RESEARCH ARTICLE

Resveratrol (3,5,4′-trihydroxystilbene) protects pregnant mother and fetus from the immunotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Scope: The ''fetal basis of adult disease'' hypothesis proposes that prenatal exposure to environmental stress can lead to increased susceptibility to clinical disorders later in life. *In utero* exposure of fetus to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) leads to alterations in T-cell differentiation in the thymus and increased susceptibility to autoimmune disease later in life. TCDD triggers toxicity through activation of aryl hydrocarbon receptor and severely affects maternal and fetal immune system during pregnancy.

Methods and results: In this study, using a mouse model, we investigated if administration of resveratrol (RES; 3,5,4′-trihydroxystilbene) would inhibit immunotoxicity induced by TCDD during pregnancy in the mother and fetus. We observed that RES protected not only normal nonpregnant mice but also pregnant mothers and their fetuses from TCDD-induced thymic atrophy, apoptosis, and alterations in the expression of T-cell receptor and costimulatory molecules as well as T-cell differentiation. In addition, there was significantly reduced expression of CYP1A1 in thymi of both the mother and the fetus when RES was used *in vivo* post-TCDD exposure.

Conclusion: In conclusion, these studies demonstrate that consumption of RES, a natural plant product, during pregnancy, may afford protection to the mother and the fetus from the toxicity induced by environmental pollutants that mediate their effects through activation of aryl hydrocarbon receptor.

Keywords:
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin / Aryl hydrocarbon receptor / Fetus / Immunotoxicity / Resveratrol

1 Introduction

Recent epidemiological and experimental evidence has led to the advancement of ''fetal basis of adult disease'' hypothesis, which suggests that malnutrition or exposure to environmental stress during pregnancy, may have a long lasting impact on the developing fetus, leading to increased susceptibility to a wide range of diseases later in life, including cancer, hypertension, cardiovascular, and autoimmune diseases [1, 2]. We and others have shown that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental pollutant, during pregnancy severely affects the immune system of the mothers and their fetuses by triggering apoptosis in thymic T cells, altering T-cell subsets and functions, as well as expression of costimulatory molecules [3, 4]. Majority of biological effects of TCDD leading to immunotocixity and associated deleterious effects are mediated by aryl hydrocarbon receptor (AhR) [5]. The AhR, also known as the dioxin receptor, is a basic helix-loop-helix (bHLH)-Per Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) Sim (PAS) transcription factor. The

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; **AhR,** aryl hydrocarbon receptor; **ARNT,** Aryl hydrocarbon Receptor Nuclear Translocator; **DES,** diethylstilbestrol; **DN,** double negative; **DP,** double positive; **DRE,** dioxin response element; **mAb,** monoclonal antibody; **MFI,** mean fluorescence intensity; **NF-κB,** nuclear factor-κB; **Resveratrol (RES),** trans-3,5,4′-trihydroxystilbene; **TCR,** T-cell receptor

binding of TCDD to the AhR allows nuclear import and exchange of chaperones for the AhR partner protein Aryl hydrocarbon Receptor Nuclear Translocator (ARNT). The AhR/ARNT heterodimer binds to the xenobiotic response elements in regulatory regions of target genes to initiate transcription [5].

Currently, there are no treatment modalities available to prevent and/or reverse the toxic effects of TCDD and its congeners. However, because most of the toxic effects of TCDD are mediated through AhR, the general consensus is that blocking TCDD–AhR interactions would be the most effective way to inhibit TCDD-induced toxicity. Recent studies have indicated that plant-derived polyphenols exhibit AhR binding activity and furthermore can inhibit the induction of CYP1A1. Reseveratrol (RES; 3,5,4′-trihydroxystilbene) is a polyphenolic compound present in plants including grapes, red wine, nuts, berries, and other foods. Resveratrol possesses anti-inflammatory, anti-oxidant, and anti-cancer properties [6–10]. RES can act as an AhR antagonist and inhibit TCDD-induced CYP1A1 expression [11–14]. Interestingly, RES was shown to attenuate the induction of CYP1A1 in the lung and kidney of rats treated with a mixture of benzo[*a*]pyrene/7,12-dimethylbenz[*a*]anthracene [13]. More recently, RES was shown to attenuate TCDD-induced toxicity against several organs *in vivo* in adult mice [15].

Previous studies from our laboratory have suggested that during pregnancy, the mother and fetus are highly susceptible to immunotoxicity induced by TCDD [3, 16]. Thus, we believed it would be interesting to test if plant polyphenols such as RES would attenuate the toxicity and protect the mother and the fetus from immunomodulation. Our studies demonstrate for the first time that RES may play a crucial role in preventing TCDD-mediated toxicity during pregnancy as well as developmental immunotoxicity by acting as an AhR antagonist.

2 Materials and methods

2.1 Mice

Timed pregnant (vaginal plug day 0) and nonpregnant C57BL/6 (H-2^b) mice were purchased from the National Institute of Health (Bethesda, MD). All animals were housed in the University of South Carolina Animal facility. Care and maintenance of the animals were in accordance with the declaration of Helsinki and according to guide for the care and use of laboratory animals as adopted by Institutional and NIH guidelines.

2.2 Chemicals

RES was purchased from Sigma-Aldrich. RES suspended in DMSO was used in *in vitro* studies and suspended in sterilized water was used in the *in vivo* studies, as described earlier [17]. TCDD was provided by Dr. K Chae of NIEHS (Research Triangle Park, NC). TCDD suspended in DMSO was used in *in vitro* studies and suspended in corn oil was used in *in vivo* studies. The following reagents including L-glutamine, HEPES, gentamicin, RPMI 1640, penicillin/streptomycin, 2-mercaptoethanol, Epicentre's PCR premix F, and Platinum *Taq* Polymerase kits, PBS, and FBS were purchased from Invitrogen Life Technologies (Carlsbad, CA). RNeasy Mini kit and iScript cDNA synthesis kit were purchased from Qiagen (Valencia, CA). TUNEL kits were purchased from Roche (Indianapolis, IN).

2.3 *In vivo* TCDD exposure and RES treatments

To determine the effect of TCDD on thymus in normal adult mice, a single dose of TCDD (10 μg/kg) was administered (i.p.) into pregnant C57BL/6 mice on GD 14. These mice next received vehicle or RES (100 mg/kg body weight) by oral gavage on daily basis till GD 19. On day 2 or 5, mice were sacrificed, thymi were harvested, and thymic weight and cellularity were determined. To determine the effect of RES on TCDD exposure during pregnancy, pregnant mice on gestational day (GD) 14 were exposed *i.p.* with a single dose of 10 μg/kg TCDD or the vehicle control (corn oil). For each treatment group, at least three pregnant mice were used and from each pregnant mother, we obtained an average of seven to nine pups. To reduce the variability among the pups in each litter, we combined the 3 L from each treatment group to generate a pool of 21–27 pups. Due to low thymic cellularity in the fetus, thymi from five pups were randomly pooled *per* sample, and approximately five replicate pools were used for statistical analysis.

To this end, a single dose of TCDD (10 μg/kg) was administered (i.p.) into pregnant C57BL/6 mice on GD 14. Next, these mice received vehicle or RES (100 mg/kg body weight) by oral gavage on daily basis till GD 19. Thymic cellularity of both mothers and fetuses was determined on day 2 (GD 16) and day 5 (GD 19) post-TCDD exposure.

2.4 Preparation of thymocytes

Thymi from mice were harvested and placed in complete RPMI-1640 medium. Single-cell suspensions of thymi were prepared as described earlier [3, 16]. Cell viability was determined on a hemacytometer 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, five to six replicate pools were compared from each treatment group and depicted as mean ± SEM.

2.5 Detection of phenotypic markers on thymocytes

Thymocytes (1×10^6) were washed with (Invitrogen, Grand Island, NY) and incubated for 30 min on ice with 0.5 μg of the following primary monoclonal antibodies (mAbs): FITC-anti CD4 (L3T4), PE-anti CD8 (Ly-2), FITC-CD3 (α-chain), FITC-CD8 Ly-2), PE-CD44 (IM7), FITC-αβTCR (H57597) (TCR, T-cell receptor), or FITC-IL-2R (7D4) (BD Pharmingen, San Diego, CA). For double-staining studies, directly conjugated mAbs were simultaneously added to the sample. Detection of J11d marker was done by staining cells with J11d mAb (BD Pharmingen) followed by FITC-anti-rat IgM mAbs. After incubation with the mAbs, cells were washed once with PBS. Negative controls consisted of cells that were stained with appropriate isotype-specic antibodies. Flow cytometric analysis was performed by a Cytomics FC 500 (Beckman Coulter) and analyzed using CXP software (Beckman Coulter). Twenty thousand cells were analyzed *per* sample. Dead cells, clumps, and debris were excluded electronically by gating on forward *versus* side scatter. Flow cytometric data were analyzed as described earlier [18]. In general, CD4/CD8 phenotypic data were represented as percentage positive cells expressing the surface marker. For the analysis of various phenotypic markers, the mean uorescence intensity (MFI) that represents the density of expression of the surface marker was determined for the control, TCDD, and TCDD+RES-treated cells. The data from five to six replicate pools were depicted as percentage change in MFI ± SEM.

2.6 Detection of apoptosis in thymocyte

Thymocytes from mice exposed to TCDD followed by vehicle or RES were analyzed for apoptosis using the TUNEL assay kit (Roche) and as described previously [3, 16, 19, 20]. In brief, thymocytes (1×10^6) 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 present in thymus, liver, and lung were quantified as average number of TUNEL positive cells from at least 30 foci of each section.

2.7 *In situ* detection of apoptosis in thymus, liver, and lung

Thymi, livers, and lungs were collected on day 3 from treatment groups and fixed in 10% formalin. Paraffin blocks were prepared, microtome sections were generated, and *in situ* TUNEL assays on tissues sections were performed using DeadEnd Colorometric TUNEL System and following the protocol of the company (Promega, Madison, WI). The sections were examined for apoptosis under a microscope. Apoptotic cells present in thymus, liver, and lung were quantified as average number of TUNEL positive cells from at least 30 foci of each section.

2.8 RT-PCR to determine the expression of CYP1A1 in thymocytes

Total RNA from thymus or liver from mice was prepared using RNeasy Mini Kit (Qiagen, Maryland). To detect the expression of CYP1A1, mouse-specific forward (5′-CCAC-AGCACCACAAGAGATA-3′) and reverse (5′-AAGTAG-GAGGCAGGCACAATGTC-3′) primers were used and PCR was performed for 30 cycles using the following conditions: 30 s at 95°C (denaturing temperature), 40 s at 60°C (annealing temperature), and 60 s at 72°C (extension temperature), with a final incubation at 72°C for 10 min. The PCR products, generated from mouse CYP1A1 primer pairs, were normalized against PCR products generated from mouse 18S-specific primers after electrophoresis on 1.5% agarose gel and visualization with UV light. The band intensity of PCR products was determined using Bio-Rad image analysis system (Bio-Rad, Hercules, CA).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism software (San Diego, CA). Student's *t*-test was used for paired observations if data followed a normal distribution to compare TCDD-induced apoptosis in T cells, and expression and quantification of CYP1A1 in thymocytes. Multiple comparisons were made using one-way analysis of variance test and Turkey–Kramer Multiple Comparisons Tests. *p*-Values of ≤ 0.05 were considered to be statistically significant.

3 Results

3.1 RES effectively reverses TCDD-induced thymic atrophy, alterations in thymic cellularity, and T-cell subpopulations in naive mice

TCDD is well known to cause thymic atrophy and alter expression of costimulatory molecules [3, 16]. In this context, we investigated whether RES would reverse TCDD-induced toxicity against thymus. To this end, nonpregnant normal C57BL/6 mice were given a single dose (10 μg/kg) of TCDD or vehicle by i.p. route followed by treatment with vehicle or RES (100 mg/kg) by oral gavage on a daily basis. On day 5, thymi mice were harvested, weighed, and thymic cellularity was determined. TCDD caused a significant decrease in thymic weight and cellularity which was reversed following RES treatment (Figs. 1A and B). Next, we analyzed the ability of RES to reverse TCDD-induced alterations in TCR and costimulatory molecule

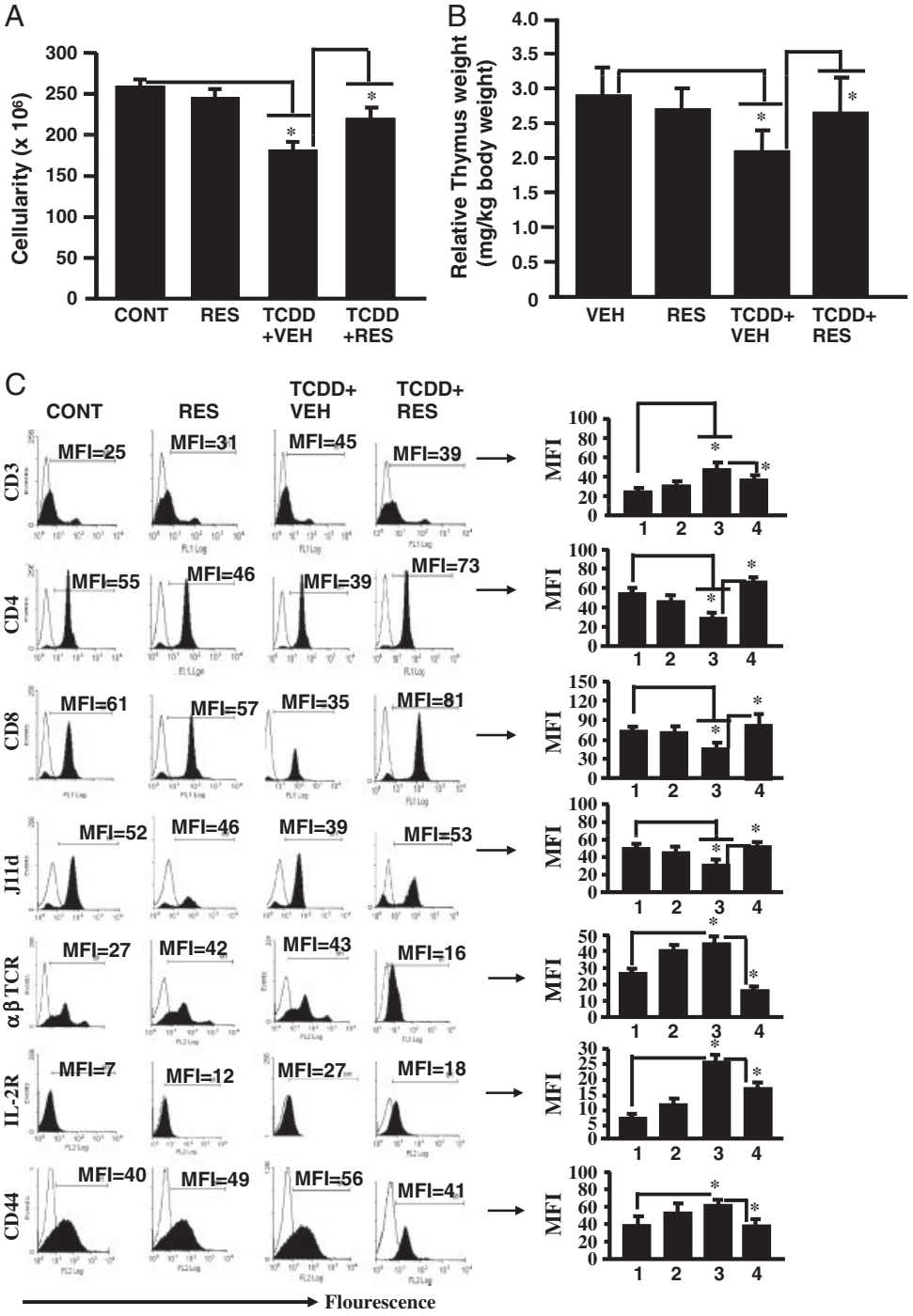

Figure 1. Thymic cellularity, thymic weight, and expression of various surface markers in thymocytes of mice post-TCDD exposure and treatment with RES or vehicle. Groups of five naive nonpregnant mice (C57BL/6) were injected with vehicle or TCDD (10 μg/kg) and treated with vehicle or RES by oral route (100 mg/kg body weight). On day 5, post-TCDD and vehicle or RES treatment, the thymi were analyzed for weight (A), cellularity (B), and expression of various surface markers (C). Vertical bars in (A) and (B) represent mean ± SEM. In (C), representative histograms (left side) are shown for each experimental group, with empty histogram representing cells stained with control antibodies and the filled histogram depict cells stained with various mAbs against surface markers. Data from groups of five mice are depicted as mean ± SEM in right panels. The numbers in right panels; 1, 2, 3, and 4 represent mice that received vehicle alone, RES alone, TCDD+vehicle and TCDD+RES, respectively. Asterisk (*) indicates statistically significant ($p<0.05$) differences between indicated groups.

expression on thymocytes, as previously noted [3, 18, 20]. As shown in Fig. 1C, TCDD-treated mice, when compared with the controls, showed phenotypic alterations characteristic of apoptotic cells including upregulation of CD3, αβ TCR, IL-2R, and CD44 markers and downregulation of CD4, CD8, and J11d as indicated by alterations in mean fluorescence intensity (MFI) values (Fig. 1C). Interestingly, thymocytes from TCDD-treated mice that received RES treatment exhibited minimal or no phenotypic changes when compared with TCDD-exposed mice treated with vehicle (Fig. 1C). Treatment with RES alone did not have a significant impact on the expression of these molecules. Together, these data suggested that RES effectively blocked TCDD-induced alterations in the expression of TCR and costimulatory molecules.

3.2 Detection of apoptosis in thymus, liver, and lung of naive mice following TCDD exposure and treatment with RES

Previous studies from our laboratory have shown that TCDD causes apoptosis in thymic T cells through activation of AhR [3, 16, 18, 21]. To investigate whether RES could block TCDD-induced apoptosis in thymic T cells, we performed TUNEL assays as described earlier [3, 16, 19, 22, 23]. To this end, thymocytes from mice exposed to TCDD and treated with vehicle or RES were analyzed for apoptosis using TUNEL assay. As shown in Figs. 2A and B, RES was able to reverse the apoptosis induced by TCDD in thymocytes. Also, *in situ* apoptosis was measured in various organs (thymus, liver, and lung) postexposure to TCDD and treatment with RES, by performing *in situ* TUNEL assays. As shown in Figs. 2C and D, there was significant apoptosis in all the organs that were harvested from mice exposed to TCDD when compared with vehicle. There was, however, significantly low frequency of apoptosis in the examined organs from mice that received RES treatment post-TCDD exposure when compared with mice that received vehicle treatment post-TCDD exposure (Figs. 2C and D). These data demonstrated that RES significantly reversed the TCDD-induced apoptosis in organs such as thymus, liver, and lung.

3.3 RES effectively reverses TCDD-induced thymic atrophy, alterations in thymic cellularity, and T-cell subpopulations in pregnant mice and their fetuses

Next, we determined the effect of RES on TCDD-induced thymic atrophy and alterations in T-cell subpopulations in pregnant mothers and their fetuses. To this end, a single dose of TCDD (10 μg/kg) was administered (i.p.) into pregnant C57BL/6 mice on GD 14. Next, these mice received vehicle or RES (100 mg/kg body weight) by oral gavage on daily basis till GD 19. Thymic cellularity of both mothers and fetuses was determined on day 2 (GD 16) and day 5 (GD 19) post-TCDD exposure. We have shown previously that pregnant mice exhibit dramatic decrease in thymic cellularity and furthermore, exposure to TCDD further decreases their numbers [3, 16]. We noted that unlike nonpregnant mice that showed ~250 million

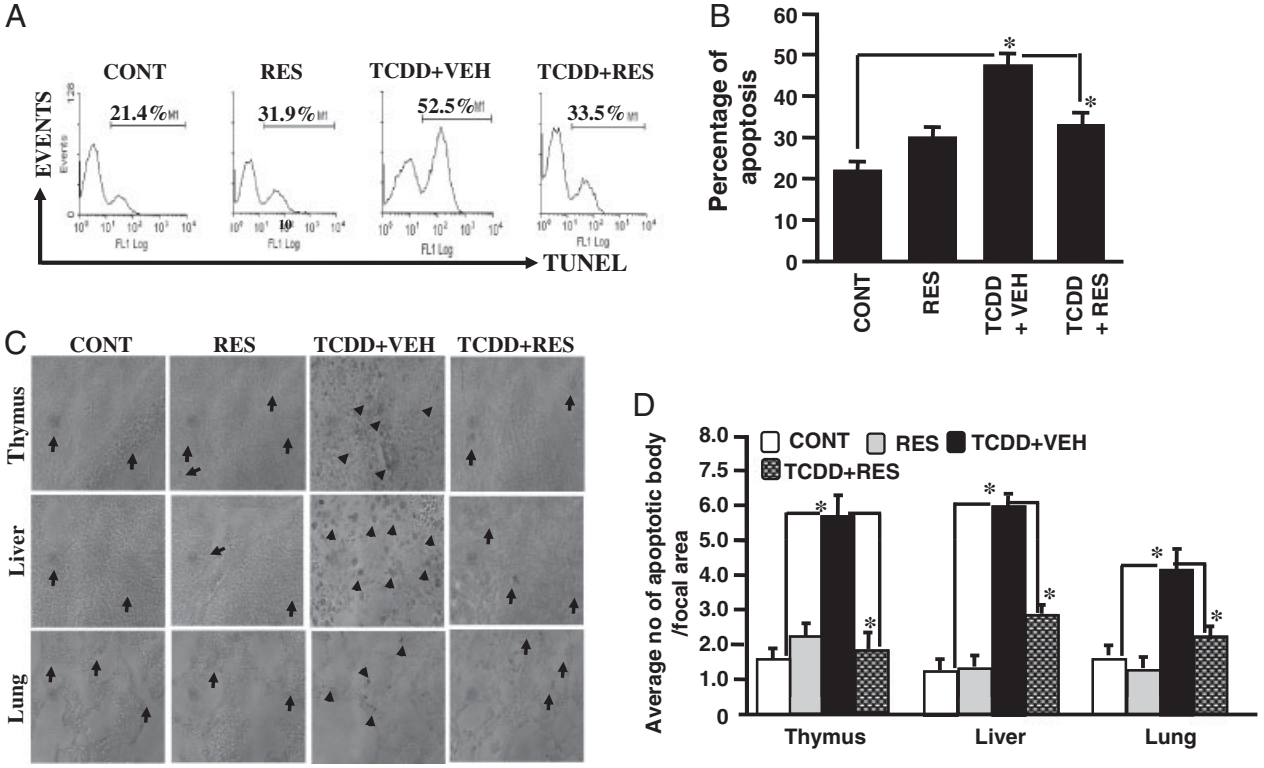

Figure 2. RES blocks TCDD-induced apoptosis *in vivo*. C57BL/6 mice were injected with TCDD and treated with RES as described in Fig. 1. On day 5, the thymocytes were analyzed for apoptosis using TUNEL assay. (A) A representative histogram, and data from groups of five mice (mean ± SEM) are shown in (B). Asterisk (*) in (B) indicates statistically significant ($p < 0.05$) difference in apoptosis between indicated groups. (C) A representative *in situ* TUNEL assays for various organs as determined using DeadEnd colometric TUNEL system, with arrows indicating apoptotic cells and quantitative data from groups of three sections of each organ (mean ± SEM) are shown in (D). Asterisk (*) in (D) indicates statistically significant ($p < 0.05$) difference in apoptosis between indicated groups.

thymocytes/mouse (Fig. 1A), pregnant mice on GD 16 had ~10 million cells/mouse. This cellularity was further decreased following treatment with TCDD and it was reversed following RES treatment (Fig. 3A). When we pursued similar studies in the fetus, TCDD was found to alter thymic cellularity in the fetus on GD 19 but not on GD 16. Also, RES administration in the fetus was able to reverse TCDD-mediated loss of fetal thymic cellularity (Fig. 3A).

In the above experiments, we also analyzed T-cell subpopulations using various markers as described earlier [3, 16]. Exposure of pregnant mice and their fetuses to TCDD led to phenotypic alterations in the thymus characteristic of apoptotic cells such as upregulation of CD3 and downregulation of CD4 and CD8 markers as indicated by alterations in MFI values, when compared with vehicle controls (Figs. 3B–D). However, thymocytes from TCDD-treated mothers and their fetuses that received RES treatment exhibited minimal or no phenotypic changes when compared with TCDD-treated mice that received vehicle treatment (Figs. 3B–D). Together, these data suggested that RES effectively blocked TCDD-induced phenotypic alteration in thymic T-cell subpopulations in mothers as well as their fetuses.

Next, we investigated the CD4 and CD8 T-cell differentiation in the thymus in TCDD-exposed mothers and fetuses following RES or vehicle treatment. The results from a representative experiment have been shown in Fig. 4A and data from multiple experiments have been plotted in Figs. 4B and C. On GD 16, thymocytes of control mothers comprised primarily of double-negative ($CD4^-$ $CD8^-$ or DN; ~36%) and double-positive ($CD4^+$ $CD8^+$or DP; ~52%) T cells (Fig. 4A). Treatment with TCDD into such mice led to marked decrease in DP T cells (~19%) and increase in DN T cells (~63%). Furthermore, treatment of TCDD-exposed mice with RES led to complete reversal of the alterations induced by TCDD in DP and DN T-cell populations. Overall, similar results were seen on GD 19 in pregnant mothers. When the differentiation of $CD4^+$ and $CD8^+$ T cells was studied in the fetus, we noted that overall, both on GD 16 and 19, TCDD inhibited the differentiation of DP T cells leading to increased accumulation of DN T cells when compared with the controls. Also, treatment with RES reversed these effects of TCDD.

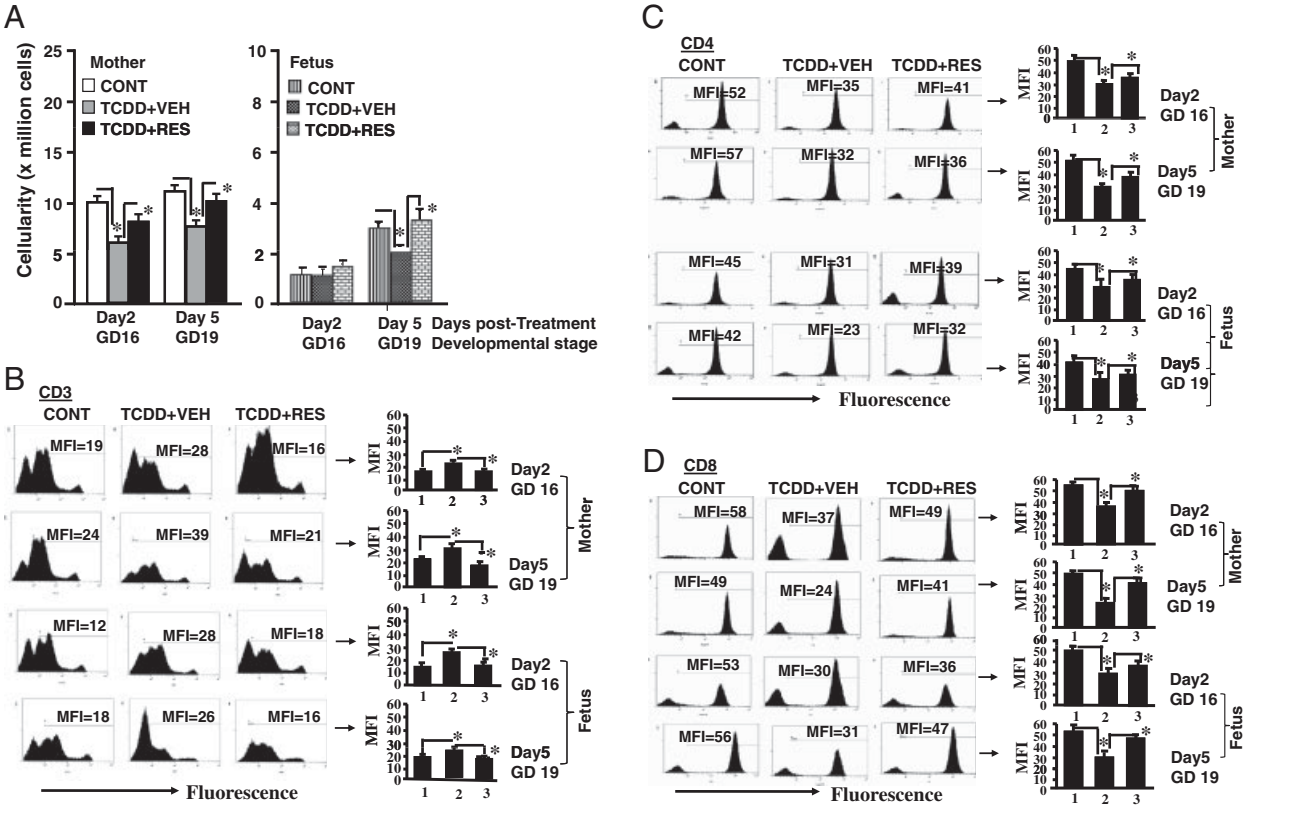

Figure 3. Effect of RES on TCDD-induced alterations in the thymus of pregnant mice and fetuses. Pregnant (gestational day = 14) mice were injected i.p. with solvent (control) or TCDD (10 μg/kg). On the same day, the mice received vehicle or RES (100 mg/kg body weight) by oral gavage. On days 2 and 5 post-TCDD exposure and vehicle or RES treatment, the thymi from mothers and their fetuses were harvested to determine thymic cellularity (A) and expression of CD3, CD4, and CD8 (B–D). Data were analyzed as described in Fig 1 legend. Asterisk (*) in (D) indicates statistically significant ($p<0.05$) differences between indicated groups.

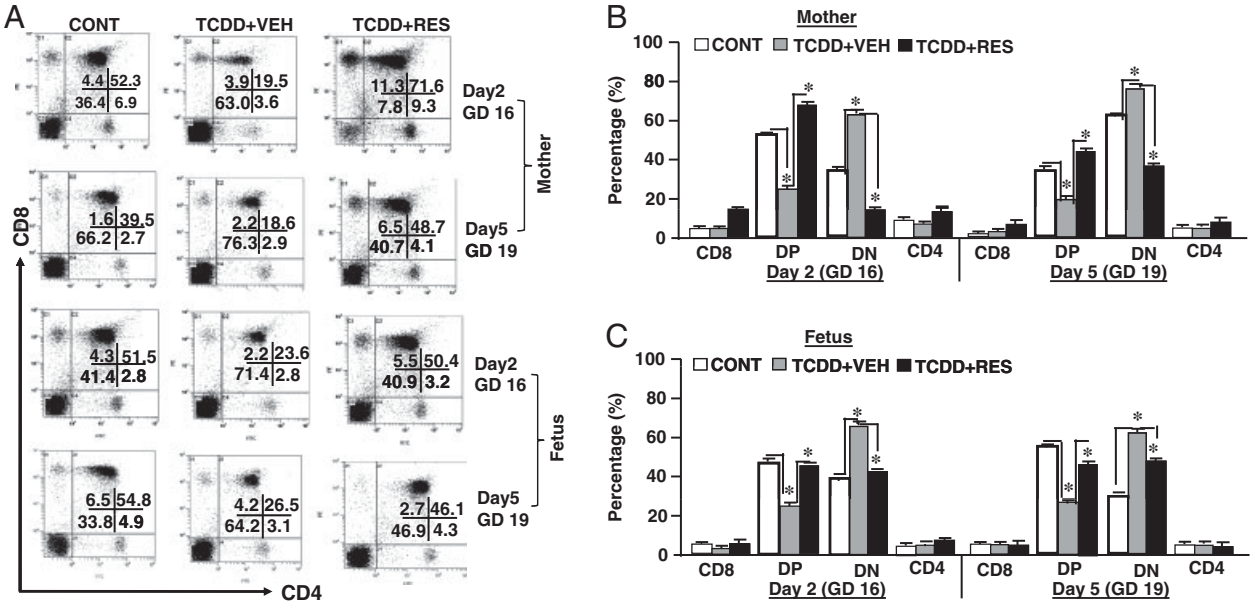

Figure 4. Effect of RES on thymic T-cell subsets of pregnant mothers and their fetuses post-TCDD exposure. C57BL/6 pregnant (gestational day = 14) mice were treated with TCDD and RES as described in the legend of Fig 3. On day 2 (GD 16) and day 5 (GD 19) post-TCDD and vehicle or RES treatment, the thymi from mothers and their fetuses were harvested and double-stained with FITC-anti-CD4 and PE-anti-CD8 mAbs. Representative dot-plots (A) and mean ± SEM from multiple experiments (B–C) have been shown. Asterisk (*) in (B) and (C) indicates statistically significant ($p < 0.05$) differences between groups compared.

3.4 Detection of apoptosis in thymocytes of pregnant mice and their fetuses following TCDD exposure and treatment with RES

Thymi from TCDD-exposed pregnant mice and fetuses were evaluated for apoptosis as described earlier. The results from a representative experiment have been shown in Fig. 5A and data from multiple experiments have been plotted in Fig. 5B. The thymocytes from TCDD treated groups consistently showed signicantly higher percentage of apoptosis in both the mothers and the fetuses on GD16 and GD19, when compared with the nontreated control groups (Figs. 5A and B). However, there was significantly reduced apoptosis in thymocytes of both mothers and fetuses that received RES treatment post-TCDD exposure (Figs. 5A and B).

3.5 RES decreases TCDD-induced upregulation in CYP1A1

TCDD is well known for its ability to induce CYP1A1 through activation of AhR [24–26]. In this context, we sought to examine the expression of mouse CYP1A1 gene in thymus post-TCDD exposure and following RES administration. To this end, expression of CYP1A1 in thymi from mothers and fetuses was determined by performing RT-PCR using mouse CYP1A1-specific sets of primers. As shown in Figs. 6A and B, thymi of both mothers and fetuses showed constitutive expression of CYP1A1. It was interesting to note that RES alone was able to suppress this constitutive expression of CYP1A1 in the thymus of both mothers and fetuses (Figs. 6A and B). Furthermore, there was a significant increase in CYP1A1 expression in thymi of both mothers and fetuses that were exposed to TCDD (Figs. 6A and B). However, the expression of CYP1A1 was significantly reduced in the thymi of mothers and fetuses when the mothers received RES treatment post-TCDD exposure (Figs. 6A and B). These data suggested that RES may act as an antagonist of AhR thereby preventing TCDD-induced expression of CYP1A1.

4 Discussion

Developmental immunotoxicity has gained significant attention recently because the "fetal basis of adult disease" hypothesis proposes that many chronic diseases including autoimmune diseases seen during adult stage of life may result from prenatal exposure to environmental contaminants [1, 2, 27]. Recent epidemiological and experimental studies have provided increasing evidence to support this hypothesis. Thus, it is critical to protect the fetus from environmental insults and dietary supplements with such properties would be an ideal choice that can be used during pregnancy. In this study, we demonstrate for the first time that RES can neutralize the developmental immunotoxicity of TCDD as well as block the changes induced by TCDD in the thymus of pregnant mice. These assertions are based on the following data that we obtained from this study: (i) RES reversed the

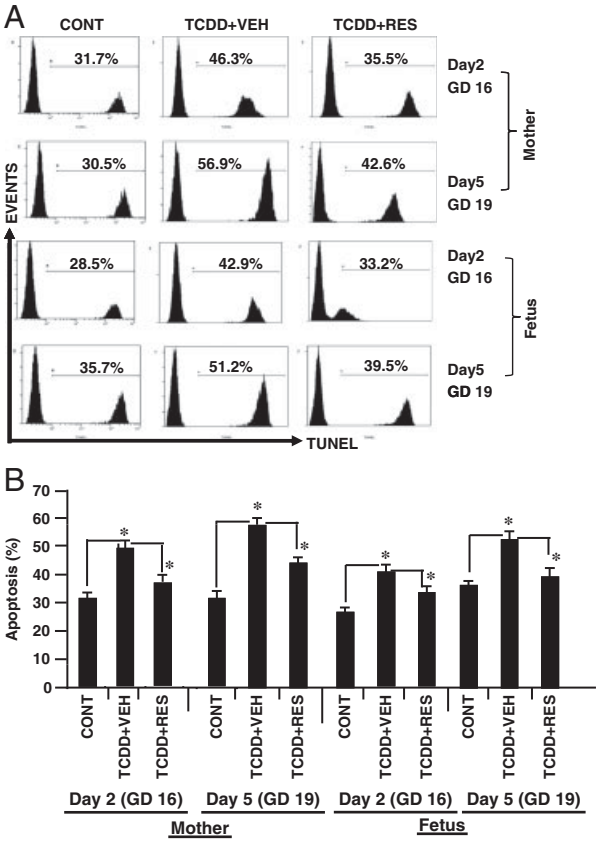

Figure 5. RES blocks TCDD-induced apoptosis in thymocytes of pregnant mother and fetuses *in vivo*. C57BL/6 pregnant (gestational day = 14) mice were injected with TCDD and RES as described in legend of Fig. 3. On day 2 (GD 16) and day 5 (GD 19) post-TCDD exposure and vehicle or RES treatment, the thymocytes were analyzed for apoptosis. (A) A representative histogram and (B) Data (mean ± SEM) from five samples. Asterisk (*) in (B) indicates statistically significant ($p < 0.05$) difference between groups compared.

effects of TCDD on thymic atrophy in nonpregnant mice (Fig. 1), pregnant mice, and their fetuses (Fig. 3), (ii) TCDD-induced alterations in T-cell subpopulations and costimulatory molecules were significantly blocked by RES (Figs. 1C and 3B–D), (iii) RES ameliorated TCDD-induced apoptosis in thymic T cells of naive mice (Figs. 2A and B), pregnant mothers, and their fetuses (Figs. 5A and B), (iv) TCDD caused major alterations in T-cell differentiation in the fetus particularly decreasing the proportion of DP T cells and increasing the percentages of DN cells, thereby suggesting that TCDD was inhibiting the differentiation of DN T cells into DP T cells. RES was able to restore the T-cell differentiation by promoting DP T-cell differentiation (Figs. 4A–C), and (v) RES inhibited TCDD-induced expression of CYP1A1 in both mothers and fetuses (Figs. 6A and B).

A well-established epidemiological finding that supports the ''fetal basis of adult disease'' stems from the prenatal exposure to diethylstilbestrol (DES), a synthetic estrogen that was used in an estimated 5–10 million Americans, between 1938 and 1975, to prevent miscarriages or premature deliveries. Exposure to DES has been associated with an increased risk for breast cancer in ''DES mothers'' and a lifetime risk of cervicovaginal cancers in ''DES daughters'' [28]. Exposure to DES has also been linked to a wide range of abnormalities in DES sons and daughters including immune system disorders such as increased incidence of autoimmunity, cancer, and certain infections [28]. Furthermore, experimental studies from our laboratory demonstrated that DES alters T-cell differentiation in the thymus by interfering with positive and negative selection processes, which in turn modulates the T-cell repertoire in the periphery [29]. In addition, we noted that TCDD alters the process of thymic selection, possibly by enhancing negative thymocyte selection, whereas at the same time allowing autoreactive T cells to escape deletion in the thymus and immigrate to the periphery [30]. Similarly, using genetically predisposed mice, developmental exposure to TCDD was shown recently to alter humoral immune functions and exacerbate a type III hypersensitivity lupus-like autoimmune disease [4]. Together, such studies suggest that exposure to TCDD during development may have a long lasting impact on the immune functions leading to altered susceptibility to infections, autoimmune disease, and hypersensitivity reactions.

Previous studies from our laboratory have demonstrated that TCDD-induced thymic atrophy in the adult and fetus may result, at least in part, from induction of apoptosis [16, 19–21]. TCDD triggered the expression of several apoptotic genes, including Fas and FasL involved in the extrinsic pathway of apoptosis. We noted the presence of a dioxin response element (DRE) and five nuclear factor-κB (NF-κB) motifs on Fas promoter, and no DRE but two NF-κB motifs on FasL promoter [31]. Further studies revealed that TCDD regulates Fas and FasL promoters through DRE and/or NF-κB motifs *via* activation of AhR [31]. We have also demonstrated that apoptotic thymocytes including those exposed to TCDD, on one hand, upregulate the expression of CD3, αβTCR, IL-2R, and CD44 markers while downregulating the expression of CD4, CD8, and J11d markers [16, 18, 20]. Based on the ability of RES to inhibit CYP1A1 induction by TCDD, we suggest that RES may act as an AhR antagonist *in vivo*, thereby blocking TCDD-mediated alterations in the TCR and costimulatory molecules, T-cell differentiation, apoptosis, and thymic atrophy. These data are consistent with other reports demonstrating RES-mediated downregulation of CYP1A1 expression *in vitro* and *in vivo* [11, 12, 32].

It should be noted that the previous studies, including those from our laboratory, have suggested that RES can act as an AhR agonist/antagonist [13, 17, 33]. This may depend on the dose of RES used and the target cells that may express varying levels of AhR. For example, we have noted that RES at higher concentrations acts as an AhR agonist and promotes apoptosis in activated T cells [17]; however, at

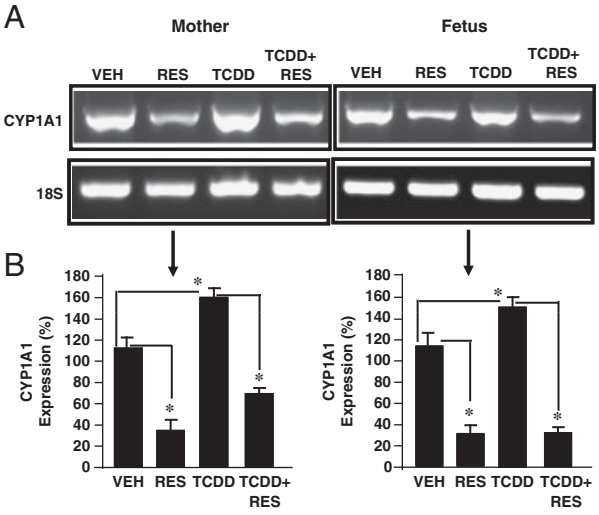

Figure 6. Expression of CYP1A1 in thymus of mothers and fetuses post-TCDD exposure and treatment with vehicle or RES. (A) Expression (RT-PCR) of CYP1A1 in thymi of mothers and fetuses. (B) RT-PCR data are presented as percentage of 18S expression with the latter being considered as 100%. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) in (B) indicates statistically significant ($p < 0.05$) difference between groups compared.

lower concentrations, it acts as a an AhR antagonist and blocks TCDD-mediated apoptosis in activated T cells (unpublished data). Numerous *in vitro* studies using different cell lines have also suggested the AhR antagonistic activity of RES. RES was shown to inhibit the CYP activity by competing for the substrate binding site [13, 32]. In both HepG2 cells [14] and human mammary epithelial cells [34], RES was shown to inhibit the induction of CYP1A1 expression by TCDD. RES acted as an AhR antagonist and inhibited the dioxin effects on bone formation *in vitro* [12]. Similar to RES, α-naphthoflavone has also been shown to act as both AhR agonist and antagonist based on the concentration [35].

Although majority of the published studies have investigated the effect of RES on the induction of CYP1A1 produced by TCDD, two recent studies also evaluated the effect of RES to neutralize dioxin-induced toxicity *in vivo* [15, 36]. In one study, TCDD-induced wasting syndrome was alleviated by treating mice for 28 days with subcutaneous injection of RES. However, in this study, RES failed to alleviate TCDD-induced hepatomegaly and thymic atrophy [15]. This may be because C57BL/6 mice were given a very high dose of TCDD (100 μg/kg body weight) while treating them with lower doses of RES (20 mg/kg), when compared with this study. Also, Jang *et al.* recently reported that the pretreatment of pregnant mice with oral resveratrol (50 mg/kg body weight) significantly reduced the incidence of cleft palate and the severity of renal malformations in the pups caused by *in utero* exposure to TCDD [36]. In this study, however, the effect on the immune system was not analyzed.

It should be noted that for effective neutralization of TCDD-induced toxicity *in vivo*, the dose and route of administration of RES may be critical. In this study, we administered RES by oral route because purified form of RES is readily available in the market for human consumption in the form of capsules. Also, oral route reflects the natural dietary exposure to RES. However, RES given orally shows poor bioavailability due to its high rate of degradation by metabolism [37]. This may be the reason why in a previous study, RES administered orally was not as effective as subcutaneous injection [15]. Bioavailability of RES in the mouse is also short (10–60 min) and varies upon dose quantity [38–40]. Yu *et al.* reported that RES at a lower dose (20 mg/kg) when administered into mice by i.p. injection was converted to resveratrol glucuronide and resveratrol sulfate metabolites within 15 min that was detectable in the serum [40]. No RES or its metabolites were detected after 1 h in the serum. In another set of experiments, when 60 mg/kg resveratrol was administered i.g. into mice, the same metabolites were detected in the sera at 30 min. No trace of RES in sera was observed after 30 min but the metabolites of RES were still detectable after 3 h. Further studies are necessary to address if metabolites of RES can mediate some of the beneficial effects of RES.

In an earlier study, we noted that 100 mg/kg body weight dose of RES was necessary to prevent inflammation in experimental autoimmune encephalomyelitis [17] as well as colitis models [41]. In this study, we observed that a similar dose of 100 mg/kg body weight effectively ameliorated TCDD-induced immunotoxicity. RES has been used at high concentrations such as 500, 1000, and 1500 mg/kg body weight for 10 days, to inhibit tumor growth in BALB/c mice in a dose-dependent manner [8] or 100 mg/kg body weight to delay tumorigenesis in rats [42]. It should be noted that the dose tested in this study is feasible to achieve in humans because the human equivalent dose of 100 mg/kg in mouse is 486 mg, considering an average human weight of 60 kg. Currently, there are several nutraceutical companies selling purified resveratrol in 500-mg quantities in capsule form. Thus, the dose used in this study reflects the potential pharmacological/dietary supplement dose that is currently available in the market.

In summary, this study suggests that RES may serve as an effective AhR antagonist *in vivo* and therefore may be an attractive candidate to prevent immunotoxic effects induced by environmental contaminants during pregnancy, thereby protecting the fetus from deleterious effects.

N. P. S. designed and performed experiments, analyzed results, prepared the figures, and wrote the manuscript; U. S. S. helped in design of experiments and discussion, M. N. designed the experiments, edited the manuscript, and provided the resources, and P. S. N. assisted in project conception, design of

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