Low 17β-estradiol Levels Are Better Inducers of Regulatory Conditioned T Cells *In-vitro*

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ABSTRACT

Background: 17β-estradiol (E2) has been known to modulate immune response. Recent studies indicate that E2 at pregnancy level plays a role in regulating T cell response. **Objective:** To investigate the optimum dose of E2 (from 10^{-9} to 10^{-7} M) in mediating the generation of regulatory T cells (Tregs), using naïve human $CD4^+$ T cells from healthy women. **Methods:** Naïve peripheral T cells were purified and conditioned with soluble anti-CD28 in anti-CD3-coated plates in the presence or absence of E2. Flow cytometry was employed to assess the expression pattern of forkhead boxP3 (FOXP3) and programmed death-1 (PD-1). Proliferation and cytokine secretions were analyzed, using XTT and ELISA assays. **Results:** In the presence of different doses of E2, the expression levels of anti-CD3/CD28 antibody-stimulated CD25/FOXP3 and FOXP3/PD-1 in conditioned T cells (cT) were peaked at 1 ng/ml (early pregnancy level, $E2_{(1)}$) (47.14% (37.3-74.9) and 32% (27.7-52.5), respectively) and a slight, but not significant, increase after declining at 36 ng/ml (late pregnancy/pharmaceutical, $E2_{(36)}$) (19.4% (15.2-24.5) and 15.8% (10.6-26.8), respectively). $E2_{(1)}$ cT showed a significantly reduced proliferation capacity ($p<0.05$) and secretion of IL-10 was enhanced in supernatants of $E2_{(1 \text{ and } 36)}$ cT ($p<0.05$). In contrast to decreased TNF-α and IFN-γ secretions in $E2_{(1)}$ cT supernatants, $E2_{(36)}$ stimulated TNF-α and IFN-γ secretions ($p<0.05$ and $p<0.01$, respectively). **Conclusion:** Our results indicate that the differential effect of E2 on generation of Tregs is consistent with the possibility that lower levels of pregnancy E2 are most efficient in induction of Tregs.

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Key words: Cytokines, 17β-estradiol, Proliferation, Peripheral blood mononuclear cell, Regulatory T cells

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INTRODUCTION

Effects of 17β-estradiol (E2) on cells of the immune system may modulate their cytokine secretion patterns (1) and with the potential to influence on the outcome of CD4+T cell-mediated immune responses (2). E2 at pregnancy levels increase interleukin (IL)-4 and IL-10 secretions, and in contrast decrease Tumor necrosis factor (TNF)-α secretion.

During first trimester of pregnancy, T cells consist of 10% of immune cells localized in deciduas (3) and represent an activated /memory phenotype (4). Decidual T cells secrete more in IL-4 and less interferon (IFN)- γ compared to blood T cells (5). Studies on immune modulation during human pregnancy have reported a decrease in IFN-γ and IL-2 secretion levels and an increase in IL-4 secretion from stimulated normal human pregnancy peripheral blood mononuclear cells (PBMC) (6,7). However, others have reported that Th1/Th2 responses change equivalently without any one-sided superiority (8,9) during normal pregnancy. It has also been reported that Th2 cytokines IL-4 and IL-10 are not critical for the allogeneic pregnancy in mice (10), which could suggest that Th2 arm of adaptive immunity might not be the crucial factor in successful pregnancy (11). Moreover, a decreased number of CD4+ T cells in the second and third trimesters (12) and of CD8+ T cells and NK cells in late pregnancy (12,13), may suggest that systemic activity of the maternal immune system is dampened during pregnancy.

Recent reports indicated that in decidua, which is in direct contact with paternal antigen, around 14% of CD4+ T cells are phenotypically CD4+CD25+ expressing large amounts of intracellular Cytotoxic T-lymphocyte-associated antigen (CTLA)-4 (14). The high frequency of Treg in human decidua suggests their crucial effect on alloreactive immune responses against the implanted fetus at the feto-maternal interface.

However, regulatory T cells (Tregs) are addressed to be important players in maintaining maternal tolerance towards the alloantigen-bearing fetus (15-19). Arruvito *et al.* showed expansions of Tregs in fertile, non-pregnant women in the late follicular phase, which was associated with serum levels of E2, while this increase in Treg numbers was followed by a decrease during luteal phase when E2 levels dropped down (20). The effect of E2 on regulatory elements that influence on Treg suppression involves programmed death-1 (PD-1) molecule (21). Interactions between PD-1, known as a negative co-stimulatory molecule in T cell activation, and its ligands PD-L1 and PD-L2 are crucial in the regulation of immune tolerance and has been shown to have a function in Treg-induced fetal protection in abortion prone mouse models (22). Furthermore, the potential of pregnancy levels of E2 in promoting expansion of CD4+CD25+ regulatory T cells has been discussed in several studies (23,24). A study by Xiang *et al.* reported that Treg levels altered with changes in estrogen levels during pregnancy and the proportions of Tregs in middle and late pregnancy were significantly higher than that in early pregnancy (25), whereas in other studies, the accumulation of functionally active Tregs in deciduas of the 1st trimester pregnant women (26,27) was followed by a reduction in the number of Tregs in the 2nd trimester of pregnancy (28). In addition, the impact of fetal alloantigen on the frequencies of Tregs during pregnancy is highlighted in several studies (29). In the present study, we investigated the effects of E2 on induction of Tregs using an *in vitro* culture system. E2 was tested at concentrations ranging from 0.4 ng/ml to 54 ng/ml (from 10-9 to 10-7 M; covering preovulatory to pregnancy and pharmaceutical doses of E2). This study highlights the *in*

vitro functional activity of, early pregnancy level, E2-conditioned T cells in human female subjects.

MATERIALS AND METHODS

Subjects. Healthy non-pregnant female volunteers (n=19) with no history of autoimmune diseases were asked to complete a questionnaire regarding their menstrual status and reproductive history. Women with regular menstrual cycles were included in the study and those taking hormone-containing medications were excluded. The mean age of the participants (n=8) was 28.4 ± 1.1 years (ranging from 25 to 32). Peripheral blood samples were drawn on the first few days of their menstrual cycles, when the blood level of estrogen is known to be the lowest as reported by Zittermann *et al.* (30) and our own observations. The study was approved by the local ethical committee of Avicenna Research Institute, Tehran, Iran and all subjects signed an informed consent to participate in the study.

Reagents. Mouse anti-human CD3 (Clone OKT3) and CD28 (Clone CD28.2) monoclonal antibodies (mAbs) (Biolegend, Munro House, UK) were used in cell stimulation assays. The following mAbs were purchased from e-Bioscience (San Diego, CA, USA) and used for flow cytometric analyses; fluorescein isothiocyanate (FITC)-anti human CD4 (Clone RPA-T48), phycoerythrin (PE)-anti-human CD25 (clone BC96), phycoerythrin-cyanin-5 (PE-Cy5)- anti-human Foxp3 (Clone PCH101), isotype-matched mAbs; FITC mouse IgG1 (Clone Mopc-21), PE mouse IgG2a (Clone Mopc-173) and PE-CY5 rat IgG2a (Clone eBR2a). FITC-anti human PD-1 (Clone EH12.2H7) was purchased from Bio legend.

Naive T cell isolation. PBMC were isolated from 25 ml of heparinized blood using Ficoll-Hypaque (Sigma Aldrich, Seelze, Germany) density gradient centrifugation. CD4+CD25- naïve T cells were then negatively selected using magnetic-activated cell sorting (MACS) kit (Miltenyi biotech, Teterow, Germany) according to the manufacturer's instructions. Briefly, PBMC were incubated with a cocktail of biotinylated antibodies against CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, CD235a, TCR γ/δ and HLA-DR to deplete memory $CD4^+$ T Cells and non-CD4 T Cells. Then the cells were magnetically labeled with anti-biotin microbeads and passed through LS columns (Miltenyi biotech). Cells in the flow through were collected as intact naïve T cells. The purity of isolated cells was evaluated by flow cytomtry using monoclonal antibodies against human CD4, CD25 and FOXP3.

E2 treatment of naïve T cells. Results of laboratory exams showed that the serum E2 levels of participants in the study before drawing blood was 31.3 ± 3.1 pg/ml. To determine the effect of E2 (Sigma Aldrich) on expression of FOXP3, representative E2 concentrations ranging from those found in the peripheral circulation during normal menstrual cycle to those present during pregnancy and pharmaceutical doses (1.5×10^{-9} – 2×10^{-7}M), including; 0.4 ($E2_{(0.4)}$), 1 ($E2_{(1)}$), 4 ($E2_{(4)}$), 7 ($E2_{(7)}$), 15 ($E2_{(15)}$), 30 ($E2_{(30)}$),36 ($E2_{(36)}$) and 54 ($E2_{(54)}$) ng/ml were used in our experiments.

Purified naïve T cells ($CD4^+CD25^-$) were cultured in anti-CD3-coated (5 µg/ml) 24 well plates (5×10^5 cells/well) containing complete DMEM medium (Sigma Aldrich) (supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum) and treated with soluble anti-CD28 (1 µg/ml) and different concentrations of E2 (dissolved in aceton). The same batch of FBS was used with no

significant concentration of E2 as determined by enzyme immunoassay (EIA) (Axis Shield, Dundee, UK). Naive T cells cultured in medium alone were referred to as non-stimulated cells. Cells that were stimulated with anti-CD3/CD28 and received acetone served as $E2_{(0)}$. Cultures were incubated in CO2 incubator at 37˚C for 96 h. All cells except those in the non-stimulated group were referred to as "conditioned T cells (cT)" throughout the study.

Flow cytometeric analysis. Freshly isolated naïve T cells or cT were stained with anti-CD4-FITC and anti-CD25-PE or their isotype control antibodies for 30 min at 4˚C in the dark. Cells were then washed twice with phosphate buffered saline (PBS), fixed in 0.5% paraformaldehyde and stained for intracellular expression of FOXP3, using FOXP3 staining protocol (ebioscience) (URL: http://www.ebioscience.com/foxp3-staining-buffer-set.htm). Intracellular co-staining of cells with FOXP3-PE-Cy5 and PD-1-FITC was done using the same protocol. Naïve or cultured cells were gated on lymphocytes, identified by forward- and side scatter parameters.

To determine levels of apoptosis, cells were stained with Annexin V-FITC and Propidium Iodide (PI), using a staining kit according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, USA). Cells were gated to determine cell death within the population. Stained cells were assessed by Partec PAS flow cytometer (Partec, Nuremberg, Germany) and data was analyzed by FloMax analysis software program (Partec).

XTT Proliferation Assay. After the initial conditioning for 96 h (see above), T cells were washed twice with PBS and cultured for an additional 96 h in a 5% CO2 incubator at 37˚C. Cell proliferation was investigated using a commercially available proliferation kit (XTT, ATCC, Manassas, VA, USA). Briefly, all groups of T cells were separately cultured (2×10^5/well in a final volume of 200 µl) in medium without phenol red (Sigma Aldrich) supplemented with 10% FBS, L-glutamine,penicillin /streptomycin complete medium in 96-well flat-bottom plates. At the end of the incubation, 100 µl of the medium was collected for cytokine analysis and 50 µl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent and 50 µl of electron coupling reagent, was then added to each well. After 4 h of incubation in a 37°C and 5% CO2 incubator, the absorbance was measured on an ELISA reader (Molecular Devices, CA, USA) at a test wavelength of 450nm. All experiments were performed in triplicates and mean values were determined.

Cytokine measurement. ELISA assays for IL-10 (eBiosince), TNF-α (BD) and IFN-γ (eBiosince) were performed in duplicates according to the manufacturer's instructions. The minimum detection levels for each cytokine were: IL-10, 2.0 ρg/mL; IFN-γ, 4.0 ρg/mL and TNF-α, 4.0 ρg/mL. Optical densities were read in a micro plate reader at 450 nm. Standard curves were generated for each cytokine using the cytokine standards supplied with each cytokine measurement kit and the amounts of cytokines were calculated accordingly.

Statistical analyses. Statistical comparisons between groups were determined by non-parametric Kruskal-Wallis and if the test was statistically significant, the Dunn's multiple comparisons or Mann-Whitney U test was applied to perform post hoc pair wise comparisons of Treg markers, cytokine expression and analysis of proliferation. P values< 0.05 were considered as statistically significant. Statistical analyses were done using the graphpad prism 5 software program (Graphpad Software. San Diego, CA).

RESULTS

$E2_{(1)}$ was the most effective dose in generation of $CD4^+CD25^+FOXP3^+$Tregs.

In order to test the impact of E2 on induction of Tregs, naïve T cells were first isolated from PBMC. Using flow cytometry, the purity of magnetic bead-separated $CD4^+CD25^-$ naïve T cells was estimated to be 96.6% ± 0.85 (95-98.5 %).

We evaluated the frequencies of CD25 and FOXP3 expressions in anti-CD3/CD28 stimulated naïve T cells treated with different concentrations of E2 (10^{-9} to 10^{-7}M). Flow cytometric analysis revealed that 28% (17.1-37.2) (median (range)), 43.3% (32.6-54.3), 47.1% (37.3-74.9), 23.2% (16-23.2), 18.0% (15.2-19.4), 4.4% (1.9-6.7), 10.3% (7.2-14.1), 19.4% (15.2-24.5) and 14.4% (12-17.3), of cells within cT co-expressed CD25 and FOXP3 in the presence of $E2_{(0)}$, $E2_{(0.4)}$, $E2_{(1)}$, $E2_{(4)}$, $E2_{(7)}$, $E2_{(15)}$, $E2_{(30)}$, $E2_{(36)}$ and $E2_{(54)}$, respectively. However, only 3.3% (1.7-10.7) of non-stimulated T cells expressed these markers (Fig.1A). Co-expression of CD25 and FOXP3 was significantly higher in $E2_{(0.4)}$ and $E2_{(1)}$ cT than that in $E2_{(0)}$ cT cells ($p<0.01$ and $p<0.01$, respectively). The expression was significantly lower in non-stimulated and $E2_{(4, 7, 15, 30, 36, 54)}$ than that in $E2_{(0)}$ cT cells. Multiple intergroup comparison between different E2 treatments showed significant changes between $E2_{(0.4 \text{ and } 1)}$ and $E2_{(15)}$ ($p<0.001$ and $p<0.0001$, respectively) and between $E2_{(0.4 \text{ and } 1)}$ and $E2_{(30)}$ ($p<0.05$ and $p<0.01$, respectively) treatments.

Further analysis revealed that the frequencies of $FOXP3^+PD\text{-}1^+$ T cells were 3.2% (1.6-7.2), 18.9% (15.4-41.8), 26.9% (26.4-29.8), 32% (27.7-52.5), 16.1% (13.9-31.7), 11.8% (10.0-13.6), 4.4% (1.9-7.9), 10.3% (7.2-14.1), 15.8% (10.6-26.8) and 14.4% (12.0-17.3), median (range) of cells within non-stimulated cells, $E2_{(0)}$, $E2_{(0.4)}$, $E2_{(1)}$, $E2_{(4)}$, $E2_{(7)}$, $E2_{(15)}$, $E2_{(30)}$, $E2_{(36)}$ and $E2_{(54)}$ cT, respectively (Fig.1B). In this regard, a significant difference was detected between $E2_{(7,15,30)}$ treated cT and $E2_{(0)}$ cT cells in their co-expression of FOXP3 and PD-1. Multiple intergroup comparison between different E2 treatments showed significant changes between $E2_{(1)}$ and $E2_{(15)}$ ($p<0.001$) treatments.

Although co-expressions of CD25/FOXP3 and FOXP3/PD-1 were detected in the absence or presence of all doses of E2, they peaked at 1 ng/ml and a slight, but not significant, increase after declining at 36 ng/ml. These doses could be referred as early pregnancy and late pregnancy/pharmaceutics, respectively (31). Accordingly, cT treated cells with these doses were selected to measure their activity, namely, proliferation ability and cytokine release in supernatants.

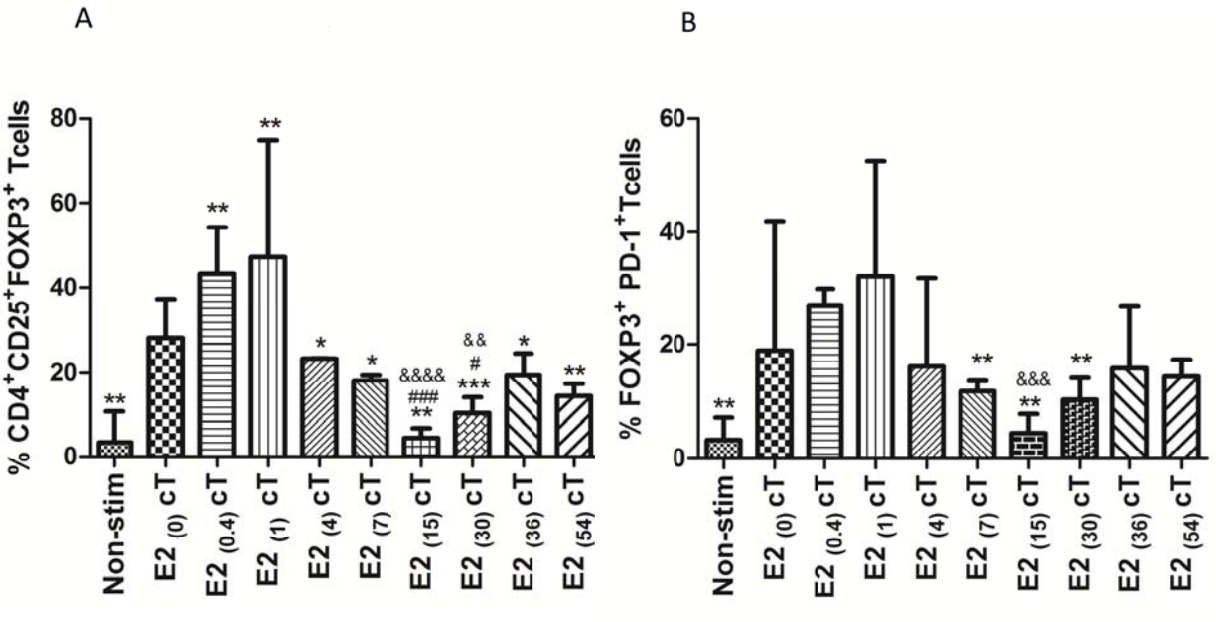

Figure 1. *In vitro* conversion of human naive T cells in to Tregs. $CD4^+CD25^-$ T cells were sorted by MACS, conditioned in the presence of anti-CD28 (1 µg/ml) in anti-CD3 (5 µg/ml) coated plates (named as conditioned T cells; cT) and co-incubated following administration of different E2 doses: 0 ng/ml, 0.4 ng/ml, 1 ng/ml, 4 ng/ml, 7 ng/ml, 15 ng/ml, 30 ng/ml, 36 ng/ml and 54 ng/ml. Non stimulated cells were the $CD4^+CD25^-$ T cells without treatment. After culturing for 96 h, the intracellular expressions of FOXP3 and PD-1 as well as CD4 and CD25 molecules were detected, using flow cytometry. **A)** Frequencies of $CD4^+CD25^+FOXP3^+$ T cells in the presence of different concentration of E2 and **B)** frequencies of $CD4^+PD\text{-}1^+$ $FOXP3^+$ T cells are shown. The data was analyzed by non-parametric Kruskal-Wallis and *Mann-Whitney U test. The values are* presented as median (range) of five independent experiments. All comparisons have been made with $E2_{(0)}$ cT cells. *p<0.05, **<0.01 and *** p<0.001. Additionally, multiple intergroup comparison between E2 treatments was analyzed by *Dunn's multiple comparison test* (# vs $E2_{(0.4)}$ and & vs $E2_{(1)}$). cT: conditioned T cells, E2: 17β-estradiol, FOXP3: Forkhead box p3, PD-1: programmed death-1.

Low proliferative ability of $E2_{(1)}$ cT cells was independent of apoptotic activity.

The proliferation rates of cT were then measured and $E2_{(1)}$ cT showed a significantly reduced proliferation capacity in comparison to $E2_{(0)}$ cT and $E2_{(36)}$ cT ($p<0.05$) (Fig. 2A). Such reduced proliferation capacity was shown to be independent of apoptosis rate (Fig. 2B). There were no significant differences in apoptosis rate between groups.

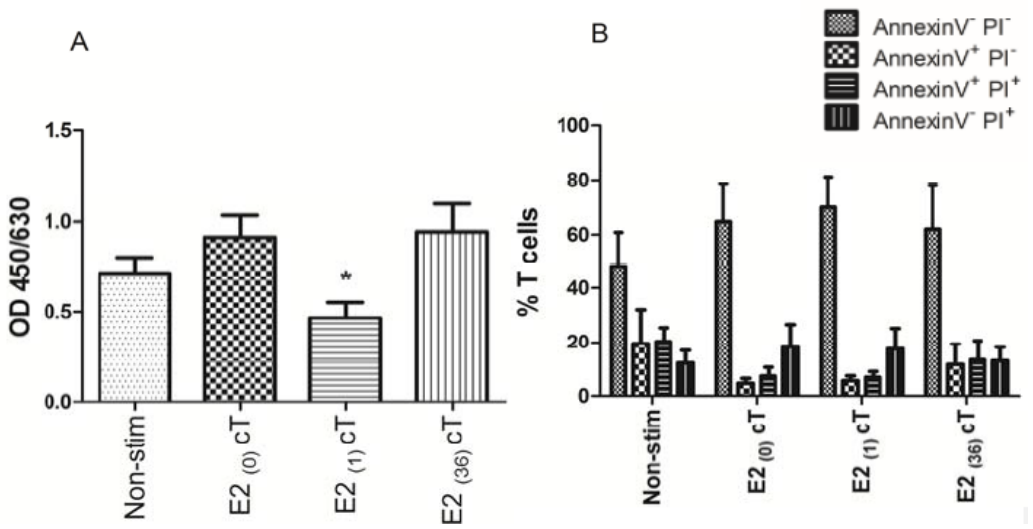

Figure 2. **Proliferation and apoptosis rates of conditioned T cells.** After the initial conditioning in the presence of anti-CD28 (1 µg/ml) in anti-CD3 (5 µg/ml) coated plates (cT) and co-incubation with $E2_{(1)}$ and $E2_{(36)}$ for 96 h, T cells were washed and cultured ($2x10^5$/200 µl /well) for additional 96 h. **A)** XTT assay was used to monitor proliferation ability of cT cells and **B)** cells were stained with PI-/Annexin-V to monitor apoptosis rates (n=4). All experiments were run in triplicates. The data was analyzed by non-parametric Kruskal-Wallis, followed by *the Dunn's multiple comparison test and* presented as mean ± SEM. * $p<0.05$ vs. $E2_{(0)}$ cT and $E2_{(36)}$ cT. cT: conditioned T cells, E2: 17β-estradiol.

Increased IL-10 and decreased IFN-γ production in $E2_{(1)}$ cT cultures.

The levels of released cytokines, IL-10, TNF-α and IFN-γ in cell culture supernatants of cT cells were measured. Significantly higher levels of IL-10 were produced by $E2_{(1 \text{ and } 36)}$ cT groups compared to non-stimulated cells ($p<0.05$) (Fig. 3A). $E2_{(36)}$ cT released significantly higher TNF-α levels than $E2_{(1)}$ cT into the cell culture supernatants ($p<0.05$) (Fig. 3B). Significantly lower levels of released IFN-γ were observed in $E2_{(1)}$ cT compared to $E2_{(0)}$ cT cell supernatants ($p<0.001$). Similarly, $E2_{(1)}$ cT produced significantly lower levels of IFN-γ than $E2_{(36)}$ cT ($p<0.01$) (Fig. 3C). The IFN-γ to IL-10 ratio was found to be significantly decreased in $E2_{(1)}$ cT compared to $E2_{(0)}$ cT cells ($p<0.05$) (Fig. 3D).

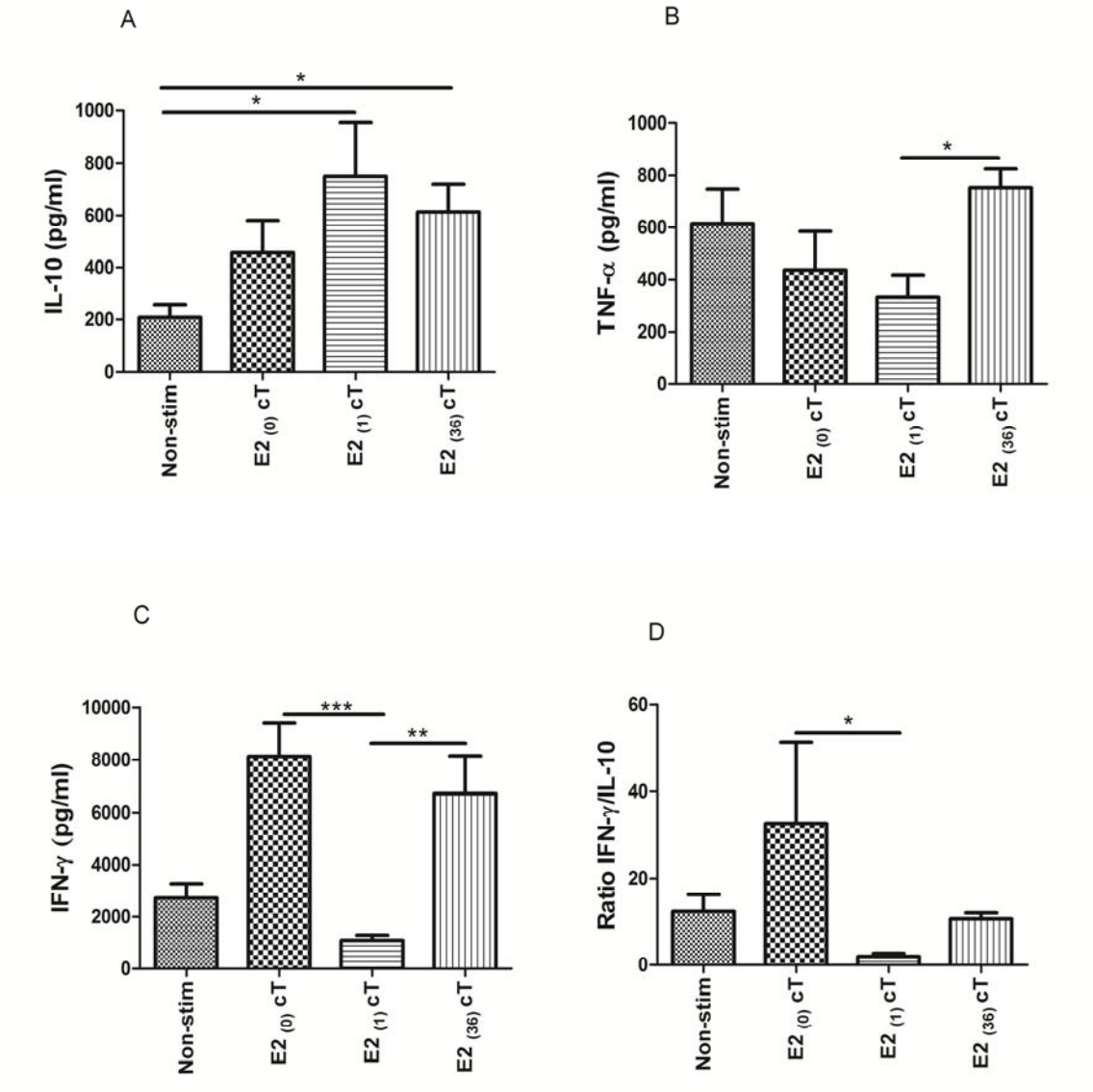

Figure 3. The expression levels of IL-10, TNF-α and IFN-γ in supernatants of conditioned T cells. After the initial conditioning in the presence of anti-CD28 (1 µg/ml) in anti-CD3 (5 µg/ml) coated plates and co-incubation with E2(1) and E2(36)for 96 h; for cytokine induction, T cells were washed and cultured ($2x10^5$/200 µl /well)for additional 96 h. The expression levels of **A)** IL-10, **B)** TNF-α, **C)** IFN-γ and **D)** IFN-γ/ IL-10 ratios in supernatants of cT cells are shown. Using ELISA, cytokine expressions were measured in the supernatants of cT cells. All experiments were run in duplicates. The data was analyzed by non-parametric Kruskal-Wallis, followed by the Dunn's multiple comparison test, and presented as mean ± SEM (n=4).*p<0.05, ** p<0.01 and ***p<0.001. cT: conditioned T cells, E2: 17β-estradiol, IFN: interferon,IL: interleukin, TNF: tumor necrosis factor.

DISCUSSION

This study focused on the effect of E2 (10^{-9} to 10^{-7} M) on the conversion of naïve T cells to $FOXP3^+$ T cells, by conditioning with anti-CD3 and anti-CD28, after 4 days of culture. Our experiments showed that E2 at 1 ng/ml (3.6×10^{-9}M) stimulated conversion of about 47% of $CD4^+CD25^-$ T cells into $CD25^+FOXP3^+$ and 32% of $CD4^+CD25^-$ T cells into $FOXP3^+PD\text{-}1^+$ T cells. However, when the concentration of E2 exceeded 1 ng/ml, the frequencies of converted $CD4^+CD25^-$ T cells decreased. Interestingly, treatment with 36 ng/ml (1.3×10^{-7} M) E2 led to an apparent but not significant increase in $CD25^+FOXP3^+$/$FOXP3^+PD\text{-}1^+$ T cells. We were interested in analyzing whether these two populations showed comparable cellular effects. In this regard, further studies showed that $E2_{(1)}$ cT proliferated poorly and released reduced amounts of pro-inflammatory cytokines in culture supernatants than $E2_{(36)}$ cT cells. These data may suggest that lower levels of E2, found during early pregnancy, are an important mediator in generating functional Tregs.

In recent years, besides the therapeutic potential of natural Tregs in diminishing the uncontrolled T cell responses of autoimmune diseases (32-34), application of E2 as a potential means of hormone therapy to induce Tregs has been discussed in treatment of inflammatory disease conditions (23,35-37). Reports from basic research and clinical studies indicates the therapeutic potential of estrogens in treatment of multiple sclerosis (MS) (38). Hormone replacement therapy (HRT) may be used to protect against osteoporosis. However, HRT is also known to increase the risk of breast cancer and heart disease. Therefore it should be administered for the shortest time and lowest dose to be helpful.

Recent published reports have demonstrated that E2 influences on the conversion of naïve T cells into Tregs during early pregnancy (39). In the present study about 47% of cells expressed FOXP3 after 96h incubation, while in Tai *et al.* study (with different concentrations of activating stimuli and after 48h incubation) only 3% of cells expressed FOXP3. The discrepancy in frequency of converted Tregs could be due to several factors, such as the source of cells, the dose of activating stimuli and culture conditions.

Depending upon the administrated dose, E2 may cause various effects on T cell cytokine production. Cytokines such as IL-2, IFN-γ and TNF-α are assigned to Th1, and IL-4, IL-5 and IL-10, to Th2 response. In addition, regulatory T cells, which drive certain anti-inflammatory responses, produce transforming growth factor (TGF)-β and IL-10 (40,41). Antigen stimulation and E2 treatment (at doses below 10 ng/ml), of human proteolipid-specific T cell clones derived from MS patients, stimulated production of IFN-γ and TNF-α in these cells, while E2 treatment of doses above 10 ng/ml, stimulated IL-10 production (2). In our experiments, upon anti-CD3/anti-CD28 activation healthy human naïve $CD4^+$ T cells treated with E2 (at both 1 and 36 ng/ml), produced IL-10, while at 1 ng/ml, production of IFN-γ and TNF-α was inhibited. In line with our findings, a previous study reported that E2 replacement therapy (at early pregnancy levels) in ovariectomized mice inhibited T cell production of TNF-α (42). To explain the mechanism why Tregs peaked at 1 ng/ml of E2, before declining, it may be suggested that at the very early stage of pregnancy, when immunologic rejection mostly occurs, there is a need for Treg support to prevent rejection of embryo. It is also documented by earlier reports (27, 28) that functionally active Tregs are accumulated in deciduas of the first trimester pregnant women. The molecular mechanism by which E2 affects T cells is uncertain. While E2 binds to estrogen receptors (ER); ERα and ERβ in

the nuclei of cells and mediates genomic effects (43), its binding to G-protein coupled estrogen receptor (GPER) mediates nongenomic effects (44). E2 induction of FOXP3 has been shown to be mediated through ERα (23). However, reports have shown that ERβ may function as negative regulator of ERα activity (45). It may be speculated that different concentrations of E2 may modulate ER expression with consequent effects on expression of other molecules. These effects might influence on the levels of $FOXP3^+$ T cell induction as reported in this study. Such investigation remains to be studied in regard to FOXP3 expression.

Offner *et al.* (37) reviewed the beneficial effects of using both E2 and TCR-peptide therapy on clinical course of experimental autoimmune encephalomyelitis (EAE). They suggested that E2 increased the number and activity of $FOXP3^+$ T cells, while TCR-peptide therapy only increased the frequency of $FOXP3^+$ T cells (37). Here, it was shown that combined treatment of naïve T cells with $E2_{(1)}$ and anti CD3/CD28 exerted an anti-inflammatory activity as compared to anti CD3/CD28 alone. Earlier in human, TCR activation using antibodies against CD3 and CD28 molecules could induce FOXP3 expression in $CD4^+CD25^-$ cells (46-52). Although in most of those studies, FOXP3 induction did not confer their suppressive function (46,48,52), which may suggest that these cells arise by transient, activation- induced expression of FOXP3 in $CD4^+CD25^-$ T cells.

Existing data (37,39) suggest that conversion or expansion of functionally active Tregs during pregnancy could be affected by E2 in combination with other stimuli. In this regard, Treg inducing agents like TGFβ and prostaglandin E that are present in seminal fluid may facilitate induction of Tregs during conception (53).

Pregnancy hormones, E2 and progesterone (P4) have been shown to cause Treg cell induction and expansion. In addition, human chorionic gonadotropin (hCG) which is secreted right after fertilization by the blastocysts, attracts Tregs to the uterus to direct immune tolerance toward the fetus (54). The expression of negative co-stimlatory molecules; like PD-1 on decidual $CD8^+$ suppressor T cells and Tregs might also play a role in immune tolerance and the maintenance of pregnancy (55).

Multiple mechanisms may contribute to suppression mediated by Tregs *in vivo* and *in vitro*. Accordingly, the roles of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, CD25 and cytokines such as TGF-β and IL-10 in Treg function have been discussed over a decade (56-59).

From evolutionary point of view, during the course of pregnancy from implantation of the fetus to the delivery, a variable degree of immune regulation might be needed to induce the relevant immune tolerance to the semi allograft embryo/fetus. In this regard different levels of E2, as reported at different stages of pregnancy might be needed to induce such immune regulation (probably through generation of Tregs) to protect the embryo from immunological rejection.

In conclusion, our findings showed that E2 levels at early pregnancy could enhance generation of Tregs with anti-inflammatory cytokine profiles. Further studies are necessary to define the mechanism of the interplay between E2 and other hormones, effector T cells and regulatory T cells. These data may extend our knowledge toward the search for an immunomodulatory therapeutic method in inflammatory disease situations.

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