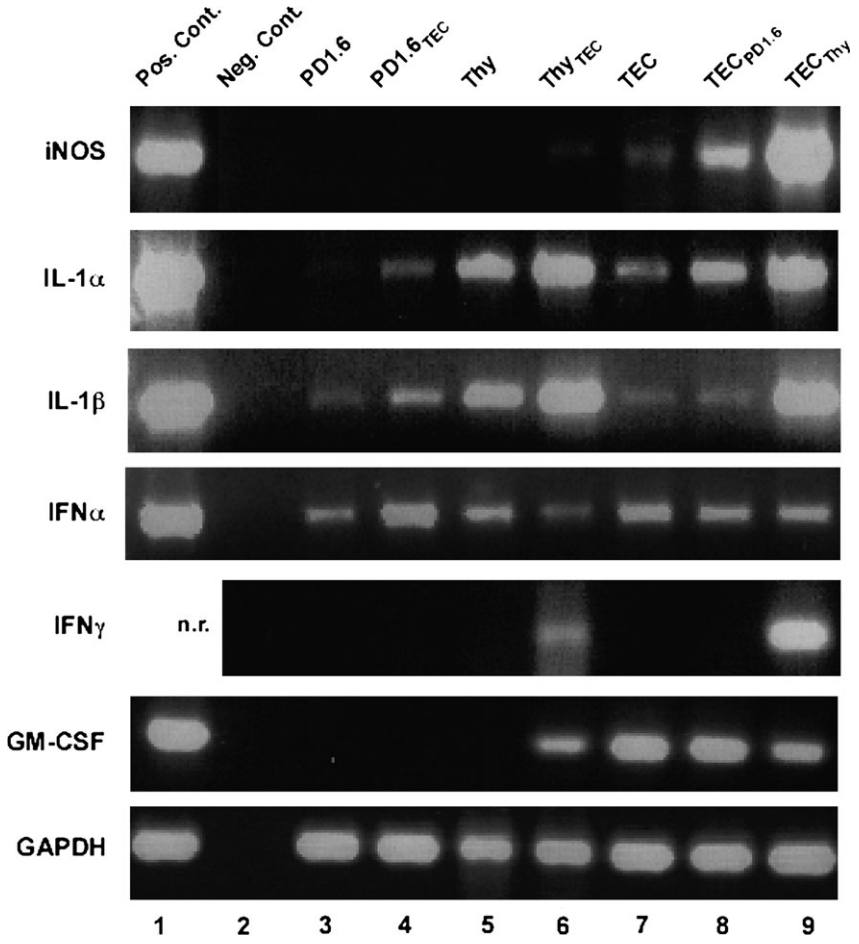

Fig. 4. Co-cultivation of DP thymic cells with TEC led to the induction of iNOS-regulating cytokines. Reverse transcription–PCR analysis of IL-1α, IL-1β, IFNα, IFNγ and GM-CSF cytokines, iNOS and GAPDH mRNA expression. Single and co-cultures were performed as described in Fig. 2. RAW 264.7 macrophages treated with 1 μg ml^{-1} LPS for 24 h served as a positive control for iNOS, IL-1α, IL-1β, IFNα and GM-CSF. n.r. is a short for not relevant. A representative of three separate experiments is shown.

Neutralizing IFNγ or IL-1 decreases TEC-induced apoptosis of thymocytes

In order to verify the function of IFNγ and IL-1α/β in iNOS-mediated TEC-induced apoptosis, we used a neutralizing antibody to IFNγ or IL-1 receptor antagonist (IL-1Ra). IL-1Ra blocks the receptor and prevents it from binding both IL-1α and IL-1β. Both IL-1Ra and anti-IFNγ decreased TEC-induced apoptosis of thymocytes (Fig. 5). Anti-IFNγ was more effective in decreasing TEC-induced apoptosis, which is consistent with our hypothesis that IFNγ is a central inducer of iNOS. Since iNOS is not the sole inducer of TEC-induced apoptosis, a total abolishment of TEC-induced apoptosis was not expected.

NO· synergizes with GC in inducing apoptosis of DP thymic cells

The level of Cort secreted by TEC is far too low (1–5 nM in PD1.6–TEC co-cultures) (4) than that required for the induction of apoptosis. We therefore postulated that NO· synergizes with GC in inducing apoptotic death. To address this possibility, PD1.6 cells were incubated with Cort or Dex alone or together with the NO· donor GSNO. Indeed, GSNO had a synergistic effect on both Dex- and Cort-induced apoptosis of PD1.6 (Fig. 6A–C). The effect of apoptosis in the presence of GSNO and GC is higher than the sum of the effect of each reagent alone. A dose-response curve (calculated for 1–5 nM Dex) shows a steeper slope (a = 5.03) in the presence of 100 μM GSNO than in its absence (a = 1.01) (supplementary Figure 1, available at *International Immunology* Online). GSNO and Cort also act in concert in inducing apoptosis of thymocytes (Fig. 6D). When gating on the DP subset of thymocytes, a synergistic effect is clearly evident (Fig. 6E). It should be noted that GSNO had no apoptotic effect on thymocytes when added alone, but it enhanced the apoptotic effect of Cort on these cells (Fig. 6E). Altogether, these data suggest that NO· produced by TEC synergizes with TEC-derived GC in inducing death of premature thymocytes.

Discussion

T cell development in the thymus involves a dynamic interaction between thymocytes and stromal cells. The fate of the

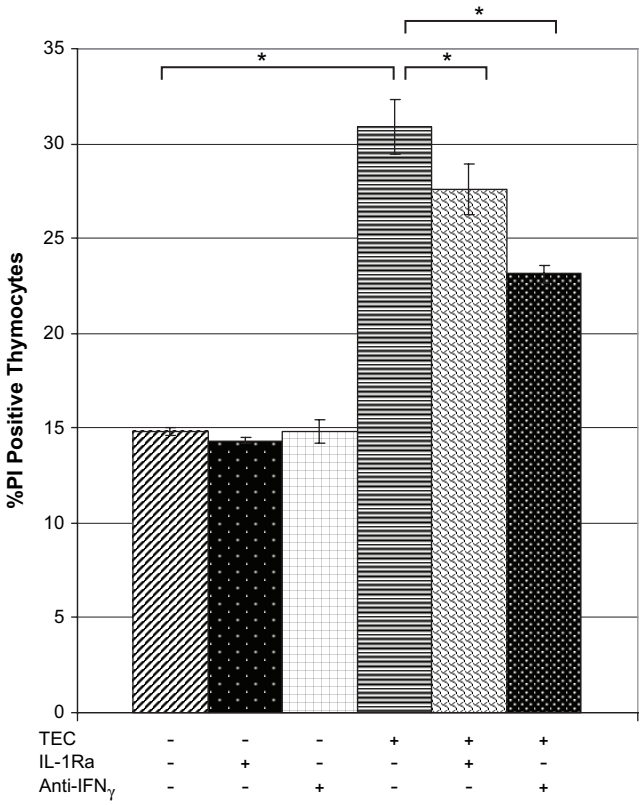

Fig. 5. Neutralizing anti-IFNγ or IL-1Ra reduces TEC-induced apoptosis of thymocytes. Thymocytes were harvested after an o.n. incubation with or without TEC, in the presence or absence of 100 nM anti-IFNγ or 100 μM IL-1Ra. Percentage of PI-positive cells was measured by PI-uptake assay. A representative of three separate experiments is shown. Statistical bars shown in the graphs correspond to mean ± SD. Significant differences were defined as *P* value <0.05 and are marked by an asterisk (*).

thymocytes is largely determined by the intensity of TCR interaction with MHC molecules on the stromal cells. Death by neglect ensures that thymocytes expressing TCR with low or no avidity to self-MHC are effectively eliminated. Such 'useless' thymocytes were considered to die in a passive form of cell death occurring in the absence of TCR signaling (28). However, the data presented in this paper together with previous studies (3, 4) suggest that this type of death is, in fact, an active process requiring specific pro-apoptotic signals delivered by TEC.

The objective of this research was to further study which TEC-derived factors are involved in executing death by neglect. In this paper, we provide evidence that NO· plays a role in TEC-induced apoptosis. We have shown that DP thymic cells induce iNOS expression in TEC, most likely through the production of iNOS-up-regulating cytokines. NO· is detected in TEC–DP thymic cell co-cultures. Cardinally, NO· synthase inhibitors decrease TEC-induced apoptosis of DP thymic cells, which demonstrates the involvement of TEC-derived NO· in TEC-induced apoptosis.

In the present study, NO· was detected by the intracellular probe DAF-FM diacetate, rather than by the routinely used Griess Reagent technique, that detects NO_2^-, an end product of NO· metabolism. The amount of NO· produced in TEC is far lower than that of activated macrophages (data not shown) and is below the threshold of the Griess reagent detection (2 μM NO_2^-). The low NO· production as well as its regulation by thymocytes interacting with TEC seem to be instrumental in the fine-tuning of the apoptotic threshold of death by neglect. Both NO· and GC are produced at low levels by TEC, insuring specific targeting of the most sensitive DP thymocytes, not protected by a TCR mediated survival signal. Higher concentrations would be harmful for T cell development, as reflected by the involution of the thymus following GC therapy (29).

Production of NO· by TEC is inducible and depends on interaction with DP thymic cells. We have shown that co-cultivation of DP thymic cells with TEC leads to elevation of IL-1α and IL-1β mRNA level in both TEC–PD1.6 and TEC–thymocyte co-cultures. IFNγ, however, is only up-regulated in TEC–thymocyte co-cultures and not in TEC–PD1.6 co-cultures. This correlates with a stronger induction of iNOS in TEC by thymocytes than by PD1.6. Neutralizing IL-1α/β or IFNγ decreased TEC-induced apoptosis of thymocytes, thus verifying the role of these cytokines in TEC-induced apoptosis. Since the decrease in apoptosis was greater when using anti-IFNγ than when using IL-1Ra it seems that IFNγ contributes more to this process. iNOS was also shown by others to be expressed in the thymus and thymic iNOS expression level to be regulated by IL-1α/β and IFNγ (16, 20).

The data suggest that IL-1α, IL-1β and IFNγ induce iNOS expression in TEC following co-cultivation. Thus, a dynamic cross-talk may exist between these cells, which determines the extent of NO· production, and consequently the strength of the pro-apoptotic signals delivered to the sensitizing DP thymic cells. A symbiotic relationship between thymocytes and TE has already been demonstrated, where TE support the differentiation of premature T cells, and at the same time, thymocytes provide signals regulating TE development (30–32). Our study sheds further light on the cytokine network persisting in the thymus (33).

In the present paper, we also demonstrate that NO· and GC cooperate in promoting death by neglect. Hence, small fluctuations in their production may have amplifying effect on the apoptotic response. The mechanisms by which NO· amplifies GC-induced apoptosis deserves further study. Since GC-induced apoptosis is accompanied by the formation of ROS such as superoxide (O_2^-·) (34), it could be that NO· reacts with superoxide to form the potent apoptotic radical peroxinitrite. The TEC-thymocytes cross-talk may involve additional levels of regulation. For instance, GC can increase the expression of some cytokine receptors, such as IL-1 receptor and IFNγ receptor (35, 36). By this virtue, TEC-produced GC may indirectly augment cytokine-mediated signals that up-regulate iNOS.

Simultaneous blocking of NO· production and the GC receptor did not completely abolish TEC-induced apoptosis, suggesting that additional TEC-derived factors contribute to death by neglect. In *iNOS-knockout* mice, the thymus, spleen and lymph nodes were histologically normal. Also, thymic and splenic cells from *iNOS*$^{-/-}$ mice and wild-type (wt) mice were indistinguishable when analyzed for immunoglobulins, CD3, CD4 and CD8 expression. Moreover, the

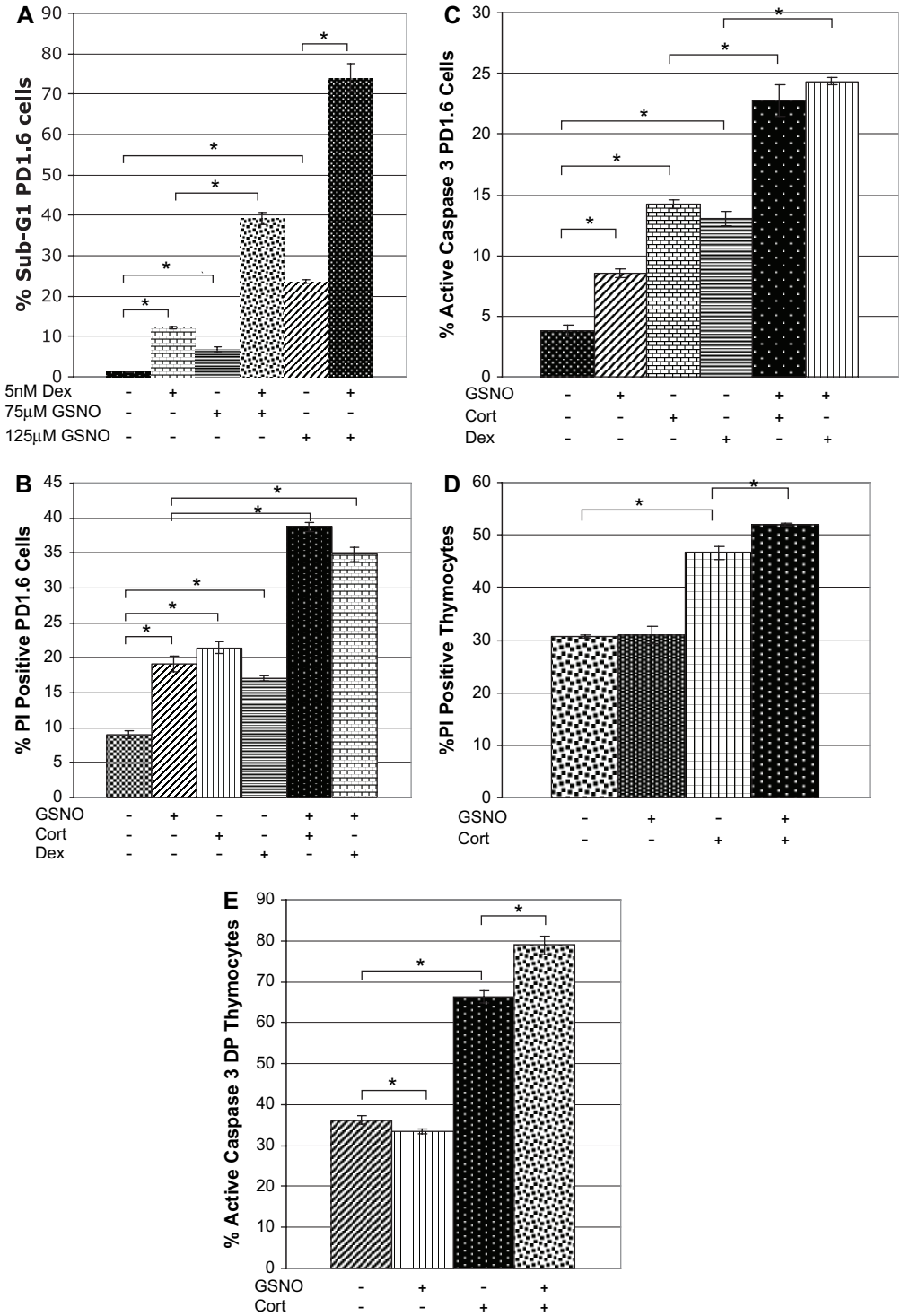

Fig. 6. NO· synergizes with GC in inducing apoptosis of DP thymic cells. (A–C) PD1.6 cells were incubated o.n. with Cort, Dex and/or GSNO. (A) The extent of apoptosis was measured by cell cycle analysis. The concentration of the reagents is as indicated in the graph (these were optimal concentrations for a synergistic effect). A representative of eight separate experiments is shown. (B) Apoptosis was measured by PI-uptake assay. A total of 75 μM GSNO, 100 nM Cort and 5 nM Dex were used. A representative of three separate experiments is shown. (C) Apoptosis was measured by active caspase 3 analysis. A total of 75 μM GSNO, 100 nM Cort and 5 nM Dex were used. A representative of three separate experiments is shown. (D and E) Thymocytes were incubated o.n. with Cort, Dex and/or GSNO. (D) Apoptosis was measured by PI-uptake assay. A total of 50 μM GSNO and 50 nM Cort were used. A representative of three separate experiments is shown. (E) Apoptosis was measured by active caspase 3 analysis after gating on the $CD4^+CD8^+$ population of thymocytes. A total of 25 μM GSNO and 100 nM Cort were used. A representative of three separate experiments is shown. Statistical bars shown in the graphs correspond to mean ± SD. Significant differences were defined as *P* value <0.05 and are marked by an asterisk (*).

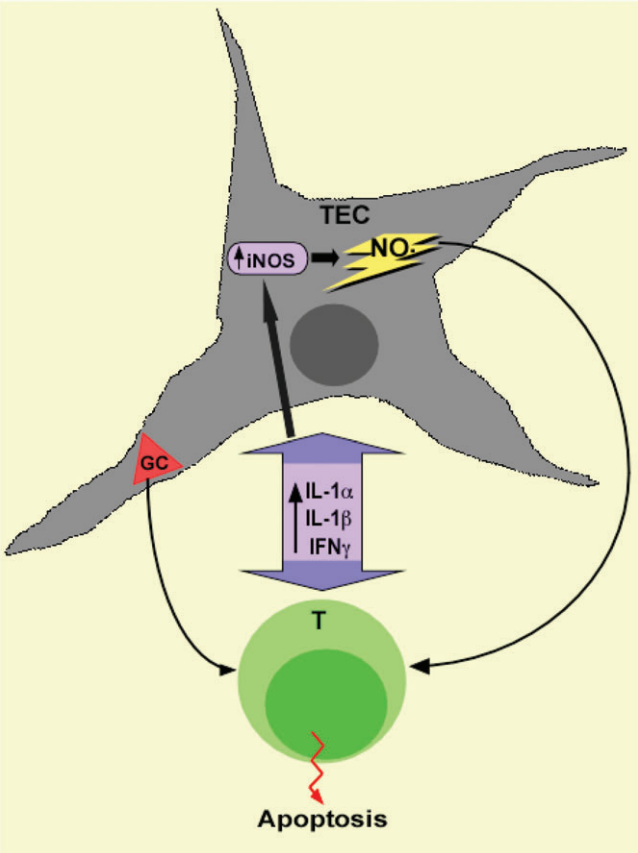

Fig. 7. A model showing the cross-talk between DP thymocytes and TEC leading to the apoptosis of the former. Interaction between DP thymocytes and TEC leads to the elevation of IL-1α, IL-1β and IFNγ cytokines. These cytokines induce iNOS expression in TEC with subsequent NO· production. NO·, in turn, cooperates with TEC-produced GC in inducing 'death by neglect' of DP thymocytes that have not received a survival signal through TCR.

same types and numbers of leukocytes accumulated in the peritoneal cavity of *iNOS*$^{-/-}$ mice during immune responses as in wt mice (37). Similarly, GR$^{-/-}$ mice showed no variance from wt mice in thymocyte development, as well as in T cell stimulation by staphylococcal enterotoxin B or anti-CD3/CD28 treatment (9). Thus, it seems that several and redundant factors mediate death by neglect, which may compensate for the lack of GC and NO·. This explains the apparent normal thymic phenotype of *iNOS*$^{-/-}$ and GR$^{-/-}$ mice. The fact that mice in which the GR level is conditionally modulated show altered T cell composition (10, 11), indicates that although thymocyte development may occur in the absence of GC and NO·, the presence of these factors affects the T cell selection process.

In conclusion, our findings suggest that death by neglect is an active process involving a dynamic cross-talk between TEC and the developing thymocytes. It requires the maintenance of a cytokine network regulating NO· production through the induction of iNOS, which synergizes with GC in promoting apoptosis. A model depicting this intercellular interaction is illustrated in Fig. 7. Accordingly, we propose that 'death by instruction' is a term better reflecting this process.

Supplementary data

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

Funding

Israel Cancer Research Fund; Israel Cancer Association; Concern Foundation; AMEN foundation.

Acknowledgements

We want to thank Dr Hella Lavi-Schottelndreier and Dr Robert Goldstein for technical assistance with confocal microscopy.

Abbreviations

cDNA	complementary DNA
CFSE	carboxyfluoroscein succinimidyl ester
Cort	corticosterone
Dex	dexamethasone
DP	double positive
DP thymic cells	Thymocytes and PD1.6 cells
GC	glucocorticoid
GM-CSF	granulocyte macrophage colony-stimulating factor
GR	glucocorticoid receptor
GSNO	*S*-nitrosoglutathione
IL-1Ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
L-NMMA	N^G-methyl-L-arginine
NO·	nitric oxide
o.n.	overnight
PD1.6	A DP thymic lymphoma cell line
PI	propidium iodide
ROS	reactive oxygen species
TE	thymic epithelial cells
TEC	thymic epithelial cell line
wt	wild-type

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