JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121

Prenatal exposure of mice to diethylstilbestrol (DES) disrupts T cell differentiation by regulating Fas/FasL expression through ERE and NF-kB motifs

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JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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Running Title: DES regulates Fas/FasL via ERE and NF-kB

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Number of Text Pages: 38
Number of Tables: 0
Number of Figures: 8
Number of References: 45
Number of words in Abstract: 250
Number of words in Introduction: 583
Number of words in Discussion: 1464

Abbreviations used in this paper: DES, diethylstilbestrol; ER, estrogen receptor; DRE, dioxin responsive element; ERE, estrogen receptor element; NF-kB, nuclear factor kappa B; RLU, relative light unit, TAM, tamoxifen

Recommended Section Assignment

Cellular and Molecular

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ABSTRACT

Prenatal exposure to diethylstilbestrol (DES) is known to cause altered immune functions and increased susceptibility to autoimmune disease in humans. In the current study, we investigated the effect of prenatal exposure to DES on thymocyte differentiation involving apoptotic pathways. Prenatal DES exposure caused thymic atrophy, apoptosis, and upregulation of Fas and FasL expression in thymocytes. To examine the mechanism underlying DES-mediated regulation of Fas and FasL, we performed luciferase assays using T cells transfected with luciferase reporter constructs containing full length Fas or FasL promoter. There was significant luciferase induction in the presence of Fas or FasL promoter following DES exposure. Further analysis demonstrated the presence of several cis-regulatory motifs on both Fas and FasL promoters. When DES-induced transcription factors were analyzed, ERE, NF-kB, NF-AT, and AP-1 motifs on Fas promoter as well as ERE, NF-kB, and NF-AT motifs on FasL promoter showed binding affinity with the transcriptions factors. EMSAs were performed to verify the binding affinity of cis-regulatory motifs of Fas or FasL promoter with transcription factors. There was shift in mobility of probes (ERE or NF-kB2) of both Fas and FasL in the presence of nuclear proteins from DES-treated cells and the shift was specific to DES as these probes failed to shift their mobility in the presence of nuclear proteins from vehicle-treated cells. Together, the current study demonstrates that prenatal exposure to DES triggers significant alterations in apoptotic molecules expressed on thymocytes which may affect T cell differentiation and may cause term effects on the immune functions.

Introduction

Diethylstilbestrol (DES; 4,4'-(3*E*)-hex-3-ene-3,4-diyldiphenol) is a potent synthetic estrogen. From the 1940s to the 1970s, pregnant women were administered DES to treat "pregnancy-associated" problems. Shortly after the link of prenatal DES-exposure with adult-onset of reproductive abnormalities was established, the use of DES was banned. However, by the time the use of DES in pregnant women was banned, nearly 2–5 million pregnant women had already been exposed to DES (Marselos and Tomatis, 1992a; Marselos and Tomatis, 1992b). Previous studies have demonstrated that prenatal exposure to DES can cause thymic atrophy and apoptosis in T cells (Brown et al., 2006a; Brown et al., 2006b). There are also reports that neonatal exposure to DES caused effects on the reproductive system (Golden et al., 1998; Herbst et al., 1971). However, precise mechanism(s) by which DES causes thymic atrophy or the fetal immune dysfunctions has not been thoroughly explored.

The murine thymus has been shown to be sensitive to DES when exposed for a short time during neonate and adult stage of mice (Besteman et al., 2005; Brown et al., 2006a; Brown et al., 2006b; Forsberg, 2000). Inasmuch as the thymus is the primary organ for the development of T cells that are long-lived and vital for immune competence, any alterations in this organ may have notable immunological consequences. DES initiates early signaling primarily through estrogen receptor (ER) and regulate expression of various genes (Brown et al., 2006a; Brown et al., 2006b; Miyagawa et al.). Previous studies have demonstrated that DES caused decrease in prothymocyte stem cells (Holladay et al., 1993), decrease in double positive $CD4^+CD8^+$ cells (Brown et al., 2006a;

Smith and Holladay, 1997), as well as induce cell death in thymocyte subsets $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ (Brown et al., 2006b; Calemine et al., 2002). DES has also been shown to induce apoptosis of double negative $CD4^-CD8^-$ cells in a fetal thymic organ culture system (Lai et al., 2000). One of the key mechanisms of apoptosis is the extrinsic pathway involving Fas and FasL. Nair et al have shown that DES caused apoptosis in spermatogenic cells of rat via Fas and FasL pathway (Nair and Shaha, 2003). In this study, therefore, we investigated the molecular mechanisms leading to regulation of Fas and FasL by DES.

Regulation of a gene is controlled by distinct protein-DNA interactions at its promoter. Earlier studies have identified c-myc and Nur77 as transcription factors involved in regulation of Fas and FasL expression (Davis and Lau, 1994). In recent years, several other transcription factors that include nuclear factor of activated T cells (NFAT), p53, ceramide, members of JNK/SAP stress-activated pathways, and NF-kB, have been identified to regulate the expression of Fas and FasL genes (Sharma et al., 2000). Studies from our laboratory have demonstrated the regulation of Fas and FasL involving DRE and/or NF-kB motifs present in their promoters (Singh et al., 2007). We have also shown that TCDD/AhR interaction in thymic stromal cells led to activation of NF-kB and its migration from the cytosol to the nucleus, where it participated in upregulation of FasL expression (Camacho et al., 2005). Furthermore, NF-kB has also been shown to upregulate the expression of Fas (Baba et al., 2001; Puga et al., 2000; Schlezinger et al., 2000; Sulentic et al., 2000; Zheng et al., 2001).

The precise mechanism of DES-mediated regulation of Fas and FasL genes with respect to the specific transcription factors has not been well characterized and understood.

Therefore, the aim of the present study was to elucidate the molecular mechanisms of DES-mediated regulation of Fas and FasL gene expression during the development of the immune system and examine the role of ERE and NF-kB motifs in regulation of these genes.

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Materials and Methods

Mice. Normal and pregnant (GD14) mice (C57BL/6: H-2^b) were purchased from Jackson Laboratory. The animals were housed in University of South Carolina Animal facility. Care and maintenance of the animals were carried out in accordance with the guidelines for the care and use of laboratory animals as adopted by Institutional and NIH guidelines.

Cell Line. Mouse T cell lymphoma cell line (EL4) was maintained in complete RPMI 1640 medium (10% heat-inactivated fetal bovine serum, 10 mM L-Glutamine, 10 mM HEPES, and 100 µg/ml penicillin/streptomycin) at 37°C and 5% CO_2.

Reagents and Antibodies. **D**iethylstilbestrol (DES; 4,4'-(3*E*)-hex-3-ene-3,4-diyldiphenol) was purchased from Sigma-Aldrich (St. Louis, MO). The following reagents (HEPES, L-Glutamine, RPMI 1640, PBS, and FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Mouse mAbs (anti-mouse IgG, FcBlock, anti-FasL-PE (Kay-10), and anti-Fas-PE (Jo2) were purchased from BD Pharmingen (Carlsbad, CA). TransFactor Universal Chemiluminescent kit was purchased from Clontech (Clontech Laboratory, Inc, Mountain View, CA). The following reagents: pGL3-basic vector from Promega (Madison, WI), pCMV-β-galactosidase vector from BD Clontech (Carlsbad, CA), Amaxa Cell line Nucleofector transfection kit and Mouse T Nuclefector transfection kit were purchased from Lonza (Lonza Cologne GMBH, Cologne, Germany), and the Dual-Light system were purchased from Applied Biosystems (Foster City, CA). RNeasy Mini kit and iScript cDNA synthesis kit were purchased from Qiagen (Valencia, CA). Epicentre's PCR premix F and Platinum Taq

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Polymerase kits were purchased from Invitrogen Life Technologies (Carlsbad, CA). T4 polynucleotide kinase was purchased from New England Biolabs (New England Biolabs, Boston, MA) and NICK columns were purchased from Amersham Biosciences (Amersham Biosciences). Western blot reagents: anti-Fas primary polyclonal antibody (Ab-1) was purchased from Calbiochem (Darmstadt, Germany) and anti-FasL primary polyclonal antibody (AB16982) was purchased from Millipore (Temecula, CA).

***In vivo* exposure of pregnant mice with DES.** To determine the effect of DES on thymus in normal adult mice and their fetuses, a single dose of DES (5 μg/kg) was administered (ip) into pregnant C57BL/6 mice on GD 14. We chose to use GD 14 pregnant mice for all our experiments as by this time, fetuses are not fully developed and so the effects of DES can fully be exploited. Also, we used DES (5 μg/kg) based on previous studies from our laboratory and studies from other laboratories (Brown et al., 2006a). On day 2 (GD 16) and day 4 (GD 18) post-DES treatment, thymic weight and cellularity of both mothers and fetuses were determined. Mice treated with vehicle (VEH; corn oil) were used as control. For each treatment group, at least three pregnant mice were used and from each pregnant mother, we obtained an average of 8 pups. To reduce the variability among the pups in each litter, we combined the three litters from each treatment group to generate a pool of ~24 pups. Due to low thymic cellularity in the fetus, thymi from ~5 pups were randomly pooled per sample, and the replicate pools were used for statistical analysis.

Preparation of thymocytes and detection of apoptosis in thymocytes. Thymi from mice and fetuses were harvested and placed in complete RPMI-1640 medium. Single cell suspensions of thymi were prepared as described earlier (Camacho et al.,

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2004a; Camacho et al., 2004b). Cell viability was determined on a hematocytometer by staining the cells with trypan blue dye and using an inverted phase contrast microscope. For calculating thymic cellularity, the data were expressed as total number of thymocytes/mice. For statistical analysis, 5-6 replicate pools were compared from each treatment group and depicted as mean ± SEM.

Thymocytes from mice exposed to DES or VEH were analyzed for apoptosis using the TUNEL assay kit (Roche, Indianapolis, IN) and as described previously (Camacho et al., 2004a; Camacho et al., 2004b; Camacho et al., 2005; Singh et al., 2008). In brief, thymocytes ($1x10^6$) from various groups of mice were cultured for 24 h in complete RPMI 1640 medium and the following day, the cells were washed twice with PBS and analyzed by TUNEL assay. Apoptotic cells were quantified as average number of TUNEL positive cells from at least 30 foci of each section.

Determination of Fas and FasL expression in fetal thymic cells. Expression of Fas and FasL in fetal thymic cells was determined by performing RT-PCR. In brief, single cell suspension of fetal thymic cells post VEH or DES exposure was prepared as described above. Expression of Fas and FasL was determined by RT-PCR as described previously (Singh et al., 2007)and by Western blotting. For RT-PCR, total RNAs from VEH- or DES-treated groups were isolated using RNeasy Mini Kit and following the protocol of the company (Qiagen, Maryland). First strand cDNA synthesis was performed in a 20 µl reaction mix containing 2 µg total RNA using iScript Kit and following the protocol of the manufacturer (Bio-Rad). PCR was performed using mouse FasL or Fas-specific sets of forward and reverse primers as described earlier (Singh et al., 2008). The PCR products, generated from mouse Fas and FasL primer pairs were

normalized against PCR products generated from mouse 18S-specific forward 5'-GCCCGAGCCGCCTGGATAC-3' and reverse 5'-CCGGCGGGTCATGGGAATAAC-3' primers after electrophoresis on 1.5% agarose gel and visualization with UV light. The band intensity of PCR products was determined using BioRad image analysis system (BioRad). Expression of Fas and FasL in thymi was also confirmed by performing Western blotting using anti-Fas primary polyclonal antibody (Ab-1; Calbiochem, Darmstadt, Germany) and anti-FasL primary polyclonal antibody (AB16982; Millipore, Temecula, CA).

Determination of Fas and FasL expression in unactivated and activated T cells. Expression of Fas and FasL in unactivated and activated T cells was analyzed by using flow cytometry and RT-PCR. In brief, unactivated purified T cells or ConA (2 µg/ml overnight)-activated T cells from C57BL/6 mice were cultured in the presence of VEH or DES (10 µM) or Tamoxifen (TAM) (10 µM) or TAM+DES (10 µM) for 8 hrs. We chose to use 10 µM DES or TAM based on previously established studies (Lai et al., 2000), including those from our laboratory (Brown et al., 2006a; Brown et al., 2006b), where various doses (1-20 µM) of DES were used. We found that 10 µM DES or TAM used in this study was the optimal concentration for examining the molecular mechanisms. Expression of Fas and FasL was determined by flow cytometry or RT-PCR as described previously (Singh et al., 2007). For flow cytometry, cells were stained with FITC-labeled anti-mouse Fas and PE-labeled anti-mouse FasL antibodies and their expression was analyzed by flow cytometry (FC500, Beckman coulter). For RT-PCR, total RNAs from various treated groups were isolated using RNeasy Mini Kit and following the protocol of the company (Qiagen, Maryland). First strand cDNA synthesis

and PCR was performed using mouse FasL or Fas-specific sets of forward and reverse primers as described earlier (Singh et al., 2008). The PCR products, generated from mouse Fas and FasL primer pairs, were normalized against PCR products generated from mouse 18S after electrophoresis on 1.5% agarose gel and visualization with UV light. The band intensity of PCR products was determined using BioRad image analysis system (BioRad).

Generation of the mouse Fas and FasL promoter reporter constructs. The mouse Fas or FasL promoter was cloned in pGL3 vector and have previously been described earlier (Singh et al., 2007). To elucidate the role of various cis-regulatory motifs present in Fas or FasL promoter involved in regulation of Fas or FasL expression, pGL3-Fas-Luc or pGL3-FasL-Luc reporter constructs were used.

Transfection of EL4 cells and luciferase assays. Freshly cultured EL4 cells ($5x10^6$) were transfected with 5-10 µg of the indicated reporter plasmid and 1 µg of the pCMV-βGal control vector using Amaxa Nucleoector instrument and kits from Lonza and following the protocol of the company (Lonza Cologne GMBH, Cologne, Germany). Two days post transfection, EL4 cells were replated in triplicate in 96-well plate and the cells were treated with VEH or various doses of DES (1, 5, 10, and 20 µM/ml) and incubated for 24 h at 37 °C, 5% CO_2. Following incubation, cells were harvested, washed with PBS, centrifuged, and extracts were prepared by lysing cells in 70 µl of reporter lysis buffer (Promega Corporation, Madison, WI). Luciferase activity was determined by mixing 20 µl of extract with 100 µl of luciferase substrate (Promega corporation, Madison, WI) and immediately reading the sample in Victor^2 (Perkin Elmer). Similarly, Beta-galactosidase activity was determined by mixing 20 µl of extract with galactolyte

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Beta-galactosidase substrate (Tropix, Bedford, MA), incubating for 1 h at 25 °C, adding 300 µl of galactolyte accelerator (Tropix, Bedford, MA), and reading the sample with Victor^2. Under similar conditions, TAM (10 µM/ml), an antagonist for DES, was used in the culture at least 1 hr prior to DES treatment and luciferase and Beta-galactosidase assays were performed. Luciferase activity was normalized by dividing the mean luciferase RLU by the mean Beta-galactosidase RLU and the normalized luciferase RLU from the stimulated samples were divided by the normalized RLU of the untreated sample, and values were expressed as "normalized-fold induction."

Identification of transcription factor binding motifs in Fas and FasL promoters. Each promoter possesses several transcription binding motifs with which transcription factors bind to regulate gene expression. To identify various transcription factors binding motifs present in the Fas and FasL promoters, we analyzed Fas or FasL promoters using Matinspector 8.0 software from Genomatrix (Genomatrix GMBH, Munich, Germany), This software allows to identify various transcription factor binding sites present in a promoter.

Generation of transcription factor binding site-specific probes for Fas and FasL promoters. Probes for various transcription factors binding sites present in Fas or FasL promoter were generated. At the 5' end, the probes were biotinylated. Probes and complementary strand were synthesized by Integrated DNA Technologies (Coralville, Iowa). The details of the sequence of various probes containing transcription factors binding sites present in Fas or FasL promoters are as described below.

Probes for Fas promoter

ERE:5'-CTCCCCCACTTGATCTTCCCATCATACTACC-3'
3'-GAGGGGGTGAACTAGAAGGGTAGTATGATGG-5'
NF-KB1: 5'-ATTGTTTGGGGACTCCCATGCAAC-3'

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3'-TAACAAACCCCTGAGGGTACGTTG-5'
NF-KB2:5'-GAAGCCAGGGACATTCCCCTCATCAGGA-3'
3'-CTTCGGTCCCTGTAAGGGGAGTAGTCCT-5'
NF-KB3:5'-GCTCTGTGGAGGGACCTCCCTCATCAGAAT-3'
3'-CGAGACACCTCCCTGGAGGGAGTAGTCTTA-5'
NF-KB4:5'-CGCCCAGGATGGGGAATGCCCATTTATGCAAT-3'
3'-GCGGGTCCTACCCCTTACGGGTAAATACGTTA-5'
NF-KB5:5'-GTGAGTCAGTGGGTTTCCCCGGGAGACCAGCA-3'
3'-CACTCAGTCACCCAAAGGGGCCCTCTGGTCGT-5'
NF-AT1:5'-CCCCATCCA*GGAA*AGGAAAACTTCAT-3'
3'-GGGGTAGGTCCTTTCCTTTTGAAGTA-5'
NF-AT2:5'-ACTCTGCTAGGTTTCCATACCTCTCAAATG-3'
3'-TGAGACGATCCAAAGGTATGGAGAGTTTAC-5'
AP-1.1:5'-CTGTGTTATTTGTCATAGTCATTTACCTTATTC-3'
3'-GACACAATAAACAGTATCAGTAAATGGAATAAG-5'
AP-1.2:5'-GCAGACCTGTCACTGACAAAATATTGAAAGT-3'
3'-CGTCTGGACAGTGACTGTTTTATAACTTTCA-5'
AP-1.3:5'-GACCTGAGGGTGTGACTGTGGAAGCGCCCA-3'
3'-CTGGACTCCCACACTGACACCTTCGCGGGT-5'
DRE and SP1: 5'-GGTTTGTGCGTGCCAGGGGGCGGGCCTAGG-3'
3'-CCAAACACGCACGGTCCCCCGCCCGGATCC-5'

Probes for FasL promoter

ERE: 5'-TTTTCATCTGGTGACCAGAAGAGAGATTGC-3'
3'-AAAAGTAGACCACTGGTCTTCTCTCTAACG-5'
NF-KB1: 5'-CTCCTTGGTCTTTTCCCCATGCCTCAGCA-3'
3'-GAGGAACCAGAAAAGGGGTACGGAGTCGT-5'
NF-KB2: 5'-ACAGGAGAAAGGTGTTTCCCTTGACTGC-3'
3'-TGTCCTCTTTCCACAAAGGGAACTGACG-5'
SP1 and NF-AT1: 5'-TCAGAAATTTCTGGGCGGAAACTTCCTGGGG-3'
3'-AGTCTTTAAAGACCCGCCTTTGAAGGACCCC-5'
NF-AT2: 5'-CCTTGACTGCGGAAACCTTATAAAACT-3'
3'-GGAACTGACGCCTTTGGAATATTTTGA-5'
NF-AT3: 5'-AAGGCTGTGAAGAGGAAACCCTTTCCTGG-3'
3'-TTCCGACACTTCTCCTTTGGGAAAGGACC-5'

Identification of transcription factors post DES treatment of T cells. After identification of various transcription factor binding sites present in the Fas and FasL promoters, we sought to identify transcription factors involved in DES-mediated regulation of Fas and FasL genes, To this end, we performed *in vitro* and chemiluminescent assays to identify various transcription factors involved in DES-mediated regulation of Fas and FasL expression in T cells. In brief, we first prepared

nuclear extracts from T cells treated with VEH or DES (10 μM/ml) for 6 hrs using TranFactor extraction kit from Clontech and following the protocol of the company (Clontech Laboratory, Inc, Mountain View, CA). Next, we performed chemiluminescent assays using TranFactor Universal Chemiluminescent kit from Clontech and following the protocol of the company (Clontech Laboratory, Inc, Mountain View, CA). The binding of transcription factor(s) with various Fas or FasL probes were identified by Victor^2 (Perkin Elmer).

Electrophoretic Mobility Shift Assay (EMSA). The EMSA assays were performed using oligonucleotide probes corresponding to ERE and NF-kB motifs of Fas or FasL promoters and nuclear proteins prepared from T cells treated with VEH or DES.

Preparation of nuclear extracts. To prepare nuclear extract for EMSA assays, purified T cells from C57BL/6 mice were treated with DES (10 μM/ml) and cultured for 6 hrs at 37°C, 5% CO_2. Post DES or VEH treatments, nuclear extracts from various treated groups were isolated as described earlier (Singh et al., 2007). The protein concentration was determined using the BCA protein determination kit from Pierce, using albumin as a protein standard. The supernatant containing nuclear extract was snap frozen in liquid nitrogen and stored at -80°C.

Generation of oligonucleotide probes. To perform EMSA assays, hairpin oligonucleotide probes corresponding to ERE and NF-kB motifs of mouse Fas or FasL promoter were synthesized by Integrated DNA Technologies (Coralville, Iowa). The details of the sequence of various ligonucleotide probes for Fas or FasL promoter are as described below.

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Mouse Fas Promoter:
Hairpin loop probe for Fas ERE motif:
GAGAAAA**CTTGATCTTCCCAT**TTTAGCTAAAATGGGAAGTCAAGTTTTCT
Hairpin loop probe for Fas ERE mutant motif:
GAGAAAA**CCCTATAGGACAAT**TTTAGCTATGGAAAATTGTCCTATAGGGTTT
TCT
Hairpin loop Probe for FAS NF-kB1 motif:
GAGAAAAGGGGACTCCCTTTAGCTAAAAGGGAGTCCCCTTTTCT
Hairpin loop Probe for FAS NF-kB2 motif:
GAGAAAAGGGACATTCCCCTTTAGCTAAGGGGAATGTCCCTTTTCT

Mouse FasL Promoter:
Hairpin loop Probe for ERE motif::
GAGAAAA**TGGTGACC**AGTTTAGCTTAAACTGGTCACCATTTTCT
Hairpin loop probe for ERE mutant motif:
GAGAAAA**TGGCTGTT**AGTTTAGCTTAAAACTAACAGCCATTTTCT
Hairpin loop Probe for NF-kB1 motif:
GAGATGGTCTTTTCCCCAATTAGCTTTTGGGGAAAAGACCATCT
Hairpin loop probe for NF-kB1 mutant motif:
GAGATGGTCTTTTAAACAATTAGCTTTTGTTTAAAAGACCATCT
Hairpin loop Probe for NF-kB2 motif:
GAGAAAAGGTGTTTCCCTTTTAGCTTAAGGGAAACACCTTTTCT
Hairpin loop Probe for NF-kB2 mutant motif:
GAGAAAAGGTGTTTAAATTTTAGCTTAATTTAAACACCTTTTCT

The hairpin oligonucleotide probes were 5-end-labeled by mixing 1 pmol of hairpin oligonucleotide with 10 µCi of [γ-^{32} P]ATP (MP Biomedicals, Aurora, OH), and 8 units of T4 polynucleotide kinase (New England Biolabs) in 1X PNK buffer and incubating for 1 h at 37°C. After incubation, the end-labeled hairpin oligonucleotides were purified from free ATP by passing over a NICK column (Amersham Biosciences). 1–5 µg nuclear protein was mixed with 1 µl radiolabeled oligonucleotide (40,000 cpm) in a reaction mix containing 1 µl binding buffer (10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, 10% (v/v) glycerol) and 1 µg poly(dI-dC) (Amersham Biosciences) as a nonspecific inhibitor in a final volume of 25 µl and incubated for 30 min at 25 °C. The samples were resolved on a 6% polyacrylamide gel in Tris borate EDTA that has been pre run for 30 min. The gels were dried and exposed to X-ray film.

For the specific and nonspecific competition analyses, equimolar amounts of the cold hairpin oligonucleotide competitors were added to the binding reaction before the addition of the labeled oligonucleotide probes.

Statistical analysis. Data presented for luciferase expression were derived from at least three independent experiments and depicted as mean ± SEM. Similarly, results for RT-PCR represent at least three animals for each treatment and expressed as the mean ± SEM. Statistical analyses were performed using Student's *t*-test or two-factor ANOVA as appropriate, with a *P*-value of ≤ 0.05 considered to be statistically significant.

Results

DES induces alterations in thymic cellularity in pregnant mice and their fetuses. In the current study, we determined DES-induced changes in thymic cellularity in pregnant mothers and their fetuses. To this end, a single dose of DES (5 μg/kg) was administered (ip) into pregnant C57BL/6 mice on GD 14. On day 2 (GD 16) and day 4 (GD 18) post-DES treatment, thymic cellularity of both mothers and fetuses were determined. We have shown previously that pregnant mice exhibit dramatic decrease in thymic cellularity when compared to nonpregnant mice and exposure to environmental contaminants such as TCDD, further decreases their numbers (Camacho et al., 2004a). We noted that unlike non-pregnant mice that showed ~250 million thymocytes/mouse (Camacho et al., 2004a), pregnant mice on GD 16 had less than ~11 million cells/mouse. This cellularity was further decreased following treatment with DES (Fig. 1A). When we pursued similar studies in the fetus, DES was found to alter thymic cellularity in the fetus on GD 18 but not on GD 16 (Fig. 1B). These data demonstrated that exposure to DES during pregnancy decreases thymic cellularity in both the mothers and fetuses.

Detection of DES-induced apoptosis in thymocytes of pregnant mice and their fetuses. Thymi from VEH- or DES-exposed pregnant mice and fetuses were evaluated for apoptosis as described earlier (Brown et al., 2006a; Brown et al., 2006b; Singh et al.). The results from a representative experiment have been shown in Figure 2A and data from multiple experiments have been plotted in Figure 2B. The thymocytes from DES-treated groups showed significantly higher percentage of apoptosis in both the

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mothers and fetuses GD18 (Fig. 2A-B), when compared to the VEH-treated control groups.

DES upregulates Fas and FasL expression in fetal thymic cells. Next, we determined expression of Fas and FasL, key molecules involved in apoptosis, in fetal thymic cells exposed to VEH or DES on GD 18 by performing RT-PCR. Thymic cells from both groups showed low level expression of Fas and FasL in the presence of VEH (Fig 3A-B). However, there was significant upregulation of both Fas and FasL expression in mice prenatally exposed to DES (Fig 3A-B). Data obtained from Western blot confirmed upregulation of both Fas and FasL expression in fetal thymic cells post-DES exposure (Fig 3 C-D). These data demonstrated that DES induces expression of both Fas and FasL in fetal thymic cells.

DES upregulates Fas and FasL expression in T cells via ER. Next, we investigated if the upregulation of Fas and FasL by DES was mediated through activation of ER. To this end, unactivated or ConA-activated T cells from normal C57BL/6 mice were cultured in the presence of VEH (DMSO) or DES (10 μM/ml) in the presence of TAM (10 μM/ml) or VEH. T cells were either analyzed by flow cytometry or by RT-PCR post treatments as described earlier (Singh et al., 2007). Over 25% of all unactivated T cells and over 35% of activated T cells expressed Fas (Fig 4A-B) in the presence of VEH whereas, both unactivated and activated T cells upon treatment with DES expressed significantly higher level of Fas as indicated by an increase in mean fluorescent intensity (MFI) as well as increase in Fas expressing cells (Fig 4A-B). These data demonstrated

that DES upregulates Fas expression in both unactivated and activated T cells. In the presence of TAM (an antagonist of ER), DES failed to increase the expression of Fas in both unactivated and activated T cells demonstrating that DES regulates Fas expression via ER (Fig 4A-B). Furthermore, unactivated or activated T cells upon treatment with TAM alone did not show significant change in the expression of Fas, when compared to VEH treated T cells (Fig 4A-B). Similar to Fas expression, DES significantly increased the percentage of FasL expressing cells in both unactivated and activated T cells cultures (Fig 4C-D). DES in the presence of TAM did not affect FasL expression demonstrating that TAM blocked DES-ER-induced upregulation of FasL expression. The presence of TAM alone did not effect FasL expression in both unactivated and activated T cells (Fig 4C-D). RT-PCR data further corroborated the results generated by Flow cytometry and confirmed that DES caused upregulation of both Fas and FasL in unactivated and activated T cells (Fig 4E).

DES regulates Fas and FasL expression through their respective promoters. To determine DES-mediated regulation of Fas and FasL expression through their respective promoters, EL4 T cells were transfected with constructs containing full-length upstream region of Fas (pGL3-Fas-Luc) or FasL (pGL3-FasL-Luc) promoter. Two days post-transfection, the transiently expressing EL4 cells were treated with various doses of DES (1-20 μM) or VEH (DMSO). Luciferase assays were performed 24 hrs post-DES or VEH treatment. We observed DES-mediated increase in expression of luciferase when Fas or FasL promoter were used for transfection and the cells were treated with DES for at least 24 hrs (Fig 5A). The effect of DES on luciferase expression was very minimal at

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lower doses (1 µM; Fig. 5A). At 5 µM, the effect of DES was moderately altered. However, we observed highly significant increase (2-3 ±.5 folds) in luciferase expression when 20 µM of DES was used (Fig. 5A). We observed minimal levels of luciferase expression when EL4 cells were transfected with Fas or FasL promoter and treated with VEH as well as in pGL-3 controls (Fig. 5A). The observed upregulation of luciferase expression was Fas or FasL-promoter-specific and DES-induced, as the luciferase expression was significantly higher ($p<0.05$) in cells transfected with Fas or FasL promoter compared to untreated or VEH treated cells (Fig. 5A). Similar results were obtained in T cells transfected with Fas (pGL3-Fas-Luc) or FasL (pGL3-FasL-Luc) promoter in the presence of DES (Fig 5B). The data obtained demonstrated that DES-induced upregulation of luciferase expression was through Fas or FasL promoter.

Identification of various transcription factor binding motifs in mouse Fas and FasL promoters. To understand DES-induced regulation of Fas and FasL expression, we first identified various transcription factor(s) binding motifs in Fas and FasL promoters using Matinspector 8.0 software from Genomatrix as described in Materials and Methods (Genomatrix GMBH, Munich, Germany). We identified the following transcription factor(s) binding motifs in mouse Fas promoter: one Estrogen Receptor Element (ERE), five Nuclear Factor Kappa B (NF-kB; named NF-kB1, NF-kB2, NF-kB3, NF-kB4, and NF-kB5), three AP1 (Ap1.1, AP1.2, and AP1.3), one SP1, one DRE, and two NF-AT motifs (Fig 6A). Similarly, we identified the following transcription factor(s) binding motifs in mouse FasL promoter: one ERE, two NF-kB (NF-kB1 and NF-kB2), one SP1, and three NF-AT motifs (Fig 6B).

JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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DES-induced transcription(s) factor bind with various cis-regulatory motifs present in mouse Fas or FasL promoters. Next, we performed *in vitro* studies to examine DES-induced transcription factors and their binding affinity with various cis-regulatory motifs present in the Fas or FasL promoters. As described in Materials and Methods, nuclear extracts prepared from T cells treated with VEH or DES (10 μM/ml) were incubated together with various double stranded probes (cis-regulatory motifs) of Fas or FasL promoter as suggested by the company (Clontech Laboratory, Inc, Mountain View, CA). The binding affinity was determined by performing chemiluminescent assays (Clontech Laboratory, Inc, Mountain View, CA) and were analyzed by Victor^2 (Perkin Elmer). We observed significantly higher binding of ERE probes of both Fas and FasL with nuclear extracts isolated from DES-treated T cells, when compared to VEH-treated nuclear extracts (Fig 7A). We did not observe significant difference in binding of DRE probe with either of the nuclear extracts (Fig 7A). These results demonstrated that DES may regulate Fas or FasL expression through ERE but not through DRE. Also, we did not observe any DRE binding motif in FasL promoter (Fig 6B). Various NF-kB probes of Fas and FasL showed different binding affinity with nuclear extracts from DES- or VEH-treated T cells. NF-kB2 and NF-kB3 probes of Fas promoter showed strong binding affinity whereas NF-kB1, NF-kB4, and NF-kB5 showed no or low binding affinity with nuclear extracts isolated from DES-treated T cells when compared to VEH-treated T cells (Fig 7**B**). Similarly, NF-KB2 probe of FasL showed significant binding affinity whereas NF-kB1 probe of FasL showed low or no binding affinity with DES-treated nuclear extract when compared to VEH-treated nuclear extracts (Fig 7B). NF-AT2 probe of Fas and NF-AT1 and NF-AT2 probes of FasL showed significant binding affinity with DES-

JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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treated nuclear extract, when compared to VEH-treated nuclear extract (Fig 7C). We did not observe significant binding difference in NF-AT1 probe with DES- or VEH-treated nuclear extracts (Fig 7C). Out of three AP-1 probes of Fas promoter, two (AP-1.1 and AP-1.2) showed moderate binding affinity with DES-treated nuclear extract, when compared to VEH-treated nuclear extract (Fig 7C). The results obtained from these studies demonstrated that various motifs of Fas or FasL promoter showed variable binding affinity with nuclear extract isolated from DES-treated T cells demonstrating their role in DES-mediated regulation of Fas or FaL expression.

DES-induced transcription factors bind with ERE and NF-kB cis-regulatory motifs present in Fas or FasL promoter. To further confirm the participation of various cis-regulatory motifs present in the Fas or FasL promoter, we selected ERE, NF-kB1, and NF-kB2 motifs to verify their participation in DES-mediated regulation of Fas and FasL expression. The selection of ERE and NF-kB (NF-kB1 and NF-kB2) motifs of Fas and FasL was based on their binding affinity with DES-induced transcription factors (Fig 7A and B) that we obtained post chemiluminescent assays. ERE and NF-kB2 motifs of both Fas and FasL demonstrated high binding affinity whereas NF-kB1 showed no or very low binding affinity with DES-induced transcription factors (Fig 7A and B). We performed electrophoretic mobility shift assays (EMSA) using radiolabeled single strand loop ERE, NF-kB1, and NF-kB2 probes of Fas or FasL incubated together with nuclear extracts (NE) from vehilcle- or DES-treated T cells. We observed shift in mobility of Fas and FasL ERE probes in the presence of nuclear extracts from T cells treated with DES (Fig. 8A for Fas ERE; panel 1 and for FasL ERE; panel 2). We also observed shift in NF-KB2

probes of both Fas (Fig. 8B; panel 1) and FasL (Fig. 8B; panel 2) in the presence of NE from DES-treated T cells. No mobility shift of Fas and FasL ERE and NF-kB2 probes was observed when incubated without nuclear extract or incubated with VEH-treated nuclear extracts (Fig. 8A-B). Furthermore, mobility shift of Fas and FasL ERE and NF-kB2 probes in the presence of nuclear extract was reversed when cold (unlabeled DNA; com) ERE or NF-kB2 probes were added in the reaction mixture (Fig 8A-B). Also, when mutant ERE or NF-kB2 probes were used, there was no shift in probes (Fig 8A-B). However, when NF-kB1 probes of Fas or FasL were used, there was no mobility shift (Fig 8C). The data obtained from these studies confirmed that DES induced transcription factor(s) bound with ERE and NF-kB2 motifs of Fas and FasL promoters demonstrating their role in DES-mediated regulation of Fas or FasL expression.

Discussion

Exposure to DES has been shown to increase the risk for breast cancer in "DES mothers" and a life time risk of cervicovaginal cancers in "DES daughters" (Giusti et al., 1995). It has also been linked to a wide range of abnormalities including immune system disorders such as increased incidence of autoimmunity, cancer and certain infections in DES offspring (son or daughter) (Giusti et al., 1995). Such studies support the "fetal basis of adult disease" which states that exposure of fetus to nutritional, environmental and other forms of stress can impact the health at later stages of life (Barker et al., 2002; Phillips, 2006). Previous studies from our laboratory have shown DES-induces thymic atrophy and deceased thymic cellularity in mice (Brown et al., 2006a; Brown et al., 2006b). Frawley et al (2011) have recently reported gene expression alterations in immune system pathways in the thymus after exposure to immunosuppressive chemicals (Frawley et al.). In this study, we investigated DES-mediated thymic atrophy in neonatal mice, regulation of Fas and FasL expression, and examined mechanisms of transcriptional regulation of Fas and FasL genes. To this end, we first examined the effects of DES in thymi of mother and neonatal mice by performing *in vivo* experiments. Next, we determined the expression of Fas and FasL in fetal thymic cells and T cells in the presence or absence of DES and investigated the role of Fas and FasL promoters in DES-mediated regulation. We also examined the participation of cis-regulatory motifs in DES-mediated regulation of Fas and FasL expression.

The results obtained from the current study showed that DES induces thymic atrophy and significant changes in thymic cellularity of both mothers (Fig 1A) and

fetuses (Fig 1B). Also, there was significant apoptosis in thymic cells of both mother and fetuses post-DES exposure when compared to VEH (Fig 2A-B). Upon analysis of Fas and FasL expression in fetal thymic cells, there was significant increase in Fas and FasL expression exposed to DES, when compared fetal thymic cells exposed to VEH (Fig 3A-B). Furthermore, expression of Fas and FasL in unactivated and activated T cells in the presence of DES was significantly upregulated in comparison to VEH (Fig 4A-E). Also, DES regulated Fas or FasL expression via ER as there was complete blockade of Fas or FasL expression in the presence of TAM, an antagonist of ER (Fig 4A-E). The data from the present study demonstrate that DES adversely affects fetal thymus by causing changes in thymic cellularity, upregulating Fas and FasL expression, and causing apoptosis in thymic cells.

Previous studies from our laboratory have demonstrated that DES altered T-cell differentiation in the thymus by interfering with positive and negative selection processes, which in turn modulated the T-cell repertoire in the periphery (Brown et al., 2006a). In another study, Nair et al reported DES-induced apoptosis in spermatogenic cells through increased expression of Fas and FasL in rats (Nair and Shaha, 2003). They have also shown that DES-mediated apoptosis did not occur in Fas deficient lpr mice (Nair and Shaha, 2003). Together, these studies suggest that exposure to DES during development severely affects thymi of neonatal mice and may have a long lasting impact on the immune functions leading to various health-associated problems and ailments.

The mechanisms through which DES upregulates Fas and FasL expression in immune cells is unclear. The current study demonstrated that DES regulates Fas and FasL expression through their respective promoters as evidenced from the data that we

generated using luciferase assays in the absence or presence of DES. There was increased luciferase expression in both DES-treated EL4 cells and cells that were transfected with constructs containing full-length Fas or FasL promoter (Fig 5A-B). In an earlier study, we noted that AhR activation by TCDD upregulated of Fas and FasL expression through their respective promoter (Singh et al., 2007). Thus, it is likely that ER activation by DES also causes similar effects and regulates Fas and FasL expression through their respective promoters.

The regulation of a gene depends on cis-regulatory motif(s) present in its promoter. The number of cis-regulatory motifs present in a promoter varies from gene to gene. Also, the participation of a cis-regulatory motif depends on the transcription factor(s) generated by various mechanisms. To understand DES-mediated regulation of Fas and FasL expression, we performed sets of experiments to determine the role of their respective promoters as well as cis-regulatory motifs present in the promoter. To this end, we first identified various cis-regulatory motifs present in the Fas or FasL promoter as described in Materials and Methods. The data obtained from the analysis showed the presence of several cis-regulatory motifs in both Fas (Fig 6A) and FasL promoters (Fig 6B). Upon analysis of binding affinity of these cis-regulatory motifs of Fas or FasL promoter with DES-generated transcription factors, the results showed participation of several cis-regulatory motifs of Fas or FasL promoter in their regulation (Fig 7A-C). The important cis-regulatory motifs of Fas promoter that showed binding affinity with DES-generated transcription factors from T cells were ERE, NF-AT2, AP-1.1, AP-1.2, NF-kB2, and NF-kB3 (Fig 7A-C). Similarly, cis-regulatory motifs of FasL promoter that showed binding affinity with DES-generated transcription factors were ERE, NF-AT1,

NF-AT2, and NF-kB2 (Fig 7A-C). DRE motif of Fas promoter showed no or minimal binding affinity with DES-generated transcription factors (Fig 7A). The binding of DES-induced transcription factors with various cis-regulatory motifs of Fas or FasL promoter was further confirmed by performing EMSA assays. The shift in probes mobility obtained from EMSA assays confirmed binding of ERE and NF-kB2 motifs of both Fas and FasL with DES-generated transcription factors (Fig 8A-B). Also, the shift in mobility of the probes was specific as cold probes (non-radioactive) reduced the shift in mobility of the probes (Fig 8A-B). Moreover, the mutant probes of ERE or NF-kB2 did not show mobility shift of the probes when incubated together with DES-generated transcription factors (Fig 8A-B). Furthermore, NF-kB1 probes of Fas or FasL did not show mobility shift in the presence of DES-generated transcription factors (Fig 8C). All these results demonstrated that DES caused upregulation of Fas or FasL expression by generating transcription factors that binding with cis-regulatory motifs present in Fas or FasL promoter regulated their expression.

Payton-Stewart et al (2101) have shown ER-mediated gene expression through ERE cis-regulatory motif present in the promoter (Payton-Stewart et al.). They have demonstrated that glyceollin I enantiomers that bind with ER regulate several genes through ERE of their promoter (Payton-Stewart et al.). Frasor et al (2009), on the other hand, have shown extensive positive cross-talk between ER and NF-kB and that these factors may act together to regulates several genes (Frasor et al., 2009). In another study, Guzeloglu-Kayisli et al (2008) reported that interaction between ER and NF-kB may affect the regulation of responsive genes by downregulating NF-kappaB-dependent gene activation and directly preventing DNA binding (Guzeloglu-Kayisli et al., 2008). Safe et

al (2008) have shown ER-mediated regulation of genes through AP-1 cis-regulatory motif (Safe and Kim, 2008). All these studies demonstrate the role of ER, various transcription factors and associated cis-regulatory motifs involved in the induction of various genes.

The induction and expression of Fas and FasL on cells could constitute a double-edged sword. Underexpression or mutation in Fas or FasL can cause various disorders including autoimmune lymphoproliferative syndrome and susceptibility to lymphoid cancers (Gronbaek et al., 1998; Kovacs et al., 1997b; Straus et al., 1999). In contrast, overexpression of Fas or FasL results in uncontrolled apoptosis leading to cardioretinopathology, neurodegenerative disorders, and failure to clear pathogens (Kovacs et al., 1997a; Sharma et al., 2000). The continuous FasL expression in testes has been shown to cause autoimmunity leading to fertility problems (Hu et al., 2003) whereas, the immune privilege status of various organs (eyes and testes) is partially due to high expression of FasL in these organs (Griffith et al., 1995). FasL constitutively expressed in the thymus plays an important role in the ontogenesis and negative selection of T cells (Kabelitz, 1993), but upregulated expression of FasL in thymus also leads to development of autoimmunity (Brochu et al., 1999; Kobata et al., 1997). Also, in the thyroid gland, inflammation leading to interaction between Fas and FasL can cause thymocyte destruction and trigger Hashimotos thyroiditis (Stassi et al., 2000). In the current study, we noted that Fas and FasL were upregulated in the T cells in the presence of DES, which suggests potential deleterious effects on the development of the normal immune system.

In conclusion, the current study demonstrates that prenatal exposure to DES triggers significant alterations in apoptotic molecules expressed on immune cells that play a critical role in the regulation of autoimmunity and cancer. The mechanisms include DES-induced transcriptional regulation of Fas and FasL genes involving ER-mediated signaling and participation of ERE and/or NF-kB motifs present on their respective promoters. Furthermore, our studies suggest that such alterations in apoptotic molecules in the thymus may affect T cell differentiation and have long term consequence on the immune functions.

JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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Acknowledgements

We thank Dr. Amar A. Beg (Columbia University, New York, NY) for providing mouse Fas promoter cloned in pGL3 vector and Dr. Timothy L. Ratiff (University of Iowa, Iowa City, IA) for providing mouse FasL promoter cloned in pGL3 vector.

Authors Contribution

Narendra P Singh participated in research design, performed experiments, contributed new reagents or analytic tools, analyzed data, and wrote the manuscript.

Udai P Singh participated in research design, performed experiments, analyzed data, and contributed in writing the manuscript.

Prakash S Nagarkatti participated in research design, contributed new reagents or analytic tools, analyzed data, and contributed in writing the manuscript.

Mitzi Nagarkatti participated in research design, contributed new reagents or analytic tools, analyzed data, and contributed in writing the manuscript.

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Footnotes

This work was supported by the National Institutes of Health National Center for Complementary and Alternative Medicine [Grant PO1-AT003961, R01AT006888], the National Institutes of Health National Institute of Environmental Health Sciences [R01ES019313], and the National Institutes of Health National Institute on Mental Health [R01MH094755].

JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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Legends for Figures

Figure 1. Effect of DES on thymic cellularity of pregnant mice and fetuses. Pregnant mice (gestational day = 14) were injected i.p. with VEH (control) or DES (5 μg/kg body weight). On day 2 (GD 16) and 4 (GD 18) post-DES or VEH treatment, the thymi from mothers and their fetuses were harvested to determine thymic cellularity (A). Data from groups of 5 mice are depicted as mean ± SEM. Vertical bars in panels A represent mean ± SEM. Asterisk (*) indicates statistically significant ($p<0.05$) differences between indicated groups.

Figure 2. DES triggers apoptosis in thymocytes of pregnant mothers and fetuses. The thymocytes from mothers and fetuses on day 4 (GD 18) post-DES treatment were analyzed for apoptosis using TUNEL assay. Panel A shows a representative histogram of three independent experiments and panel B shows data from groups of 5 mice (mean ± SEM). Asterisk (*) in panel B indicates statistically significant ($p<0.05$) difference in apoptosis between DES and VEH-treated groups.

Figure 3. Expression of Fas and FasL in fetal thymic cells following prenatal exposure to DES. Expression of Fas and FasL in fetal thymic cells was determined by RT-PCR 24 hrs post-DES or VEH treatments (3A-B). Data in panel A is a representative of three independent assays which are plotted in panel B respectively as Mean +/- SEM. Asterisk (*) indicates statistically significant ($p<0.05$) difference in expression of Fas and FasL between VEH and DES-treated thymic cells. C-D, Expression of Fas and FasL in fetal thymic cells were also determined by Western blot. Data in panel C is a representative of

three independent experiments and are plotted in panel D as Mean +/- SEM. Asterisk (*) indicates statistically significant ($p<0.05$) difference in expression of Fas and FasL between VEH and DES-treated thymic cells.

Figure 4. Expression of Fas and FasL in T cells in the presence of DES. Unactivated or ConA-activated T cells were cultured in the presence of VEH (DMSO) or DES (10 μM) for 24 hrs. Expression of Fas and FasL in unactivated and activated T cells was determined by flow cytometry (A-D) or RT-PCR (E) 24 hrs post-DES treatments. Data in panels A and C are representatives of three independent assays which are plotted in panel B and D respectively as Mean +/- SEM. Data in panel B and D represent mean +/- SEM of three independent experiments and asterisk (*) indicates statistically significant ($p<0.05$) difference in expression of Fas and FasL between VEH and DES-treated groups. Data in panel E represent semi-quantitative RT-PCR. Expressions of Fas and FasL are presented as percentage of 18S expression in the Y-axis and expression of 18S was considered to be 100% for each experiment. Data in panel E represent mean +/- SEM of three independent experiments. As summarized in lower panel of panel E, Fas and FasL expression was noted to be significantly ($p<0.05$) increased. Data in lower panel of panel E represent mean +/- SEM of three independent experiments and asterisk (*) indicates statistically significant ($p<0.05$) difference in expression of Fas and FasL between VEH and DES-treated groups. In panel E, Tamoxifen (TAM), an antagonist of ER was also tested.

Figure 5. DES regulates Fas and FasL promoter activity. EL4 cells (A) or T cells (B) were transfected with luciferase reporter constructs containing full length Fas promoter (pGL-3-Fas) or FasL (pGL-3-Fas) or pGL-3 control and pcDNA3.1 containing pCMV-β-galactosidase gene. Two days post transfection, the transiently expressing EL4 or T cells were treated with various doses of DES (1, 5, 10, and 20 μM/ml) or DMSO (VEH). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized fold induction. The vertical bar represents mean ± SEM from three independent experiments.

Figure 6. Cis-regulatory motifs present in Fas and FasL promoter. Panel A shows sequence of Fas promoter and various regulatory motifs present in the promoter. Panel B shows sequence of FasL promoter and various cis-regulatory motifs.

Figure 7. Identification of binding affinity of cis-regulatory motifs present on Fas and FasL promoter with DES-generated transcription factors in T cells. A, shows binding affinity of ERE and DRE motifs of Fas promoter and ERE motif of FasL promoter with transcription factors generated by DES or VEH in T cells. ERE motif of both Fas and FasL promoters showed significantly higher binding affinity with transcription factors harvested from DES-treated T cells, when compared to transcription factors from VEH-treated T cells. DRE motif of Fas promoter showed low and insignificant binding affinity with transcription factors from DES-treated T cells, when compared to VEH-treated T cells. B, shows binding affinity of five NF-kB (NF-kB1, NF-kB2, NF-kB3, NF-kB4, and NF-kB5) motifs of Fas promoter and two NF-kB (NF-kB1 and NF-kB2) motifs of FasL

promoter with transcription factors from DES-or VEH-treated T cells. NF-kB2 and NF-kB3 motifs of Fas promoter showed significantly higher binding affinity with transcription generated from DES-treated T cells, when compared to VEH-treated T cells. NF-KB1, NF-kB4, and NF-kB5 motifs of Fas promoter showed very low and insignificant binding affinity with transcription factors from either DES- or VEH-T cells. NF-kB2 motif of FasL promoter showed moderate but significant binding affinity with transcription factors from DES-treated T cells, when compared to VEH-treated T cells. NF-kB1 motif of FasL promoter showed very low and insginicant biding affinity with transcription factors from both DES- or VEH-treated T cells. C, Panel C shows binding affinity of NF-AT (NF-AT1 and NF-AT2 motifs of Fas promoter and NF-AT1, NF-AT2, and NF-AT3 motifs of FasL promoter) and AP-1 (AP-1.1, AP-1.2, AP-1.3) motifs of Fas promoter. NF-AT2 of Fas promoter and all three NF-AT motifs of FasL promoter showed significantly higher binding affinity with transcription factors from DES-treated T cells, when compared to VEH-treated T cells. Similarly, AP-1.1 and AP-1.2 motifs of Fas promoter showed significant binding affinity with transcription factors from DES-treated T cells, when compared to VEH-treated T cells. The vertical bars represent mean ± SEM from three independent experiments. Asterisk denotes significant ($p<0.05$) difference in binding affinity of various motifs of Fas or FasL promoter with transcription factors from DES- and VEH-treated T cells.

Figure 8. EMSA analysis of ERE, NF-kB1, and NF-kB2 motifs of Fas and FasL promoters. A, Single strand hairpin loop probes containing ERE motif of mouse Fas and FasL promoters were generated. Nuclear extract was generated from DES-treated T cells

JPET Fast Forward. Published on August 10, 2012 as DOI: 10.1124/jpet.112.196121
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and 4-5 μg of nuclear extract (NE) was used in each reaction. Radiolabeled (P^{32}) ERE probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant ERE probe. In figure 8A; panel 1, arrow 1, shows Fas ERE probe DNA band, arrows 2 and 3, demonstrate ERE-NE complexes. The lanes are labeled as ERE (ERE probe without NE), ERE+VEH NE (ERE probe + VEH-treated NE), ERE+DES NE (ERE probe + DES-treated NE), COMP (ERE probe + DES NE + cold (unlabeled) ERE probe for competition), and Mut ERE+DES NE (Mutant ERE probe + DES-treated NE). In figure 8A; panel 2 represents similar lane labeling but mouse FasL ERE probes have been used. B, Single strand hairpin loop probes containing NF-kB2 motif of Fas and FasL promoters were generated.

Radiolabeled (P^{32}) NF-kB2 probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant NF-kB2 probe. In figure 8B; panel 1, arrow 1, shows Fas NF-kB2 probe DNA band, arrows 2 and 3, demonstrate NF-kB2-NE complexes. The lanes are labeled as NF-kB2 (NF-kB2 probe without NE), NF-kB2 +VEH NE (NF-kB2 probe + VEH-treated NE), NF-kB2 +DES NE (NF-kB2 probe + DES-treated NE), COMP (NF-kB2 probe + DES NE + cold (unlabeled) NF-kB2 probe for competition), and Mut NF-kB2 +DES NE (Mutant NF-kB2 probe + DES-treated NE). In figure 8B; panel 2 represents similar lane labeling but mouse FasL NF-kB2 probes have been used. C, Single strand hairpin loop probes containing various NF-kB1 motifs of Fas and FasL promoter were generated. Radiolabeled (P^{32}) NF-kB1 probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant NF-kB1 probe. In figure 8C; panel 1, arrow 1, shows Fas NF-kB1 probe DNA band, arrows 2 and 3, demonstrate NF-kB1-NE

complexes. The lanes are labeled as NF-kB1 (NF-kB1 probe without NE), NF-kB1 +VEH NE (NF-kB1 probe + VEH-treated NE), NF-kB1 +DES NE (NF-kB1 probe + DES-treated NE), COMP (NF-kB1 probe + DES NE + cold (unlabeled) NF-kB1 probe for competition), and Mut NF-kB1 + DES NE (Mutant NF-kB1 probe + DES-treated NE). In figure 8C; panel 2 represents similar lane labeling but mouse FasL NF-kB1 probes have been used.

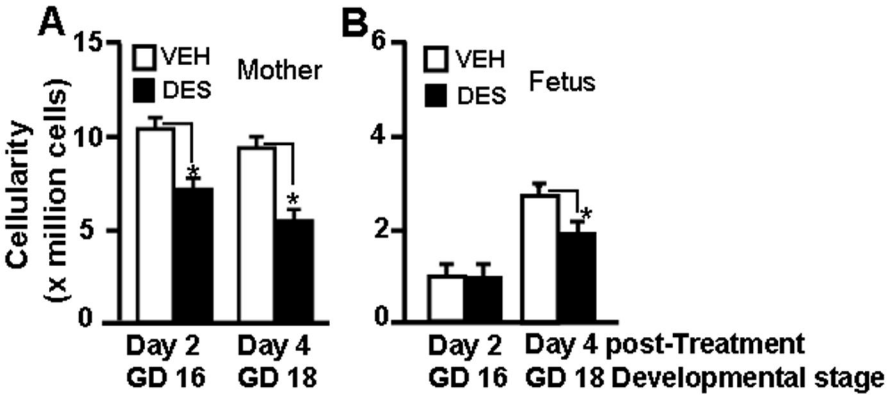
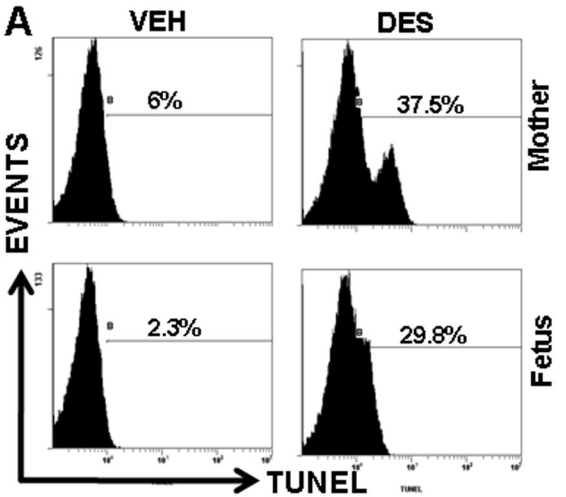

A
B
VEH
DES
Mother
Fetus
Cellularity
(x million cells)
15
10
5
0
6
4
2
0
*
Day 2
GD 16
Day 4
GD 18
Day 2
GD 16
Day 4 post-Treatment
GD 18 Developmental stage

Figure 1


A
VEH
DES
6%
37.5%
2.3%
29.8%
Mother
Fetus
EVENTS
TUNEL

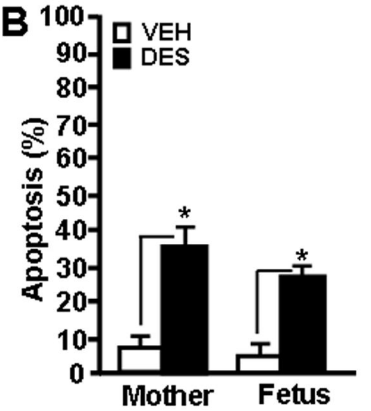

B
VEH
DES
Apoptosis (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
Mother
Fetus

Figure 2

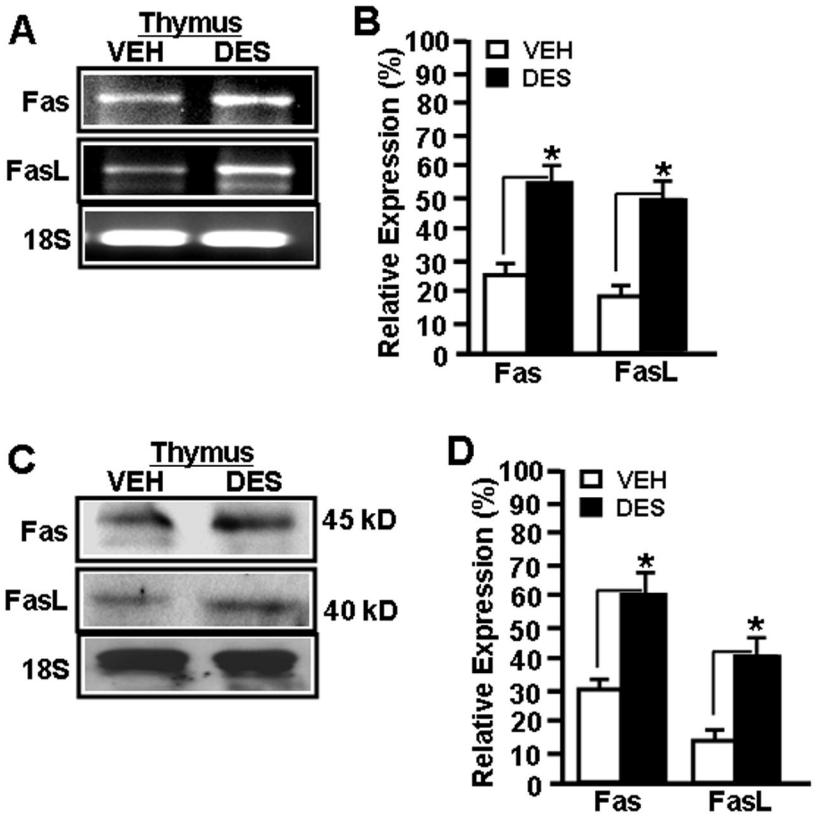

A
Thymus
VEH
DES
Fas
FasL
18S
B
Relative Expression (%)
100
90
80
70
60
50
40
30
20
10
0
VEH
DES
*
*
Fas
FasL
C
Thymus
VEH
DES
Fas
FasL
18S
45 kD
40 kD
D
Relative Expression (%)
100
90
80
70
60
50
40
30
20
10
0
VEH
DES
*
*
Fas
FasL

Figure 3

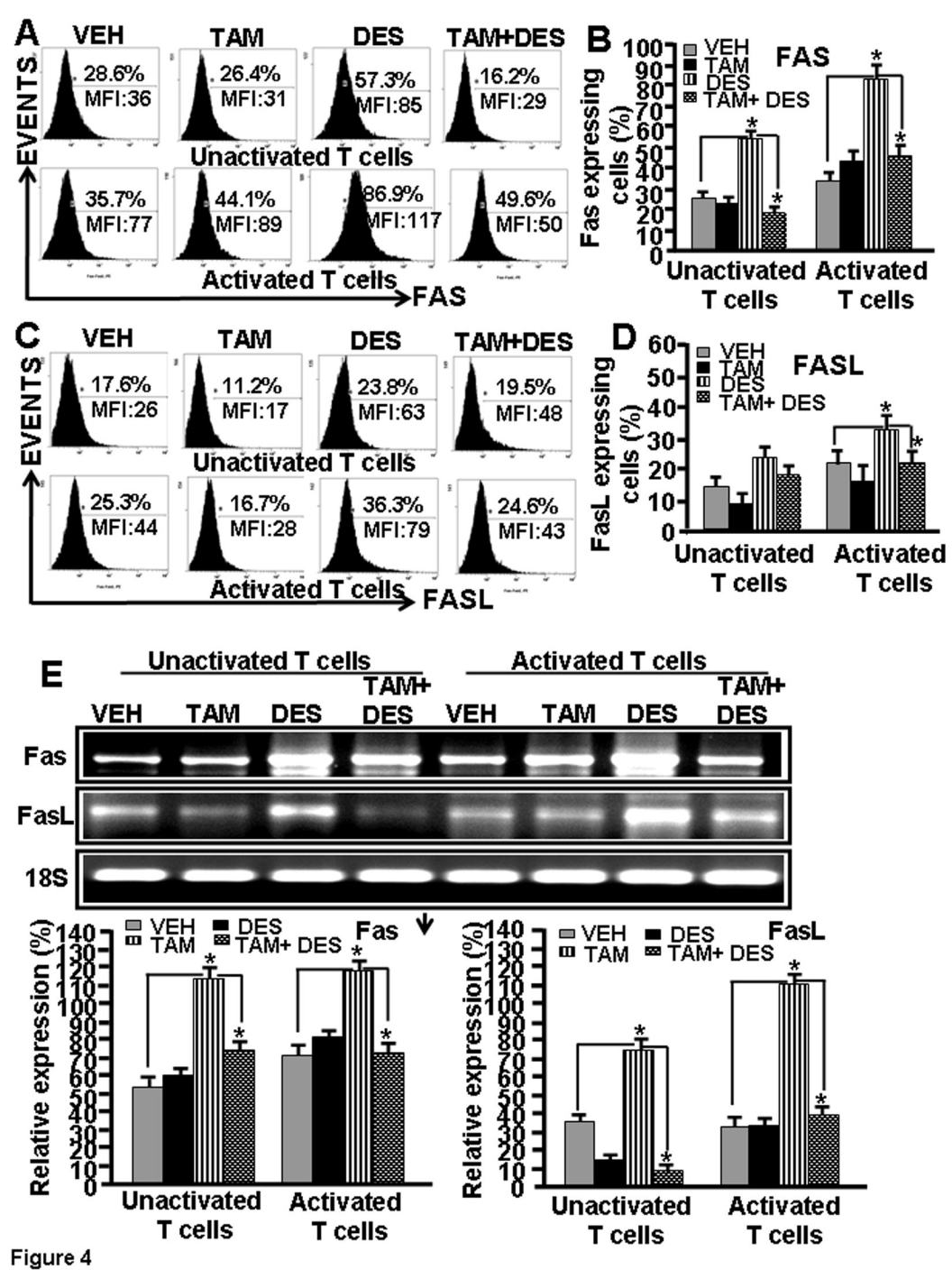

A
VEH
TAM
DES
TAM+DES
EVENTS
28.6%
MFI:36
26.4%
MFI:31
57.3%
MFI:85
16.2%
MFI:29
Unactivated T cells
35.7%
MFI:77
44.1%
MFI:89
86.9%
MFI:117
49.6%
MFI:50
Activated T cells
FAS
B
FAS
VEH
TAM
DES
TAM+ DES
Fas expressing cells (%)
Unactivated T cells
Activated T cells
C
VEH
TAM
DES
TAM+DES
EVENTS
17.6%
MFI:26
11.2%
MFI:17
23.8%
MFI:63
19.5%
MFI:48
Unactivated T cells
25.3%
MFI:44
16.7%
MFI:28
36.3%
MFI:79
24.6%
MFI:43
Activated T cells
FASL
D
FASL
VEH
TAM
DES
TAM+ DES
FasL expressing cells (%)
Unactivated T cells
Activated T cells
E
Unactivated T cells
Activated T cells
VEH
TAM
DES
TAM+ DES
VEH
TAM
DES
TAM+ DES
Fas
FasL
18S
Fas
VEH
TAM
DES
TAM+ DES
Relative expression (%)
Unactivated T cells
Activated T cells
FasL
VEH
TAM
DES
TAM+ DES
Relative expression (%)
Unactivated T cells
Activated T cells

Figure 4

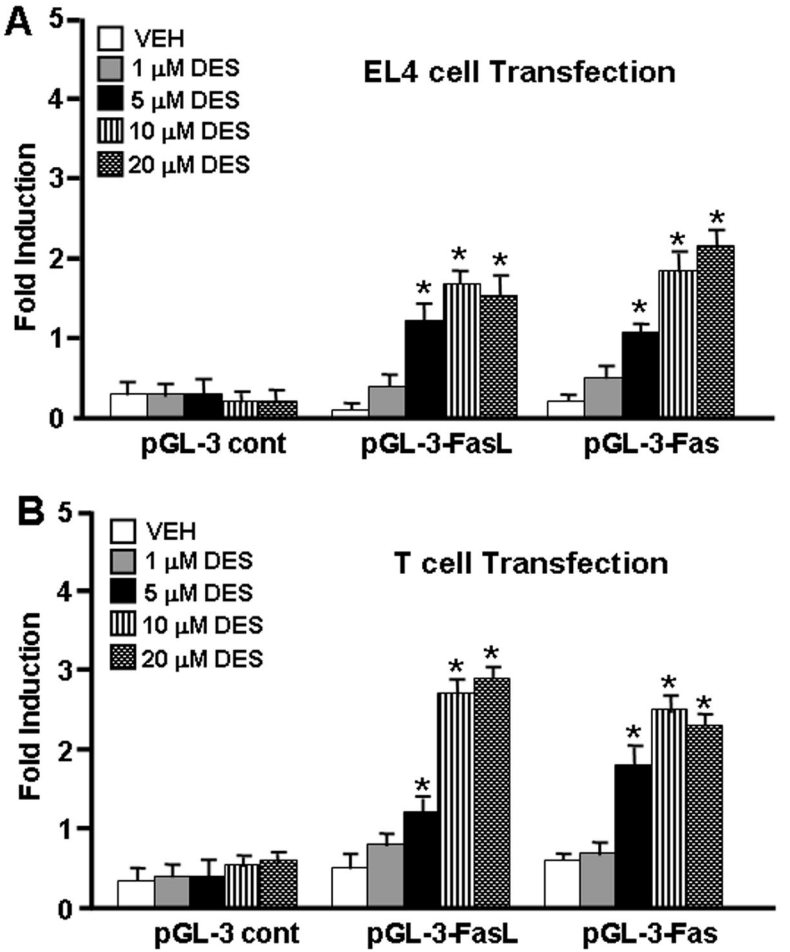

A
EL4 cell Transfection
VEH
1 μM DES
5 μM DES
10 μM DES
20 μM DES
Fold Induction
0
1
2
3
4
5
pGL-3 cont
pGL-3-FasL
pGL-3-Fas
B
T cell Transfection
VEH
1 μM DES
5 μM DES
10 μM DES
20 μM DES
Fold Induction
0
1
2
3
4
5
pGL-3 cont
pGL-3-FasL
pGL-3-Fas

Figure 5

A Identification of various regulatory elements in Fas promoter

-1272 TGTTGGCATGTAGGGATGAAGGCTCTATGTAGGCACTTTCTTCTTGCTCTCAAC
TTCTCTATATTCAATGAGTTGTGTGGGTCTCAGTTTGTGGGGAGCATCTATAGTCTTGGC
AACAGGCTGCATTGTTTGGGGACTCCCATGCAACCTTTTTGGCCAACGATTCAATTAGAT
GCAACCCCATCCAGGAAAGGAAAACTTCATTTGATGACAAGAGATGTTCACTTGTGGCT
CTGTCTCCCCCCATTATTTGGCTATAGATCACCTTCATGTATGCTTATACTTTAGGAAGAC
TCTGCTAGGTTTCCATACCTCTCAAATGCCCTTTAATTTTAGCTGTCTCTCCCTGTATTC
CCATTCATCGTCTTCCTTTCCCCTCCCCCACTTGATCTTCCCATCATAACTACCTATTTT
ATTCCTCTTCCTTAATAAGATCTTTCTGTCCCCTCCCCTAGTCCCTTACTCTATATCTAA
CCCCCGTGGTTCTAAAGATTCTGGCTTGGTCCCTGAGTTAACTGCTGTGTTATTTGTCAT
AGTCATTTACCTTATTCTCCATTTGGGAAACGAAGCCAGGGACATTCCCCTCATCAGGAT
CCCAAAGCCTATTATCTCTACCTCTGCTGTGGTGATTGTCACTTCATGTATCATATATGA
GCTCTGTGGAGGGACCTCCCTCATCAGAATCCTAGGGTGTGTACCTCCTCTGTGGTAATT
ACCATTCAGAGGCCCCAGAAGCCCCAGCAGACCTGTCACTGACAAAATATTGAAAGTGA
AGGCAAAATGGTCTTTGTCTCCACAATCAGCCAAAGTCCAATTCAATACAGAGTTCAGAA
TTCCTTCCAGGGTGAGCAGCAGGCAGAAAAAAAAATCTCACTTGACCTGAGGGTGTGAC
TGTGGAAGCGCCCAGGATGGGGAATGCCCATTTATGCAATCAAGCCCTGCTTGGCCCCC
TCCTCCCCCGCCCCCCACCCCCACCCCGCTCAGGCCGCCTGTGCAGTGGTGAGTCAGTG
GGTTTCCCCGGGAGACCAGCAGAACTTTCTCGCACCCTTGGGGCTTTACGAAGATTGTG
TTTGGTCCCTCAAAAGAAAAAAAGTGACATACACGTGTTCAAAGCGATTTCTGGGAAGA
CCTGAATAGGAGCGAAGCGGTTTGTGCGTGCCAGGGGGCGGGCCTAGGCAAAGTACAT
ACCCACAGGCAGTCTAGAGCTGGTGGGTGGGGCCGCAGGCTGCCCACACAGGCCGCCC
GCTGTTTTCCCTTGCTGCAGACATG

Red: ERE,
Black: NF-kB1 to NF-kB5,
Violet: AP1,
Blue: SP1,
Orange: DRE,
Green: NF-AT

B Identification of various regulatory elements in FasL promoter

-786GTACCTCAGTTTTCATCTGGTGACCaGAAGAGAGATTGCAAAGTTAAATGATTACA
GAGGAGGAAGCTACATTTCCCAGGGGAAGTGGGATGGATAGGGGGCAGGCACAGTG
GGGGGTTAGGGCAGCCTTGAACACCTGGCACACATTCCTGGTTGCAGCTGGCTGATGT
TCAGGGAAGGGACTTCAACAGAGGATTCAACTCCCTATGCTCAGATGTGGAGATATCT
TCTCCAGCCGAACTCCTTGGTCTTTTCCCCATGCCTCAGCACCAACAGGCCTCTCAGG
ACACACCCCAGAGCTGCGGAAGAGCTAATGTCCTCAAGGGGTATCCAGCGCTGACTT
GCTGAGTTGGACTCAGGCAGGCAAGCCTGGTTTACCAGCCTTCTCAGTTAGCACAGAG
ACGCCAATTGGAACTTCGAAGACTTGTCGTCAGAAATTTCTGGGCGGAAACTTCCTGG
GGTTGCTGTGAGCTTTTTGAGGCTTCTCAGCTTCAGATGCAAGTGAGTGGGTGTCTCAC
TGAGAAGCAAAGAGAAGAGAACAGGAGAAAGGTGTTTCCCTTGACTGCGGAAACCTT
ATAAAGAAAACTTAGCTTCTCTGGAGCAGTCAGCGTCAGAGTTCTGTCCTTGACACCT
GAGTCTCCTCCACAAGGCTGTGAAGAGGAAACCCTTTCCTGGGGCTGGGTGCCATG

Red: ERE
Black: NF-kB1 and NF-kB2
Blue: SP1
Green: NF-AT

Figure 6

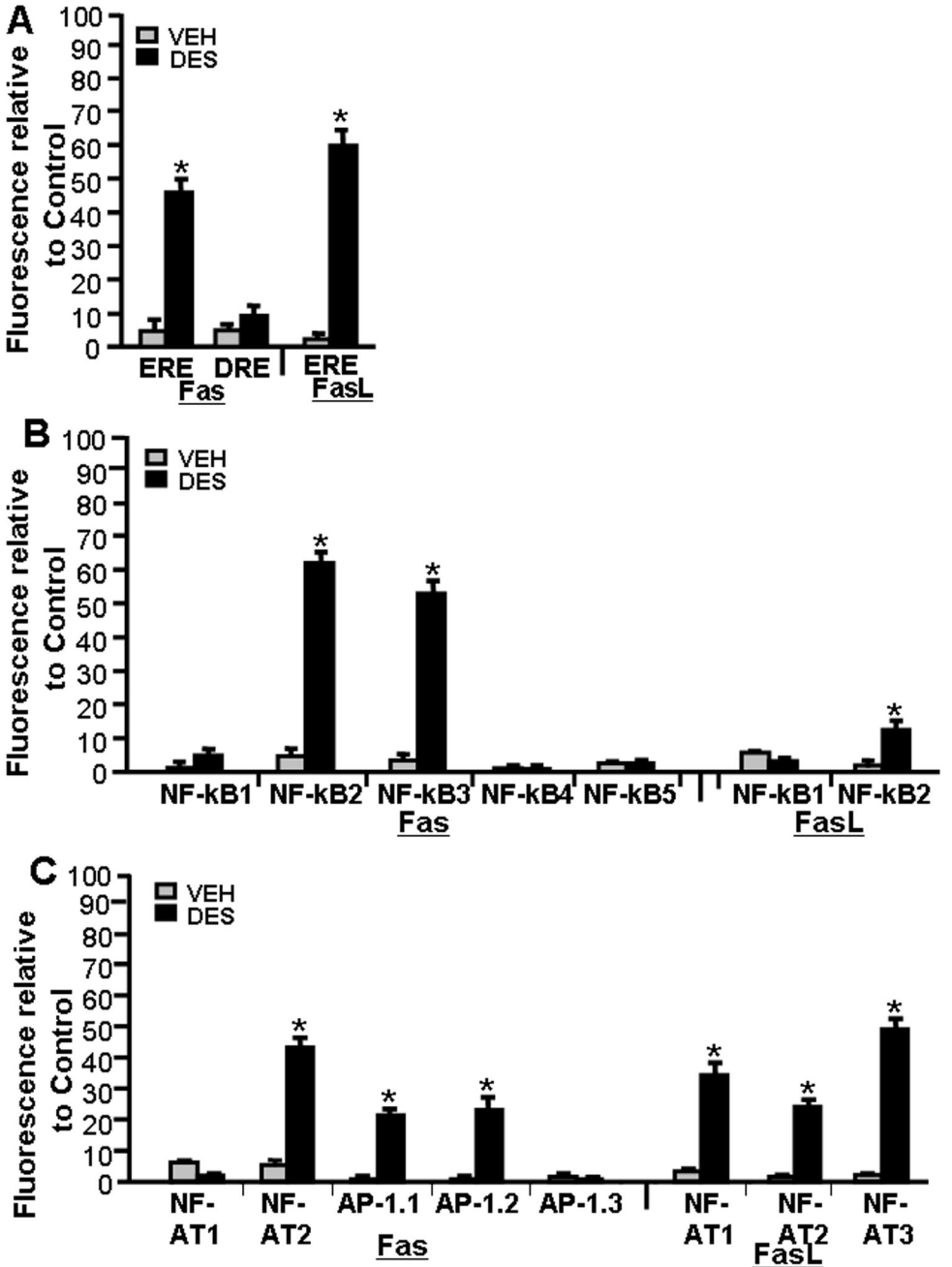

A
Fluorescence relative to Control
VEH
DES
ERE
DRE
ERE
Fas
FasL
B
NF-kB1
NF-kB2
NF-kB3
NF-kB4
NF-kB5
NF-kB1
NF-kB2
Fas
FasL
C
NF-AT1
NF-AT2
AP-1.1
AP-1.2
AP-1.3
NF-AT1
NF-AT2
NF-AT3
Fas
FasL

Figure 7

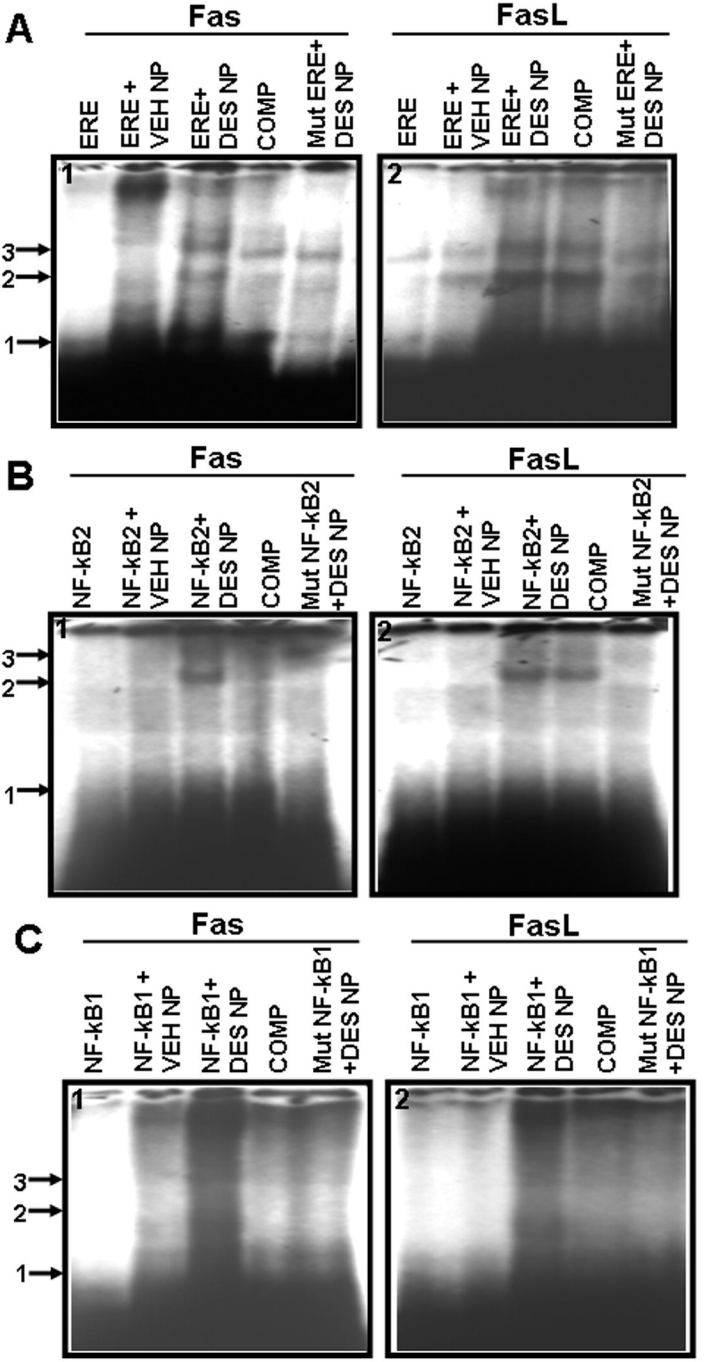

A
Fas
FasL
ERE
ERE + VEH NP
ERE+ DES NP
COMP
Mut ERE+ DES NP
1
2
3
2
1
B
Fas
FasL
NF-kB2
NF-kB2 + VEH NP
NF-kB2+ DES NP
COMP
Mut NF-kB2 +DES NP
1
2
3
2
1
C
Fas
FasL
NF-kB1
NF-kB1 + VEH NP
NF-kB1+ DES NP
COMP
Mut NF-kB1 +DES NP
1
2
3
2
1

Figure 8