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Diethylstilbestrol (DES) mediates immune suppression via modulation of microRNA expression in mice

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Diethylstilbestrol (DES)-mediated immunosuppression through modulation of microRNAs expression in mice

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ABSTRACT

Diethylstilbestrol (DES) is a synthetic estrogen and considered as an endocrine disruptor. DES was used in humans to prevent adverse pregnancy and was later on discontinued due to toxicity particularly increasing the susceptibility to certain types of cancers. DES has also been investigated for its immunosuppressive effects, while the mechanisms remain unclear. In this study, we investigated the mechanisms through which DES exposure triggers immunosuppression and affects T cell functions. To this end, we investigated the effect of DES on T cells in vitro and examined DES-induced apoptosis in T cells, expression of genes involved in apoptosis, and differentiation of T helper cells. We observed DES-mediated significant increase in apoptosis in activated T cells in dose-dependent manner *in vitro*. Upon examination of FasL expression in activated T cells, DES caused significant increase in FasL expression. Furthermore, when microRNAs (miRs) profile in activated T cells was studied, we noted dysregulation of a large number of miRS by DES. Of the total 885 miRs screened, there were more than 217 miRs showing greater than 1.5 fold and 101 miRs showing 2.0 or more than 2.0 fold differential expressions in DES group when compared to vehicle-treated group. Next, the immunosuppressive effect of DES was tested using an animal model (mouse) of delayed type hypersensitivity (DTH).

Interestingly, DES treatment decreased the DTH responses and reversed the inflammation triggered by methylated bovine serum albumin (mBSA). Also, there was significant increase in regulatory T (Tregs) cells but a decrease in both Th1 and Th17 cells in mice that received mBSA+DES treatment when compared to mice that received mBSA+ vehicle treatment. Taken together, the data obtained from this study demonstrated that DES-mediated immunosuppression may be due to DES-regulated mechanisms including apoptosis, gene expression, changes in miRs profile, and regulation of T cells differentiation.

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LIST OF ABBREVIATIONS

CD4 .....Cluster of differentiation 4

CD8 .....Cluster of differentiation 8

ConA .....Concanavalin A

UTR .....3'untranslated

region DTH .....Delayed type hypersensitivity

DES .....Diethylstilbestrol

DMSO .....Dimethylsulfoxide

FoxP3…….....Forkhead box P3

IFN-γ .....Interferon gamma

IL-17 .....Interleukin 17

KLH .....Keyhole limpet hemocyanin

LN.....Lymph node

mRNA(s).....Messenger RNA(s)

mBSA .....Methylated bovine serum albumin

MiR(s).....MicroRNA(s)

OVA .....ovalbumin

PCR.....Polymerase chain reaction

Tregs .....Regulatory T cells

RT-PCR.....Reverse Transcriptase PCR

SIRT1…..Sirtuin 1

Th .....T helper cells

Th1 .....T helper 1 cells

Th2 .....T helper 2 cells

Th17 .....T helper 17 cells

VEH.....Vehicle

CHAPTER 1: INTRODUCTION

Diethylstilbestrol (DES) is a nonsteroidal synthetic estrogen that was first synthesized in 1938. It is also classified as an endocrine disruptor (1-4). It was used in the US (from 1940s to 1975) to prevent spontaneous abortion (5, 6). Approximately 5–10 million expectant mothers and developing fetuses were exposed to DES during this time. DES exposure of mothers and fetuses caused long-term adverse effects to mothers and has been shown to cause increased risk of breast cancer (5, 7). DES-exposed daughters have also shown increased risk of cervicovaginal cancer (8, 9). There have also been reports about other abnormalities, such as immune system disorders, psychosexual effects, and reproductive abnormalities in DES daughters and sons (10). Studies have shown that prenatal exposure of DES alters immune functions in T cells in addition to other immune cells (11, 12). There have also been reports of increased incidence of autoimmune disease in adult life following prenatal DES exposure (13). All these reports suggest that the immunological effects of DES exposure may be far reaching in sons and daughters of exposed mothers.

Earlier studies have demonstrated that murine thymus is highly sensitive to DES when exposed even for a short time during neonatal or adult stage of mice (14-17). DES exposure to neonatal and adult mice has been shown to cause various abnormalities including thymic atrophy, skeletal tissues, female reproductive organs, and muscles (18-20).

In the thymus, several changes such as apoptosis in thymic cells, T cell differentiation, immunotoxicity, and immunosuppression have been reported post DES exposure (11, 14, 21). Holladay et al have shown that DES exposure caused postnatal alterations in T-cell and natural killer (NK) cell function and increased incidence of autoimmune diseases (22). DES exposure has also been shown to affect expression profile of several genes in the thymus (23, 24). The studies from our laboratory have also demonstrated DES-mediated thymic atrophy in mothers as well as in neonatal mice (14). Furthermore, we have also demonstrated DES-induced alterations in positive and negative selection of T cells in the thymus (14).

DES initiates early signaling primarily through estrogen receptor (ER) and regulates expression of various genes (14, 15, 25). 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. Previous studies have demonstrated that DES caused decrease in prothymocyte stem cells (26), decrease in double positive $CD4^{+}CD8^{+}$ cells (14, 27), as well as induced cell death in thymocyte subsets $CD4^{+}CD8^{+}$, $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ (15, 28). DES has also been shown to induce apoptosis in double- negative ($CD4^{-}CD8^{-}$) cells in fetal thymic organ culture system (29). 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 (30). In a recent study, Frawley et al have reported DES-induced alteration in gene expression profile in thymic cells (31).

The precise mechanism of DES-mediated regulation of genes 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 gene expression in T cells and understand the molecular mechanisms leading to immune suppression. MicroRNAs (miRs) are highly conserved noncoding single-stranded small RNA molecules (17–27 nucleotides) that control gene expression post-transcriptionally by binding the 3' untranslated region (UTR) of target gene mRNA. The binding of miRs with the target UTR of mRNA results in the degradation of the target mRNA or inhibition of the translation of the mRNA (32, 33). They account for 1% of the genome and play a critical role in cellular processes such as apoptosis, proliferation, and differentiation (33, 34). MiRs are conserved across species, and their expression is highly specific for tissue type. MiRs regulate 30% of the genome. In most types of cancer, miRs are in general downregulated and can function as either tumor suppressor or oncogene (32, 33). miR profiling in several human malignancies has shown that such an approach may be used to identify miRs with a role in tumor cell biology, classify tumor subtypes, and identify diagnostic and prognostic markers (33).

Since miRs play crucial role in regulation of genes, examination of miR profile in the thymus of prenatal mice post DES exposure is very important. The study will allow cataloging various miRs that are regulated by DES in T cells of mice and will throw light as to how DES-regulated miRs may play an important and significant role in immune suppression and cause long-term effects.

Understanding the long-term effects of DES in T cells will allow development of strategies to neutralize long-term effects of DES in adults. In this study, therefore, we examined miRs profile in T cells of mice post DES exposure. To further understand DES-regulated miRs profile, we analyzed miRs profile and their role in various pathways (cancer, apoptosis, cellular mechanisms etc). Furthermore, we examined some of the specific miRS involved in molecular mechanisms associated with apoptosis, immunotoxicity, and immune suppression.

Delayed-type hypersensitivity (DTH) reactions are classified as a type IV allergy and can experimentally be induced in mice and other rodents by immunization with exogenous antigens such as pathogens (*Mycobacterium*, *Leishmania* and viruses), cells (allogenic splenocytes and red blood cells from other species) and protein antigens such as methylated bovine serum albumin (mBSA), keyhole limpet hemocyanin [KLH] and ovalbumin [OVA] (35). During sensitization of mice with such antigens, antigen-specific memory T cells develop in draining lymph nodes (LNs). After challenge of the sensitized mice with the same antigens, either interferon gamma (IFN-γ) producing T helper 1 (Th1) cells or interleukin 17 (IL-17) producing T helper 17 (Th17) cells are induced. For example, mice deficient in IFN-γ or IFN-γR1 showed decreased DTH response against herpes simplex virus type (36, 37), but not *Mycobacterium tuberculosis* (38). In addition, IFN-γ-deficient (IFN-γ-/-) mice showed attenuated DTH responses to KLH (39, 40), but aggravated responses to OVA and mBSA (41, 42). On the other hand, Nakae et al recently demonstrated that IL-17-/- mice showed reduced DTH reaction induced by mBSA and *Mycobacterium tuberculosis* (43, 44). Thus, depending on the nature of antigens, Th1 or Th17 cells play crucial role in mediating DTH.

In the current study, our goal was to investigate the effect of DES in immunosuppression and examine molecular mechanisms leading to immunosuppression. To that end, we examined the effect of DES on T cells in vitro and studied the molecular mechanisms leading to generation of various T helper (Th) cells, such as Th1, Th2, Th17, and regulatory T (Tregs) cells. We also examined the effect of DES on regulation of microRNAs in T cells. Furthermore, we tested immunosuppressive effect of DES in vivo using an animal model of Th17 cell-mediated mBSA-induced delayed type hypersensitivity (DTH). Our data indicated that DES can decrease DTH reaction by suppressing Th17 cells while enhancing Tregs.

CHAPTER 2: MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from NCI. The mice were kept in University of South Carolina Animal facility. The mice were cared and maintained according to guide for the care and use of laboratory animals as adopted by Institutional and NIH guidelines.

Chemicals. DES was purchased from Sigma-Aldrich. DES suspended in dimethylsulfoxide (DMSO) was used for *in vitro* studies and DES suspended in corn oil was used in *in vivo* studies. The following reagents, RPMI 1640, L-glutamine, HEPES, gentamicin, PBS, and FBS were purchased from invitrogen life technologies (Carlsbad, CA). Concanavalin A (ConA) was purchased from Sigma-Aldrich (St. Louis, MO). Polymerase chain reaction (PCR) reagents were (Epicentre's PCR premix F and Platinum *Taq* Polymerase kits) purchased form Invitrogen (Invitrogen Life Technologies Carlsbad, CA). The following reagents including miRNeasy kit, miScript cDNA synthesis kit, miScript primer assays kit, and miScript SYBR Green PCR kit were purchased from Qiagen (Qiagen Inc, Valencia, CA). The following mAbs were purchased from eBioscience (Carlsbad, CA): anti-mouse IgG-PE, FC block, CD4-FITC, FoxP3-PE, IFN-γ-PE, IL-17-PE, and IL-4-PE.

Effect of DES on primary T cells. To determine the effect of DES on primary T cells, spleen from C57BL/6 mice were harvested and single cell suspension was prepared. T cells were purified using nylon-wool column following the protocol of the company (Polysciences, Inc., Warrington, PA) followed by depletion of B cells and macrophages. The purity of T cells was more than 90% as determined by flow cytometry (Cytomics FC 500; Beckman Coulter). Purified T cells, were first activated with ConA (2 μg/ml) overnight (12-18 hrs), and then treated the following day with vehicle (DMSO: 20 μl) or various doses (5, 10, 15, and 20 μl) of DES dissolved in DMSO for 12-24 hrs. Apoptosis in activated T cells post-DES exposure was determined by performing TUNEL assays (FITC-dUTP nick-end labeling) using in situ Cell-Death Detection kit (Roche, Indianapolis, IN) as described previously (45, 46). In brief, T cells were harvested post-DES or vehicle treatment and washed twice with cold PBS. Cells were then fixed using 4% Para formaldehyde and incubated for 30-45 min at room temperature. The cells were washed twice with cold PBS and permeabilized using freshly prepared permeabilization solution and the cells were incubated at 4°C for 2 min. The cells were washed once with PBS, resuspended in 50 μl/tube TUNEL reaction mixture and incubated for 60 min at 37°C in a humidified atmosphere in the dark. The cells were washed twice with PBS and finally suspended in 200-500 μl cold PBS and analyzed using flow cytometry (Cytomics FC 500, Beckman Coulter) and software

Reverse Transcriptase PCR (RT-PCR) to determine the expression of FasL in activated T cells post DES treatment. Total RNA was isolated from ConA-activated primary T cells treated with DMSO as VEH or DES 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 1 µg total RNA using iScript Kit and following the protocol of the manufacturer (Bio-Rad). Following first strand synthesis, 2 µl (10% of the reaction volume) was used as a template for PCR amplification. To detect mouse FasL (435 bp) expression, forward (5'-CGG TGG TAT TTT TCA TGG TTC TGG-3') and reverse (5'-CTT GTG GTT TAG GGG CTG GTT GTT-3') primers were used. PCR for FasL was performed for 35 cycles using the following conditions: 30 s 95°C (denaturing temperature), 40 s at 58°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 FasL primer pairs, were normalized against PCR products generated from mouse 18S forward (5'-GCC CGA GCC GCC TGG ATA C-3') and reverse (5'-CCG GCG GGT CAT GGG AAT AAC-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).

Effect of DES on differentiation of T helper (Th) subsets: To determine the differentiation of various Th subsets (Th1, Th2, Th17, and Tregs) in the presence or absence of DES, intracellular staining of T cells was performed. In brief, spleen cells (3×10^6 cells/well in 24-well plates) were cultured in the presence of vehicle (DMSO) or DES (10 µM/ml) at 37°C for 72 hours. In last 6 hrs of the culture, the cells were stimulated with 1µg/ml ionomycin and 100 ng/ml PMA (Sigma-Aldrich). Golgiplug (1 µM) was added to the culture during the last 4 hours. After washing twice with FACS buffer (PBS with 1% BSA), cells were pre-blocked with Fc receptors for 15 min at 4°C.

The cells were washed with FACS buffer, and then stained with FITC-conjugated anti-CD4, PE-conjugated anti-IFN-γ, PE-conjugated anti-FoxP3, PE-conjugated anti IL-17, and APC-conjugated anti-IL-4 mAbs (eBioscience, Carlsbad, CA), for 30 minutes with occasional shaking at 4 °C. The cells were washed twice with FACS staining buffer and resuspended in BD Cytofix/Cytoperm solution for 20 min. The cells were washed again with BD perm/wash solution after incubating them for 10 min at 4°C. The cells were then washed twice with FACS buffer and analyzed by flow cytometry (FC 500) and using CXP software (Beckman Coulter, Fort Collins CO).

Isolation of total miRs and miR array assays. Total RNA including miRs from T cells, treated with DES or vehicle for 18 hrs, was isolated using miRNeasy kit and according to the manufacturer's instructions (Qiagen, Valencia, CA). MiRs arrays were performed on Affymetrix GeneChip miR platform of microRNAs in core facility of Johns Hopkins University, Baltimore, MD. The data generated from miR arrays were analyzed using hierarchical clustering and pathway network analysis for the induction or repression in the expression of miRs were analyzed using 2-sample t-test. A p-value of <0.01 in the t-test was considered significant. A fold-change (FC) of more than 1.5-fold was considered positive.

Table 2.1 Sequence of the target miR

miRBase ID	Target Sequences	Qiagen Cat No
Mmu-miR-23a_st	AUCACAUUGCCAGGGAUUUCC	MS00007266
Mmu-miR-18b_st	UAAGGUGCAUCUAGUGCUGUU	MS00011326
Mmu-miR-31_st	AGGCAAGAUGCUGGCAUAGCUG	MS00001407
Mmu-miR-34a_st	UGGCAGUGUCUUAGCUGGUUG	MS00001428

Real-Time PCR to validate the expression of miRs in T cells post DES treatment. To validate the expression of some of the miRs obtained from miR arrays data in T cells treated with vehicle (DMSO) or DES (10 μM), we selected 4 miRs (miR-18b, miR-23a, miR-31, and miR-34a). Real-Time PCR assays were performed on cDNAs generated from total RNAs including miRs isolated from T cells exposed to DES or vehicle as described earlier. We used miScript primer assays kit (details in Table 1) and miScript SYBR Green PCR kit from Qiagen and following the protocol of the company (Qiagen, Valencia, CA). We used StepOnePlus Real-Time PCR system V2.1 (Applied Biosystems, Carlsbad, CA) and at the following conditions: 40 cycles using the following conditions: 15 min at 95°C (initial activation step), 15 s at 94°C (denaturing temperature), 30 s at 55°C (annealing temperature), and 30 s at 70°C (extension temperature and fluorescence data collection) were used. Normalized expression (NE) of miRs was calculated using NE ¼ 2_DDCt, where Ct is the threshold cycle to detect fluorescence. The data were normalized to various miRs against internal control miR and fold change of miRs were calculated against control miR and treatment group (DES) was compared with vehicle group. To define significant differences in miR levels in the thymi of DES- or vehicle-treated groups, ANOVA was performed using GraphPad version 4.0 (GraphPad Software, Inc., San Diego, CA). Differences between treatment groups were considered significant when: $*p < 0.05$.

MiR-messenger RNAs (mRNAs) target interactions. We identified miR-specific mRNAs (mouse-FoxP3, -SIRT1, and -IL-17) target genes using micro RNA.org, TargetScan mouse 5.1, and miRGEN (version 3) software and their database. Computational algorithms aid this task by examining base-pairing rules between miR and mRNA target sites, location of binding sites within the target's 3'-UTR, and conservation of target binding sequences within related genomes. The details of some of miRs and UTR region of their target gene (RNA targets) are described in Table 2.2.

Table 2.2 Binding affinity of miR with 3'UTR of the gene

miR-31 and FoxP3 3'UTR binding

```
3'gucgauacggUC-GUAGAACGGa5'mmu-miR-31
                || |      | | || | | |
5'gguugcucaaAGUCUUCUUGCCc 3' Foxp3
```

miR-320 and IL-17 3'UTR binding

```
3'agcgggagagUUGGGUCGAAAa5'mmu-miR-320
                |    : : || | | |||
5'auuaauggaaAUUUCAGCUUUa 3' IL17A
```

miR-34aa and SIRT1 3'UTR binding

```
3'uguuGGUCGAUUCUGUGACGGu5'mmu-miR-34a
      :| |      |      |      |: || | | | |
5'aucuUCACCACAAAUACUGCCa 3' SIRT1
```

Determination of FoxP3, IL-17, and SIRT1 expression in T cells post DES treatment.

Expression of FoxP3, IL-17, and SIRT1 in T cells treated with vehicle (DMSO) and DES (10 μM/ml) was determined by performing RT-PCR. In brief, activated T cells were treated with vehicle or DES for 18-24 hrs. T cells post DES treatment were collected and total RNA was either isolated immediately or stored at -80°C for use in future. Expression of FoxP3, IL-17, and SIRT1 was determined by RT-PCR as described previously (47). For RT-PCR, total RNAs from VEH- or DES-treated groups of T cells 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 1 μg total RNA using iScript Kit and following the protocol of the manufacturer (Bio-Rad). PCR was performed using mouse FoxP3-specific sets of forward (5'CAC CCA GGA AAG ACA GCA ACC-3') and reverse (5'-GCA AGA GCT CTT GTC CATTGA-3') primers, mouse SIRT1-specific forward (5'-GGA GCA GAT AGT AAG CGG CTTGAG GG-3') and reverse (5'-GACCTC CCA CAT TCG GGC CTC TCC-3') primers, and mouse IL-17-specific forward (5'-TCT GGG AAG CTC AGT GCC G-3') and reverse (5'-CTGAGA AAC GTG GGGGTT TCT-3') primers. The PCR products, generated from mouse FoxP3, SIRT1, and IL17 primer pairs, were normalized against PCR products generated from mouse 18 specific forward 5'-GCC CGA GCCGCCTGG ATA C-3' and reverse 5'-CCG GCGGGTCATGGG AAA AC-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).

Analysis of miRs and their association with various pathways. Post generation of heatmap and analysis of miR expression, we selected miRs that were upregulated or downregulated more than 1.5 fold in fetal thymi exposed to DES, when compared to fetal thymi exposed to vehicle.

Next, the selected miRs were analyzed for their role in expression of various genes and pathways using IPA software and database of the company (ingenuity Inc, California) and Cytoskape software.

Generation of DTH in mice and DES treatment: DTH was induced in C57BL/6 mice using methylated BSA (mBSA) and as described previously (43, 48, 49). In brief, C57BL/6 mice were sensitized with 200 µl (1.25 mg/ml) mBSA (Sigma-Aldrich, MO) emulsified in complete Freund's adjuvant (CFA; DIFCO Laboratories, MI) by subcutaneous injection at the base of the tail. Six days later, the mice were re challenged by intradermal injection of 20 µl of 10 mg/ml mBSA suspended in PBS into both footpads. The immunized mice (C57BL/6) were treated with vehicle (VEH: corn oil), or DES, dissolved in corn oil (10 µg/kg bw). We used this optimum dose (10 µg/kg bw) based on our previous studies (14, 15). In this study, we used the following groups: Control, mice that did not receive any treatment, mBSA-VEH, mice immunized and re-challenged with mBSA in footpad and received VEH treatment, mBSA-DES D0, mice immunized and re-challenged with mBSA in footpad and received DES treatment on day 0 (the day of first mBSA immunization), mBSA-DES D6, mice that received DES treatment on day 6 (day of second immunization), and mBSA-DES D0+D6, mice that received DES on day 0 and day 6. Each group contained at least five mice.

The footpad thickness was measured before (0 hours or 0 days) and after mBSA or PBS challenge using an engineer's calipers. The data were calculated as follows: increase in footpad thickness (mm) = (footpad thickness after challenge) - (footpad thickness before challenge).

Analysis of various T helper (Th) subsets in lymph nodes post mBSA and DES treatment: To determine the generation of various Th subsets (Th1, Th2, Th17, and Tregs) in draining lymph nodes (LN) of mice that were immunized with mBSA and rechallenged with mBSA on day 6 and treated with vehicle or DES on day 0 or day 6 or day 0 and day 6, intracellular staining of T cells was performed. In brief, draining LN (Popliteal LN) were harvested, single cell suspension was prepared, and the cells were cultured in complete medium for 6 hrs in the presence of 1 μg/ml ionomycin and 100 ng/ml PMA (Sigma-Aldrich). Golgiplug (1 μM) was added to the culture during the last 3 hours. After washing twice with FACS buffer (PBS with 1% BSA), LN cells were pre-blocked with Fc receptors for 15 min at 4°C. The cells were washed with FACS buffer, and then stained with FITC-conjugated anti-CD4, PE-conjugated anti-IFN-γ, PE-conjugated anti-FoxP3, PE-conjugated anti IL-17, and APC-conjugated anti-IL-4 mAbs (eBioscience, Carlsbad, CA), for 30 minutes with occasional shaking at 4 °C. The cells were washed twice with FACS staining buffer and resuspended in BD Cytofix/Cytoperm solution for 20 min. The cells were washed again with BD perm/wash solution after incubating them for 10 min at 4°C. LN cells were then washed twice with FACS buffer and analyzed by flow cytometry (FC 500) and using CXP software (Beckman Coulter, Fort Collins CO).

Statistics. 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 DES-induced apoptosis in T cells, and expression and quantification of FasL expression in T cells. Differential (upregulated or downregulated) expression of miRs was analyzed using 2-sample t-test method. The significance of analysis of microarrays was performed using Kaplan-Meier method. Multiple comparisons were made using ANOVA (one-way analysis of variance) test and Turkey-Kramer Multiple Comparisons Tests. P-value of ≤ 0.05 was considered to be statistically significant.

CHAPTER 3: RESULTS

DES triggers apoptosis in T cells in vitro. ConA-activated T cells were cultured in the absence or presence of DES for 18-24 hrs. Post DES treatment, T cells were evaluated for apoptosis as described earlier (14, 15, 50). Activated T cells treated with DES showed significantly higher percentage of apoptosis (Fig. 1A-B), when compared to the VEH-treated T cells. Also, there was a dose-dependent (5-20 µM/ml; more at higher dose and less at lower dose) DES-induced apoptosis in activated T cells. The results from a representative experiment have been shown in Figure 3.1 A and data from multiple experiments have been plotted in Figure 3.1 B. These results demonstrated that DES causes significant apoptosis in activated T cells.

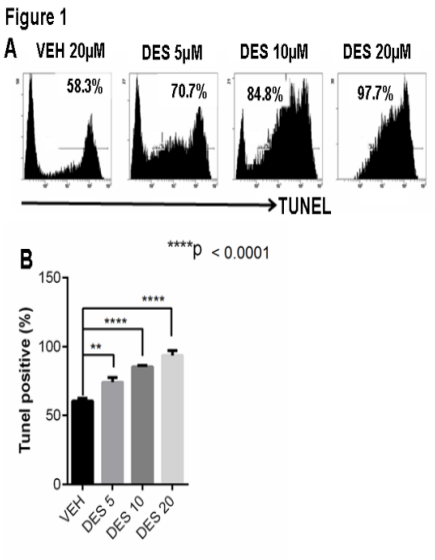

Figure 3.1 *DES causes apoptosis in T cells***.** A. DES causes apoptosis in activated T cells in dose-dependent manner. There was significant apoptosis in DES-treated activated T cells, when compared to vehicle-treated T cells. Data presented represent one of three independent experiments performed. B. The bars represent mean of three independent experiments and asterisks represent significant (**p>0.001, ****p>0.0001) apoptosis in activated T cells post DES treatment when compared to vehicle control

DES upregulates FasL expression in activated T cells. Next, we determined expression of FasL, a key molecule involved in apoptosis by performing RT-PCR. T cells treated with DES showed moderate level expression of FasL in the presence of VEH (Fig 3.2A-B). However, there was significant upregulation of FasL expression in activated T cells upon exposure to DES (Fig 3.2A-B). These data demonstrated that DES induces expression of FasL in activated T cells.

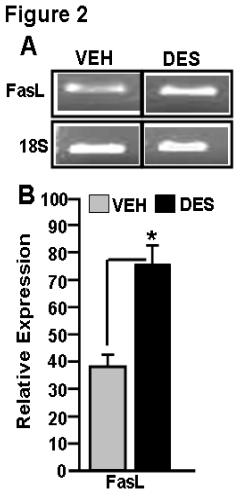

Figure 3.2 *DES upregulates FasL expression in activated T cells*. A. RT-PCR was performed on RNA harvested from vehicle or DES-treated activated T cells. The data presented represent one of the three experiments performed. B. Bars represent mean of three independent experiments and asterisk represents significant ($p>0.05$) upregulated expression of FasL in activated T cells post DES treatment.

DES triggers reciprocal differentiation of Tregs and Th17 cells *in vitro*. Next, we directly tested if DES would affect differentiation of T helper (Th) cells *in vitro* and alter differentiation of regulatory (Tregs) and Th-17 cells. To this end, T cells were cultured in the presence of purified anti-mouse CD3 and CD28 mAbs and vehicle or DES (10 µM/ml) for three days. The analysis of T cells differentiation revealed that DES caused significant induction of CD4+FoxP3+ Tregs (~10 to 11%), when compared to VEH (~4-5%) (Fig 3.3A). However, DES caused suppression of CD4+/IL-17+ Th17 cells (Fig 3.3B), when compared to VEH. Similarly, when we examined generation of Th1 ($CD4^+$/IFN-γ^+) and Th2 ($CD4^+$/IL-4^+) in the presence of DES, we observed significant suppression of Th1 cells (Fig 3.3C) and Th2 cells (Fig 3.3D). These data demonstrated that DES regulates differentiation of Th cells and allows preferential induction of Tregs while suppressing induction of Th1, Th2, and Th17 cells.

Cluster Analysis of DES-regulated miRs Profile in activated T cells. Raw data obtained from miR arrays of activated T cells post DES or vehicle exposure were analyzed for miR expression. To this end, cluster analysis of 885 miRs (Fig 3.4A), as defined by median absolute deviation in DES and vehicle-treated samples, were performed using Ward's method. Similarity measure of miRs of the two groups was done using Half Square Euclidean Distance method and ordering function of miRs was done on the basis of Input rank. In Fig 3.4A, the visualization of cluster analysis of miRs have been shown as a dendrogram (a tree graph) and their expression pattern is reflected in a range from +53.0 to -47.0 (Fig 3.4A).

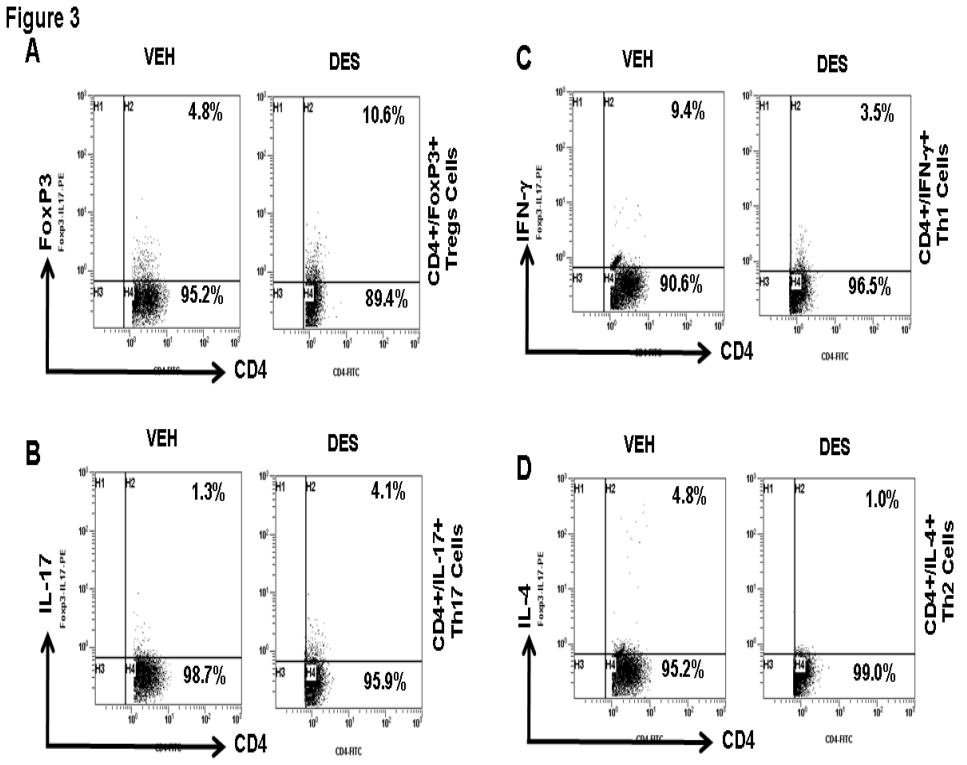

Figure 3.3 *Effect of DES on differentiation of T helper cells in vitro*. T cells from naïve C57BL/6 mice were activated using anti-mouse CD3 (1 µg/ml) and CD28 (1 µg/ml) mAbs. The cultures received VEH or DES (10 µM/ml) for 72 hrs. Panel A shows Treg and Th1 staining while panel B shows Th17 and Th2 staining. Data presented is representative of three independent experiments

Differential Expression (fold change) of miRs. Differential (upregulated or downregulated) expression of miRs was analyzed using 2-sample t-test method. The significance of analysis of microarrays was performed using Kaplan-Meier method. A p-value of <0.01 in the t-test was considered significant. Of the total 885 miRs screened, there were more than 217 miRs showing more than 1.5 fold and 101 miRs showing 2.0 or more than 2.0 fold differential expressions in DES group when compared to vehicle-treated group (Fig 3.4B and Table 3).

There were 116 (61 upregulated and 55 downregulated) miRs, whose expression varied between 1.5-1.9 folds, whereas there were 101 (58 upregulated and 43 downregulated) miRs, whose expression was 2 or more than 2 fold (Fig 3.4B). Expression of miRs, 1.5-fold or higher, was considered positive and so for all further analyses, characterization of miRs was performed based on differential expression of 1.5 or more (upregulated/downregulated). The data obtained from these studies demonstrated that DES-mediated effects on miR regulation varied from moderate (from 1 to 3 fold) to high (from 3.5 to 19.7 fold) but the majority of miRs expression fell in the range of 1.5-3.5 folds (upregulation or downregulation) as shown in (Figure 3.4B).

Table 3.1 Showing upregulated and downregulated miRs in activated T cells post DES exposure when compared to Vehicle-treated activated T cells

miRs	Downregulated	miRs	Upregulated
mmu-miR-181c-star_st	-4.4	mmu-miR-101b_st	5.8
mmu-miR-181d-star_st	-1.8	mmu-miR-143_st	3.2
mmu-miR-1899_st	-1.7	mmu-miR-145_st	6.1
mmu-miR-1927_st	-1.9	mmu-miR-155-star_st	6.1
mmu-miR-301b-star_st	-1.5	mmu-miR-1943-star_st	3.2
mmu-miR-3061-5p_st	-1.8	mmu-miR-221-star_st	2.9
mmu-miR-3074-5p_st	-1.8	mmu-miR-3060-star_st	1.6
mmu-miR-3076-5p_st	-2.6	mmu-miR-3064-3p_st	1.5
mmu-miR-449c_st	-1.8	mmu-miR-3067_st	1.7
mmu-miR-5125_st	-1.6	mmu-miR-3077_st	3.4
mmu-miR-874_st	-1.9	mmu-miR-3100-3p_st	1.6
mmu-miR-96_st	-1.5	mmu-miR-326-star_st	1.8
mmu-let-7i-star_st	-1.7	mmu-miR-331-3p_st	2.4
mmu-miR-103-2-star_st	2.9	mmu-miR-34a1_st	1.8
mmu-miR-103_st	-1.6	mmu-miR-34aa-star_st	9.2
mmu-miR-106b-star_st	-1.6	mmu-miR-34ab-3p_st	2.5
mmu-miR-129-5p_st	-2.4	mmu-miR-384-5p_st	1.8
mmu-miR-1306-3p_st	-1.6	mmu-miR-3968_st	1.6
mmu-miR-132-star_st	-1.8	mmu-miR-3971_st	1.9
mmu-miR-132_st	-1.9	mmu-miR-598_st	1.6
mmu-miR-139-3p_st	-2.4	mmu-miR-615-3p_st	1.9
mmu-miR-139-5p_st	-1.8	mmu-miR-676_st	1.5
mmu-miR-149_st	-2.5	mmu-miR-708_st	2.1
mmu-miR-150-star_st	-3	mmu-miR-99a_st	2.8
mmu-miR-152_st	-10.6	mmu-let-7d-star_st	1.7
mmu-miR-181d_st	-1.9	mmu-let-7f_st	1.9
mmu-miR-182_st	-1.5	mmu-let-7g-star_st	1.5
mmu-miR-188-5p_st	-2.3	mmu-let-7g_st	2.1
mmu-miR-1896_st	-1.6	mmu-miR-10a_st	1.6
mmu-miR-1897-5p_st	1.5	mmu-miR-1186b_st	3.4
mmu-miR-18b_st	-3.4	mmu-miR-1195_st	1.8
mmu-miR-1906_st	-1.7	mmu-miR-1247-star_st	2
mmu-miR-1934-star_st	-2	mmu-miR-125b-5p_st	4
mmu-miR-1948_st	-2.2	mmu-miR-126-3p_st	2.5
mmu-miR-1949_st	-1.7	mmu-miR-142-5p_st	1.6
mmu-miR-1971_st	-1.6	mmu-miR-146a_st	2
mmu-miR-210-star_st	-1.9	mmu-miR-147_st	1.7
mmu-miR-210_st	-3.1	mmu-miR-151-3p_st	2.2
mmu-miR-211-star_st	-1.6	mmu-miR-151-5p_st	1.9

mmu-miR-212-3p_st	-2.7	mmu-miR-155_st	2.2
mmu-miR-212-5p_st	-3	mmu-miR-1839-5p_st	1.7
mmu-miR-23b-star_st	-1.8	mmu-miR-1843-5p_st	3.6
mmu-miR-27a-star_st	-1.5	mmu-miR-1903_st	3.2
mmu-miR-291b-5p_st	-2.3	mmu-miR-1907_st	2.3
mmu-miR-296-3p_st	-1.8	mmu-miR-191-star_st	1.6
mmu-miR-298_st	-1.9	mmu-miR-192_st	2.3
mmu-miR-301b_st	-3.8	mmu-miR-1935_st	2.3
mmu-miR-3060_st	-1.8	mmu-miR-1946a_st	2.6
mmu-miR-3079-5p_st	-3.4	mmu-miR-1946b_st	2
mmu-miR-3082-5p_st	-1.5	mmu-miR-194_st	1.8
mmu-miR-3087-star_st	-1.6	mmu-miR-195_st	2.8
mmu-miR-3090-star_st	-2.2	mmu-miR-1982-star_st	1.5
mmu-miR-3096-3p_st	-2.2	mmu-miR-19a_st	1.5
mmu-miR-3096-5p_st	-2.1	mmu-miR-200a_st	1.7
mmu-miR-3096b-3p_st	-1.7	mmu-miR-207_st	1.9
mmu-miR-3096b-5p_st	-2.2	mmu-miR-214-star_st	3.1
mmu-miR-31-star_st	-5.6	mmu-miR-21_st	1.6
mmu-miR-3102-star_st	-2.5	mmu-miR-221_st	2.4
mmu-miR-3110-star_st	-3	mmu-miR-222_st	3.6
mmu-miR-31_st	-3	mmu-miR-26b_st	2.9
mmu-miR-32-star_st	-1.6	mmu-miR-28c_st	2.2
mmu-miR-324-5p_st	-2.3	mmu-miR-297c_st	2.6
mmu-miR-326_st	-19.7	mmu-miR-29a_st	2.3
mmu-miR-330-star_st	-2	mmu-miR-29b-1-star_st	3.5
mmu-miR-34a6-star_st	-2.5	mmu-miR-29b-2-star_st	4.7
mmu-miR-351_st	-4.9	mmu-miR-29b_st	4.5
mmu-miR-363-3p_st	-1.7	mmu-miR-29c_st	9.1
mmu-miR-363-5p_st	-1.6	mmu-miR-3065_st	2.3
mmu-miR-3962_st	1.5	mmu-miR-3068_st	2.4
mmu-miR-421_st	-2.1	mmu-miR-3075_st	1.9
mmu-miR-425-star_st	-2	mmu-miR-3098-5p_st	1.7
mmu-miR-449a_st	-4.7	mmu-miR-30a_st	2.5
mmu-miR-467b-star_st	-1.6	mmu-miR-30b_st	1.6
mmu-miR-467f_st	-1.6	mmu-miR-30e_st	2.4
mmu-miR-483_st	-2	mmu-miR-3109-star_st	1.5
mmu-miR-491_st	-3.1	mmu-miR-320_st	1.7
mmu-miR-500-star_st	-1.7	mmu-miR-34a70a_st	2.3
mmu-miR-503_st	-4.2	mmu-miR-34a70b_st	1.7
mmu-miR-504-star_st	-1.8	mmu-miR-34aa_st	5.9
mmu-miR-5119_st	-2.2	mmu-miR-3572_st	4.2
mmu-miR-652-star_st	-1.9	mmu-miR-362-3p_st	2.6
mmu-miR-667-star_st	-1.6	mmu-miR-429_st	2.1

mmu-miR-671-5p_st	-2.1
mmu-miR-690_st	-1.6
mmu-miR-691_st	-1.6
mmu-miR-695_st	-1.5
mmu-miR-760-3p_st	-1.5
mmu-miR-92b-star_st	-1.6
mmu-miR-93_st	-1.5
mmu-miR-98_st	2.4
mmu-miR-451_st	1.8
mmu-miR-466b-3p_st	1.7
mmu-miR-466c-3p_st	2.2
mmu-miR-466d-3p_st	5.3
mmu-miR-466e-3p_st	1.7
mmu-miR-466g_st	1.7
mmu-miR-466p-3p_st	2.2
mmu-miR-467b_st	4.4
mmu-miR-467d-star_st	1.6
mmu-miR-467e_st	3.5
mmu-miR-467h_st	2.5
mmu-miR-484_st	2
mmu-miR-669h-3p_st	1.8
mmu-miR-669h-5p_st	1.8
mmu-miR-669k-star_st	1.7
mmu-miR-669l_st	1.6
mmu-miR-669o-5p_st	1.6
mmu-miR-674-star_st	1.8
mmu-miR-680_st	1.5
mmu-miR-704_st	1.9
mmu-miR-712_st	2.4
mmu-miR-7a-1-star_st	1.9
mmu-miR-7a_st	3.5
mmu-miR-92a-1-star_st	4.1
mmu-miR-99b-star_st	-3.4

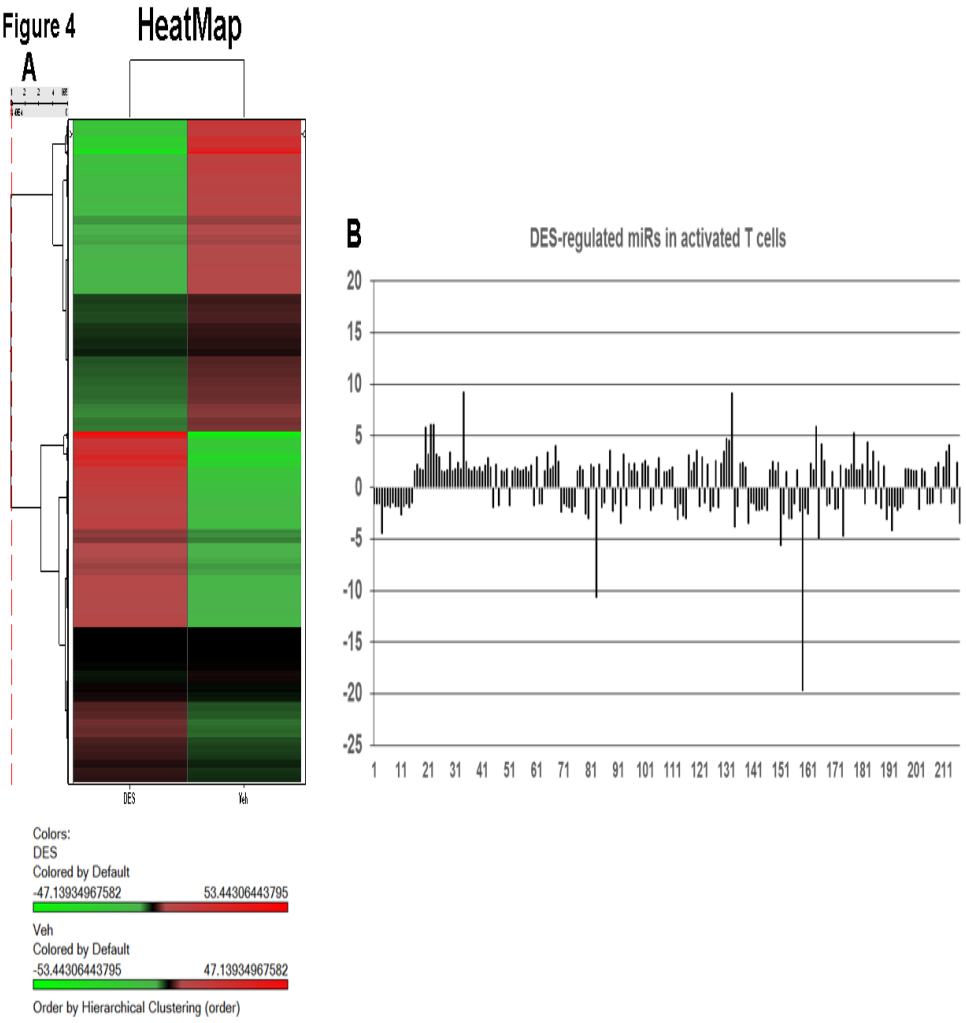

Figure 3.4 *Heat map of miR expression profile in activated T cells post DES treatment*. A. Heat map depicting miR expression profile in activated T cells post DES or vehicle treatment. The expression pattern (green to red) represents the spectrum of downregulated to upregulated expression of miRs. B. Depicts fold change expression profile of miRs post-DES exposure in comparison to vehicle.

Validation of miR Expression by Real-Time PCR. Based on the analysis of miRs array data, we chose three downregulated miRs (miR-18b, -23a, and -31) and one upregulated miR (miR-34a) to verify and validate their expression in activated T cells post DES or vehicle exposure (Fig 5). To this end, Real-Time PCR was performed on cDNAs converted from total RNAs including miRs from activated T cells treated with DES or vehicle as described in Materials and Methods. Data obtained from Real-Time PCR demonstrated significant downregulated expression of miR-18b, miR-23a, and miR-31 and upregulated expression of miR-34a in activated T cells treated with DES when compared to vehicle-treated T cells (Fig 3.5). The Real-Time PCR data validated the expression profile of selected miRs (miR-18b, -23a, -31, and 34a) obtained from miRs' array of activated T cells post DES or vehicle treatment.

DES upregulates FoxP3 expression but downregulates IL-17 and SIRT1 expression. Post validation of several miR expression in DES-treated activated T cells, we next determined the expression of FoxP3, IL-17, and SIRT1 in DES-treated activated T cells by performing RT-PCR. There was significantly upregulated expression of FoxP3 in activated T cells post DES treatment, when compared to vehicle-treated T cells (Fig 3.6 A-B). Contrary to FoxP3, DES significantly downregulated the expression of IL-17 and SIRT1 in activated T cells, when compared to vehicle-treated T cells (Fig 3.6 A-B). Data obtained from these studies clearly demonstrated that DES affects regulation of FoxP3, IL-17, and SIRT1 possibly by regulating expression of various miRs in T cells.

Figure 5

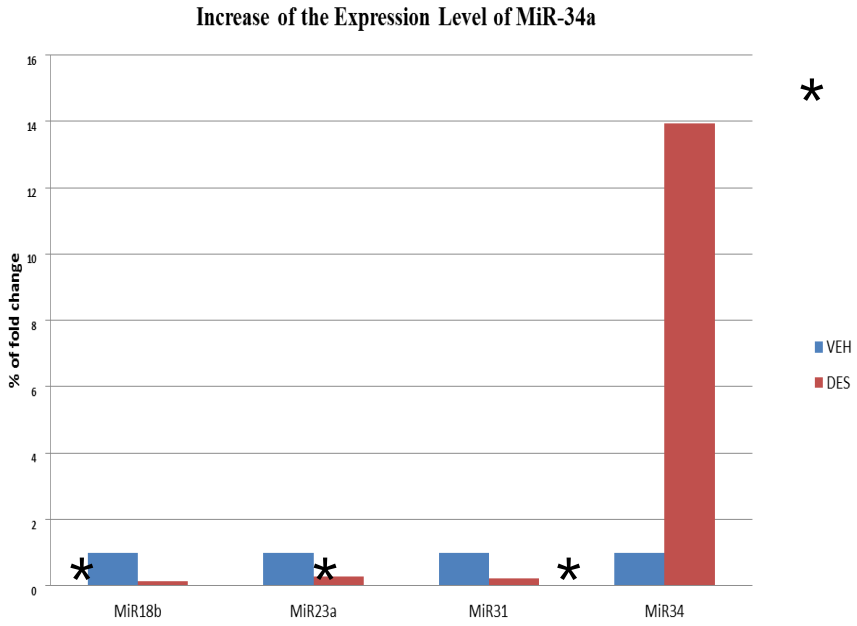

Figure 3.5 *Validation of expression profile of selected miRs in activated T cells post exposure to DES.* A, Expression profile of selected miRs (miR-18b, -23a, -31, and -34a) in activated T cells was determined using miR-specific primers and by performing Real-Time PCR. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) indicates statistically significant ($p<0.05$) difference between groups compared

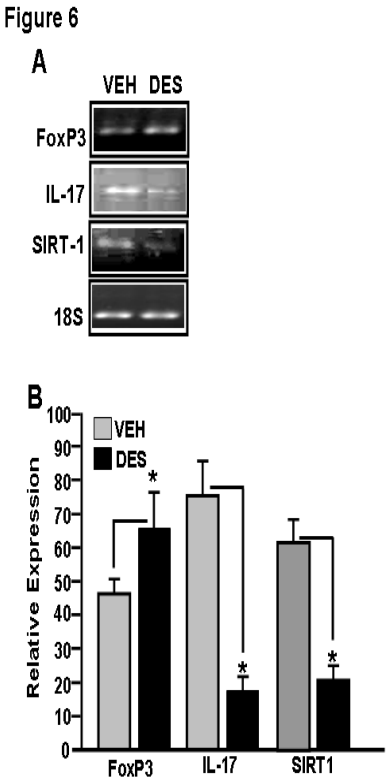

Figure 3.6 *Expression of FoxP3, IL-17, and SIRT1 in activated T cells post DES treatment*. A. Activated T cells exposed to DES or vehicle was analyzed for the expression of FoxP3, IL-17, and SIRT1 by performing RT-PCR. In panel B, RT-PCR data are presented as percentage of 18S expression with the latter being considered as 100%. Data are depicted as mean ± SEM of three independent experiments. Asterisk (*) in panel B indicates statistically significant ($p<0.05$) difference between groups compared.

DES-regulated miRs play important role in various diseases. Post analysis of miR expression in DES-treated activated T cells and comparison with vehicle-treated activated T cells, miRs expressing more than 1.5 fold (upregulated or downregulated) were selected for further analysis. To this end, 217 miRs were analyzed using IPA software and database of the company (Ingenuity Inc). Although, there were as many as 30 pathways that might be affected by DES-regulated miRs, there were at least 12-15 dominant pathways that might be affected by various miRs regulated by DES in activated T cells. Upon further analysis of DES-regulated miRs (>2 fold upregulated or downregulated expression) using Ingenuity IPA software, the following pathways were more prominent. The important pathways were inflammatory pathway (Fig 3.7A) and cancer pathway (Fig 3.7B). Upon analysis of miRs and associated genes using Cytoscape analysis software, there were at least 17 miRs that may regulate more than 100 genes (Fig 3.7C). Furthermore, we also observed DES-regulated miRs in activated T cells may regulate various pathways including immunological pathways (Fig 3.7D). Thus, DES-regulated miR (upregulated or downregulated) profile in activated T cells demonstrated that DES may affect a large number of genes that participate in various clinical disorders including cancer and inflammation.

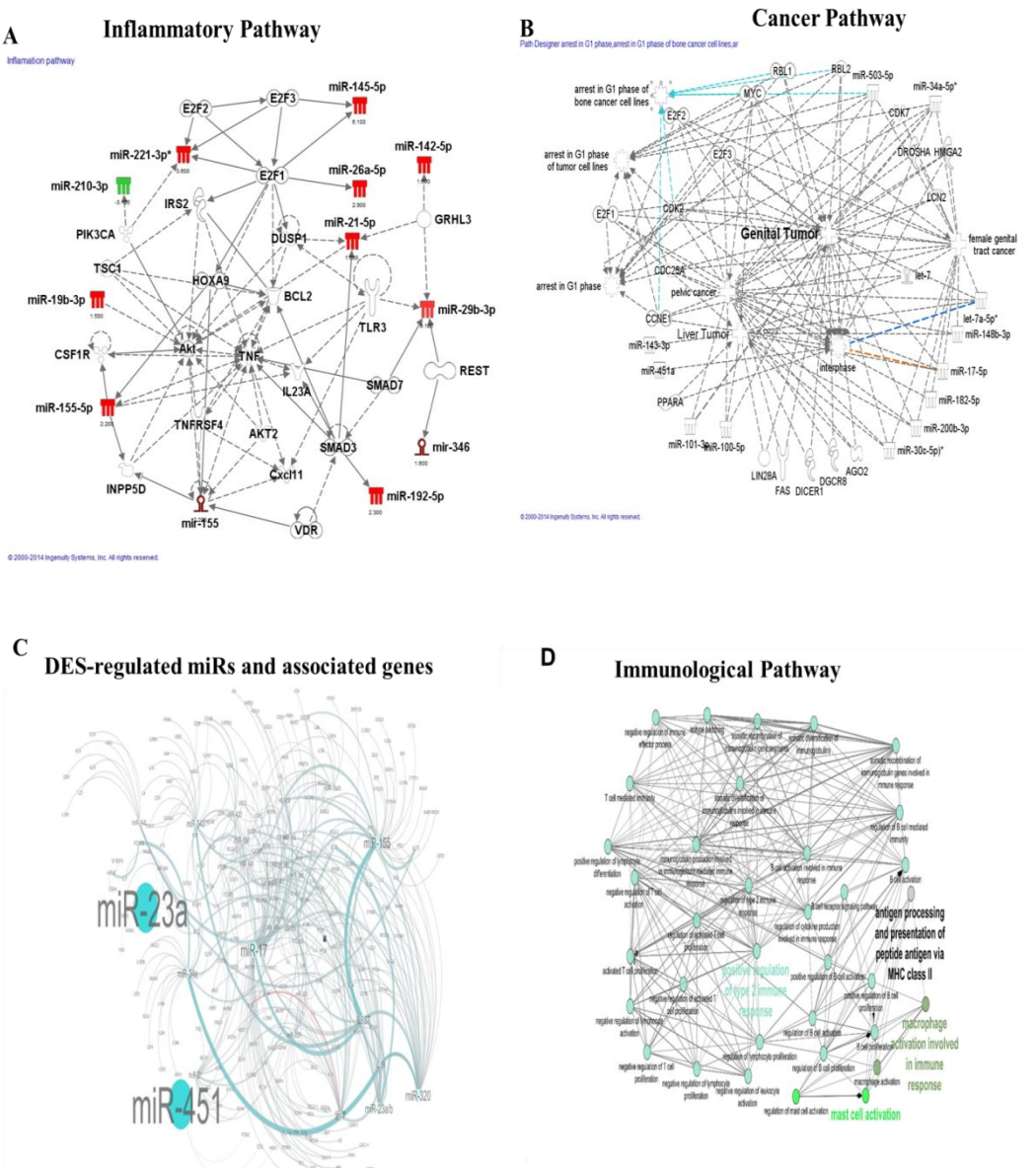

Figure 3.7 *DES-regulated miRs and their association with various pathways.* DES-regulated miRs as described in Fig 4 were analyzed using IPA software and the database (Ingenuity Ssytems, Inc). A. miRs involved in inflammatory pathway and B. miRs involved in cancer pathway. Red icons represent upregulated mature miRs and green icons represent downregulated mature miRs involved in the pathways. C. represents relationship amongst various miRs and genes post Cytoscape analysis. D. represents immunological pathways post cytoscape analysis of miRs and genes.

DES suppresses mBSA-induced DTH in mice. DTH was induced using mBSA as described in materials and methods. Throughout the study, we used 5 groups of mice; CONTROL (No treatment), mBSA+VEH, mBSA+DES/D0, mBSA+DES/D6, and mBSA+DES/D0+D6. Second challenge with mBSA in footpad six days post first mBSA immunization induced severe DTH in C57BL/6 mice, as shown by significant increase in footpad thickness (Fig 3.8A-B). There was a significant increase in IL-17 (Fig 3.8C) but decrease in IL-10 production (Fig 3.8D) in mBSA immunized mice. Interestingly, DES treatment decreased the DTH response and reversed the inflammation triggered by mBSA (Fig 3.8A-C). Also, there was significant increase in IL-10 (Fig 3.8D) cytokine in the sera of mice that received mBSA +DES treatment when compared to mBSA+VEH treatment. It is interesting to note that treatment with DES, also caused a small but significant induction of IL-10 when compared to vehicle.

DES triggers reciprocal differentiation of Tregs and Th17 cells during mBSA-mediated DTH in mice. Next, we examined the impact of treatment with DES on Tregs or Th17 cells in mBSA-induced DTH in mice. Upon analysis of Tregs ($CD4^+/FoxP3^+$) and Th17 ($CD4^+/IL\text{-}17^+$) cells in spleen post DES-treatment, we noted a significant increase in Tregs in the spleen in mBSA+DES groups of mice, when compared to mBSA+VEH group of mice (Fig 3.9A). In contrast, mBSA treatment caused a significant increase in Th17 cells and treatment with DES caused significant decrease in Th17 cells (Fig 3.9B). When Th1 ($CD4^+/IFN\text{-}\gamma^+$) and Th2 ($CD4^+/IL\text{-}4^+$) cells were analyzed, we observed significant increase in Th1 cells but decrease in Th2 cells in mice that were immunized with mBSA and received VEH treatment (Fig 3.9C-D). However, treatment with DES decreased Th1 ($CD4^+/IFN\text{-}\gamma^+$) cell generation (Fig 3.9C) but increased Th2 ($CD4^+/IL\text{-}4^+$) cells (Fig 3.9D).

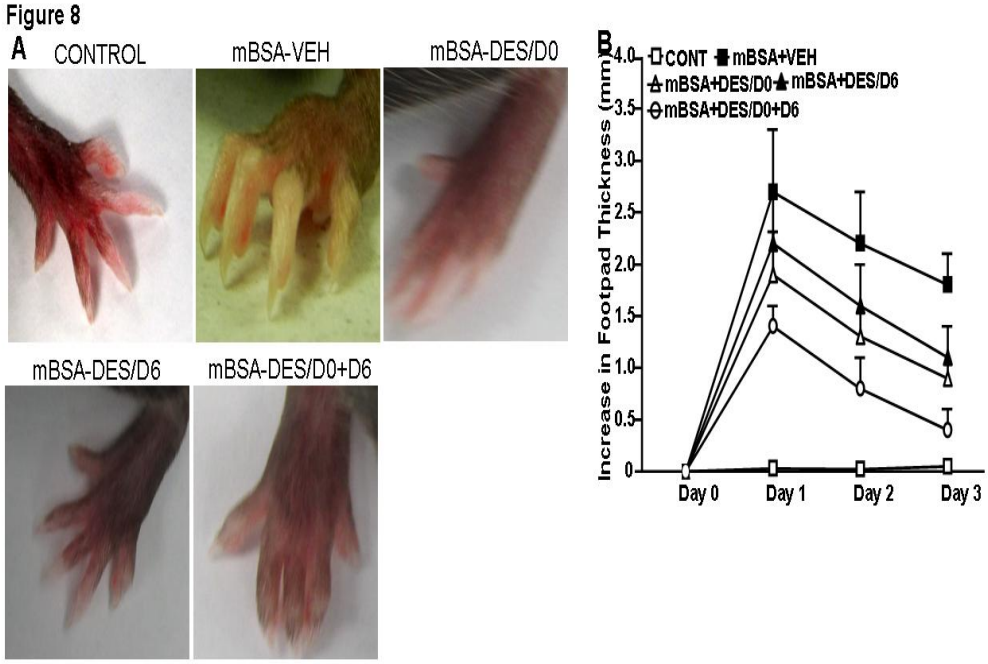

Figure 3.8 *DES attenuates mBSA-induced DTH in mice.* C57BL/6 mice were immunized subcutaneously with mBSA emulsified in CFA. Six days later, the mice were rechallenged with mBSA in footpads. We used 5 groups of C57BL/6 mice: those that received no treatment (Control), mBSA+VEH, mBSA+DES/D0, mBSA+DES/D6 and mBSA+DES/D0+D6. A. Footpads of each group of mice were shown. B. The thickness of each footpad of all groups of mice were measured daily with an engineer's calipers for 3 days (72 hrs). Data represents the mean of three experiments involving 5 mice per group. There was significant difference in footpad swelling between control versus mBSA+VEH, mBSA+VEH versus mBSA+DES/D0, mBSA+VEH versus mBSA+DES/D6, as well as mBSA+VEH versus mBSA+DES/D0+D6 groups. Asterisks indicate statistically significant differences ($p < 0.05$). The statistically significant difference between each group were assessed by using Mann-Whitney U Test.

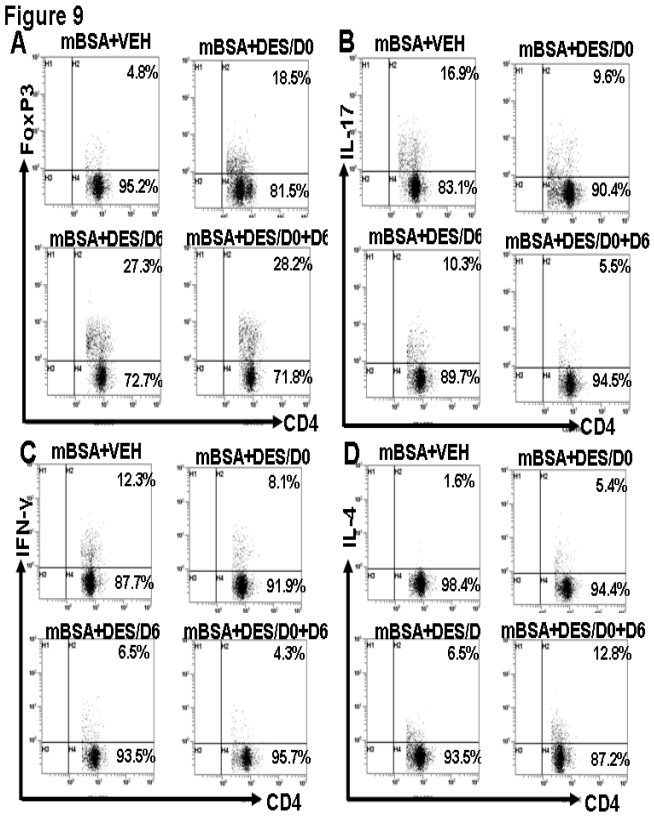

Figure 3.9 *DES regulates differentiation of Th subsets (Th1, Th2, Th17, and Tregs) in spleens during mBSA-induced DTH in mice*. Spleen cells were isolated post-secondary challenge with mBSA and various treatments as described in Fig. 8. Cells were stained for Tregs (CD4 + FoxP3), Th1 (CD4 + IFN-γ), Th17 (CD4 + IL17), and Th2 (CD4 + IL-4) and analyzed by flow cytometry. A representative staining for Tregs (CD4+/FoxP3+; panel A), Th17 (CD4+/IL-17+; panel B), Th1 (CD4+/IFN-γ+; panel C), and Th2 (CD4+/IL-4+; panel D) cells are shown from three independent experiments.

CHAPTER 4: DISCUSSION

DES is an endocrine disruptor (1-4) and has been shown to increase the risk for breast cancer in "DES mothers" and a life time risk of cervicovaginal cancers in "DES daughters" (10). It has also been linked to cause a wide range of abnormalities including immune system disorders such as immune suppression, increased incidence of autoimmunity, cancer, and certain infections in DES son or daughter (10). Previous studies from our laboratory have shown DES induced thymic atrophy, decreased thymic cellularity, and apoptosis in thymic cells in mice (14, 15). In another study from our laboratory, Singh et al have shown DES caused increased expression of Fas and FasL in fetal thymic cells and T cells and the role of transcription factors and cis-regulating elements on the promoter of Fas and FasL (45). Frawley et al (2011) have also recently reported gene expression alterations in immune system pathways in the thymus after exposure to immunosuppressive chemicals (31).

While most of the effect of DES has been investigated on thymus and mechanisms involved in thymic atrophy, there is lack of knowledge regarding the effect of DES on peripheral lymphoid organs (spleen and lymph node) and peripheral T cells. In the present study, therefore, we investigated the effect of DES on primary T cells and examined the molecular mechanisms involved.

Furthermore, we examined whether DES regulates mechanisms that affect miRs profile in activated T cells. Also, we examined DES-regulated mechanisms causing immunosuppression and examined whether DES-regulated immunosuppression can be exploited against inflammatory responses. In this context, we examined DES-mediated apoptosis in activated T cells, expression of Fas and FasL, and differentiation of T cells in the presence of DES *in vitro*. We also examined miRs profile in activated T cells post exposure to DES by performing miRs arrays and investigated relationship of miRs with their target genes (Fas, FasL, FoxP3, I-17, and SIRT1) in activated T cells post-DES exposure. Furthermore, we tested immunosuppressive properties of DES on a mouse model of DTH.

DES causes long-term effects in adults (5, 7-9) and the long-term effects of DES may be due to several mechanisms such as T cell differentiation in thymus, thymic atrophy, effects on peripheral T cells and reproductive organs etc. leading to several health-associated problems including immune suppression, immune dysfunction, development of various types of cancer including breast cancer, ovarian cancer, uterus cancer, defects in reproductive functions etc. However, DES may be a useful drug for short term treatment of inflammatory diseases. The results obtained from the current study showed that DES induces apoptosis in both unactivated and activated T cells in dose-dependent manner (Fig 3.1A-B). Upon analysis of FasL expression in activated T cells, there was significant increase in FasL expression following exposure to DES, when compared activated T cells exposed to VEH (Fig 3.2A-B).

Furthermore, upon analysis of differentiation of T cells in the presence of DES *in vitro*, there was significant increase in the number of FoxP3 expressing regulatory T cells (Fig 3A) but significant suppression of IFN-γ expressing Th1 cells (Fig 3B), IL-17 expressing Th17 cells (Fig 3.3C), and IL-4 expressing Th2 cells (Fig 3D) *in vitro*, when compared to vehicle (Fig 3.3A-D). The data from these studies demonstrated that DES adversely affects T cells by causing apoptosis and upregulating Fas and FasL expression. It should be noted that we made similar observations using immature thymic T cells (45). Furthermore, our studies 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 (14). Our previous studies also demonstrated that DES regulated Fas and FasL expression through their respective promoters (45). In another study, Nair et al reported DES-induced apoptosis in spermatogenic cells through increased expression of Fas and FasL in rats (30). They also showed that DES-mediated apoptosis did not occur in Fas deficient lpr mice (30). In the present study, we also demonstrated that DES regulates differentiation T cells *in vitro*. Upon examination of DES effects on differentiation of T cells *in vitro*, we noted a significant increase in FoxP3+ Tregs (Fig 3.3A) but significant decrease in IFN-γ+ Th1 cells (Fig 3.3C) and IL4+ Th2 cells (Fig 3.3D) *in vitro*. There was no or minimal change in the expression of IL17+ Th17 cells, when compared to vehicle *in vitro* (Fig 3.3B). Together, these studies suggested that exposure to DES severely affects peripheral T cells and may have a long lasting impact on the immune functions and immune suppression causing various health-associated problems and ailments.

DES-mediated regulation of genes has been shown to be an important mechanism influencing physiological and biological functions (23, 24). In recent years, miRs have been focus of several studies due to their direct role in gene regulation. It is possible that miRs might modulate mRNA levels of various genes, therefore, the effects by DES on miRs need to be fully explored. There is very little information in literature regarding DES-mediated regulation of miRs in thymic T cells. One study demonstrated altered expression of 82 miRs in breast epithelial cells when exposed to DES (51). In another study, it was reported that DES regulates miR-34ab, an onco-suppressor miR present in breast cancer cells and proposed new therapeutic strategies to target the complex estrogenic pathway in human breast cancer progression through miR regulation (52). In another study, PY et al reported DES-mediated epigenetic regression of miR-9-3 in breast epithelial cells and proposed that hypermethylation of this miR may be hallmark of early breast cancer development and can be exploited for future treatment of breast cancer (51). However, there is almost no information on DES-regulated miRs in peripheral T cells. Therefore, this study focused on examining the effect of DES on miRs expression profile in peripheral T cells.

MicroRNA's expression profile by cluster analysis showed clustering of a large number of miRs that are differentially expressed in activated T cells upon exposure to DES *in vitro*. We identified several miRs that were either upregulated or downregulated in the presence of DES. Of the 885 miRs analyzed, there were more than 217 miRs showing more than 1.5 fold and 101 miRs showing 2.0 or more than 2.0 fold differential expressions in DES group when compared to vehicle-treated group (Fig 3.4B).

There were 116 (61 upregulated and 55 downregulated) miRs and their expression varied between 1.5-1.9 folds, whereas there were 101 (58 upregulated and 43 downregulated) miRs, whose expression was altered 2 or more than 2 fold (Fig 3.4B). Expression of miRs, 1.5-fold or higher, was considered positive and so for all further analyses and characterization of miRs, differential expression of 1.5 or more was used as significant. The expression profile of some of the miRs (miR-18b, -23a, -31, and -34) were validated by performing Real-Time PCR. All the miRs that we analyzed by Real-Time PCR confirmed and corroborated with the miRs expression profile data obtained from miR arrays analysis. These validated miRs showed similar patterns of downregulation or upregulation in activated T cells post-DES exposure *in vitro*. Furthermore, the relationship of miRs and their target gene expression was also verified. We selected miR-18b, miR-23a, miR-31, and miR-34a to verify their relationship with their specific target gene and their expression. The reason for choosing miR-18b, miR-23a, miR-31, and miR-34a was that miR-23a possesses binding affinity with mouse Fas UTR, miR-18b possesses binding affinity with mouse FasL UTR, miR-31 possesses binding affinity with mouse FoxP3 UTR, and miR-34a possesses binding affinity with mouse SIRT1 UTR.

In this study, we observed that miR-23a and miR-18b were downregulated (Fig 3.5), in activated T cells post DES exposure when compared to vehicle. Previous studies from our laboratory have shown DES-mediated upregulation of Fas and FasL expression in thymic cells of pregnant mothers (14).

Upon examination of miRs (miR-23a and miR-18b) expression, both miRs showed downregulated expression in activated T cells in the presence of DES, when compared vehicle-treated activated T cells (Fig 3.5). Examination of FasL expression by RT-PCR showed upregulated expression of FasL (Fig 3.2A-B) in activated T cells upon exposure to DES, when compared to vehicle *in vitro*. These two miRs have also been shown to regulate genes that are involved in various physiological functions in various tissues (53). DES has been shown to cause toxicity in a wide range of tissues or organs; such as reproductive organ, breast tissue etc (11, 14, 21). MiR-23a possesses binding affinity with Fas 3'UTR region (Table 3.1) whereas, miR-18b possess binding affinity with FasL 3'UTR region (Table 3.1). 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 (45, 46, 54, 55). We have also reported that such apoptosis may be induced through the extrinsic pathway through the induction of Fas and FasL in thymocytes (47, 55). Thus, these two miRs may directly/indirectly be involved in apoptosis of T cells leading to immune suppression.

In this study, we also observed that miR-31 was downregulated (Fig 3.5) in activated T cells post DES exposure, when compared to vehicle. Upon analysis of miR-31 by microRNA.org and TargeScanMouse 5.1 database, miR-31 showed binding affinity with 3'UTR region of Foxp3 gene. Foxp3 is known to be expressed predominantly in regulatory T cells (56, 57). Regulatory T cells have well been characterized for their immunosuppressive functions (58-60). There are studies demonstrating role of miR-31 in the regulation of FoxP3 gene expression (61, 62).

The data obtained from this study demonstrated downregulation of miR-31 (Fig 3.5) and upregulated expression of FoxP3 (Fig 3.6A-B) in activated T cells in the presence of DES, when compared to vehicle. These data suggested that DES may promote differentiation of Tregs via miR-31-mediated expression of FoxP3 in T cells.

Upon analysis of miR-34a, we observed significant upregulation of miR-34 in activated T cells, when compared to vehicle-treated activated T cells *in vitro*. The computational analysis of miR-34a by microRNA.org and Target ScanMouse 5.1 showed binding affinity with 3'UTR of SIRT1, Notch ligand JAG-1, and large number of zinc finger (ZNFs) genes respectively. Expression of miR-34a has been shown to be regulated by p53, which mediates cell-cycle arrest and promotes apoptosis [17, 38]. Also, overexpression of miR34 has been shown to decrease the expression of a number of cell cycle regulatory proteins including cyclin D1, c-MET and CDK4 [15, 17, 23], and thus hampering cell cycle progression [15, 16]. MiR-34ab has been reported to act as a tumor suppressor in colorectal cancer [18], prostate cancer [25], and gastric cancer [24]. In another recent study, Lee et al have shown the role of miR-34ab in expression of ER and ER-mediated growth of breast cancer cells *in vitro* (63). They have demonstrated that the induction of miR-34ab significantly decreased cell proliferation rate *in vitro* and greatly lowered tumor growth in an orthotropic breast cancer model *in vivo*.

Although, there is no report of DES-mediated SIRT1 expression in T cells, miR-34a-regulated suppression of SIRT1 has been shown to cause apoptosis in cancer cells (64). There are also studies demonstrating miR-34aa-mediated downregulation in SIRT1 expression leads to upregulation of p53 gene in cancer cells (64, 65). Although, not much is known about DES-mediated expression of SIRT1 and p53in T cells, DES has been shown to cause immune suppression and immune dysfunction. Data obtained from this study demonstrate a possible role of DES in causing apoptosis in activated T cells via miR-34a. Taken together, these data demonstrated that a large number of miRs that are downregulated or upregulated in activated T cells by DES may control the expression of genes involved in various mechanisms and functions like apoptosis in T cells, differentiation of T cells, immune suppression, toxicity, cancer etc.

Upon examination of DES effect against mBSA-generated DTH in mice, we noted a significant decrease in DTH response in mice that received DES treatments, when compared to mice that received vehicle treatment (Fig 3.8A-B). Because mBSA causes DTH in mice by generating primarily Th17 cells (66), the effects of DES in suppression of DTH is likely caused by suppression of Th17 cells and generation of immunosuppressive Tregs as shown in data obtained from *in vitro* experiments. We also noted that there was significant generation of Tregs (Fig 3.9A) but suppression of Th17 (Fig 3.9B), Th1 (Fig 3.9C), and Th2 cells (Fig 3.9D) in mice with DTH that received DES treatment, when compared to mice with DTH but received vehicle treatment. These data correlated with significantly more induction of Tregs (Fig 3.9A) and less induction of IL-17 (Fig 3.2A), IFN-γ (Fig 2B), IL-4 (Fig 3.9D).

These data clearly demonstrated that DES preferentially generated more Tregs and suppressed generation of Th17 and Th1 cells in mice. Expression of FoxP3 has been shown to be specific to Tregs (56, 67), whereas expression of IL-17 has been shown to be associated with Th17 cells (68). Together, these data demonstrated that DES preferentially promotes differentiation of Tregs but suppress generation of Th17 and Th1 cells and thereby suppressing mBSA-mediated DTH in mice.

In summary, we demonstrate for the first time that exposure to DES can have a significant effect on peripheral T cells via regulating the miR profile of T cells and thereby influencing the regulation of genes that may cause immune suppression and affect immune functions. Identification of miRs as targets for DES-induced modulation of gene expression offers novel pathways to understand the DES-regulated immunosuppressive mechanisms and its long-term effects.

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