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Regulatory Effects of Estradiol on Peripheral Blood Mononuclear Cells Activation in Patients with Asthma

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ABSTRACT

Asthma prevalence and severity are greater in women than in men, and mounting evidence suggests this is in part related to female steroid sex hormones. Conflicting data are reported regarding pro- and anti-inflammatory properties of estradiol. This study was designed to clarify whether estradiol may contribute to enhanced T helper (Th) 17-associated cytokines production by peripheral blood mononuclear cells (PBMC) in asthmatic patients and healthy individuals.

PBMCs from patients with asthma and healthy donors were cultured with 17-β estradiol (E2) and phytohemagglutinin (PHA). The quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure IL-6, IL-17, IL-23 and TGF-β.

We observed a significant increased IL-17, IL-23 and TGF-β expression in PBMCs of patients compared to the healthy individuals. In addition, our findings indicated that IL-6 and IL-17 expressions in PBMCs were induced, following E2 treatment.

Our results identified an impact of E2 in stimulation of Th17 phenotype, and upon hormonal oscillations and hormone replacement therapy (HRT), asthma inflammation may be mediated by Th17-associated cytokines.

Keywords: Asthma; Estradiol; IL-17; IL-21; IL-23; TGF-β

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INTRODUCTION

Asthma is a chronic airway inflammatory disease. It is characterized by airway constriction that leads to wheezing, coughing, and shortness of breath. Despite significant advances in the understanding, prevention, treatment and diagnosis of this disease over the past decade, as well as the availability of comprehensive

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clinical practice guidelines for the disease, asthma control is not optimal. In asthma, T helper 2 (Th2) and cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13, that promote eosinophilic inflammation, recruitment of eosinophils into the lungs and immunoglobulin E (IgE) production by B cells, play dominant pathological roles and leading to a deterioration of lung function over time.[1]

Estrogen is responsible for the development and regulation of the female reproductive system. Estradiol is a form of estrogen, that is produced by the ovaries, adrenal gland and also the placenta during pregnancy.[2] This hormone is necessary for many processes in the body and also it is used to treat ovarian failure, symptoms of menopause, cancer treatment, prevention of osteoporosis in postmenopausal women, and replacement of estrogen in women who lack natural estrogen in the body as a hormone replacement therapy.[2,3] It has long been postulated that the woman's hormones, progesterone and estrogen, play important roles in allergic and autoimmune diseases.[4,5]

Although hormone replacement therapy (HRT) has been used for years as a therapeutic approach in millions of women to relieve from menopausal symptoms, there are several potentially harmful effects of HRT includingasthma.[5,6]

Observational epidemiologic studies indicate that mast cells are sensitive to activation or dysregulation by estradiol,[5] and although the prevalence of asthma is greater in boys than in girls during prepubescent ages, it has long been known that asthma prevalence is two to three times higher in women than in men.[7] In particular, it has been hypothesized that hormonal fluctuations during the menstrual cycle play a significant role in the pathophysiology of asthma, resulting in periodic worsening of disease severity in adult females.[5,8]Estradiol can enhance antigen presentation and production of dendritic cell populations, which promote Th2 responses, polarization by induction of IL-5 and IL-13 production, isotype switching to IgE, and mast cell degranulation via classical estrogen receptors.[5]

On the other hand, it is observed that HRT increases estrogen levels and alleviates symptoms in postmenopausal asthmatic women. Therefore, it is important to know side effects of HRT in women. In this regard, effects of HRT on the immune system has been consideredbysomestudies.[5,9,10] Recent evidence suggests that rates of new asthma diagnosis increase after HRT initiation but diminish with cessation of HRT.[11]

A more complete understanding of the activities of hormones in regulating asthma exacerbations could introduce new strategies for symptom management and decrease the disease burden associated with this phenomenon. Therefore, in view of these findings obtained in previous studies on estrogens' fluctuations at ovulation and its impact of on asthma in women, and also HRT after menopause, the aim of this study was to determine whether estradiol is able to induce the Th17-associated cytokines in vitro when cultured with PBMCs via the assessment expression of pro inflammatory and anti-inflammatory cytokines including IL-6, IL-17, IL-23, and TGF-β.

MATERIALS AND METHODS

Subjects

Seventeen asthmatic patients with mean age of 31±4 years (17 females), that confirmed using diagnostic criteria including symptoms of cough, wheezing, chest tightness or pain, and difficulty in breathing by an internist using simple sampling, were selected from Motahari Hospital of Jahrom University of Medical Sciences. Inclusion criteria were asthmatic patients diagnosed according to the Global Initiative for Asthma (GINA) guidelines;[12] patients did not have current or recent infection, were not taking any medication, and did not experience any acute asthma exacerbations or attacks; patients were only allergic to dust mites. Asthmatic patients with other chronic diseases, e.g. pulmonary diseases, lung infection (pneumonia), coronary heart disease, lung cancer, and also infectious disease were excluded from this study. Informed consent was obtained from patients and the study was approved by Jahrom University of Medical Sciences Ethics Committee. Table 1demonstrates the clinical status of Asthma patients. Blood samples from 20 healthy females with mean age of 30±2 years without a history of lung diseases, autoimmune disorders or malignancies and allergy were also obtained as the control group. During sample collection, it was ensured that subjects had neither infection nor any acute or chronic disease. Peripheral venous blood samples (10 mL) were taken by venipuncture and prepared for isolation of PBMCs.

Table 1. Demographic, clinical and immunological status of patients with asthma and control group

Group	Female	Age	IgE (kU/l)	Eos (%)
Asthma	17	31±4	453.29±284.28	4.60±2.80
Control	20	30±2	38.16±34.51	3.72±3.40

IgE: total immunoglobulin E, Eos: percentage of eosinophils in peripheral blood

Isolation of PBMCs

PBMCs were obtained from donated blood samples. In summary, blood samples were run with Ficoll (Lymphodex Inno, Germany) in a 15 mL conical tube. Samples were centrifuged at 400×g for 40 minutes. The mononuclear cell layer was transferred to a new tube and washed with phosphate buffered saline (PBS), pH 7.4 at 300×g for 10 min. The cell pellet was re-suspended in 50 mL of PBS and centrifuged at 200×g for 15 min to reduce platelet contamination.

Culture of PBMCs

PBMCs culture were established in three experimental groups in DMEM, 10% FBS (Gibco, USA) and penicillin-streptomycin with and without 17-β estradiol (E2)(Sigma-Aldrich, Germany), and one group as untreated PBMCs that were considered for both healthy and patient individuals: involving PBMCs,$E210^{-8}$ M, and phytohemagglutinin (PHA)(Gibco, USA);PBMCs,$E210^{-7}$ M and PHA; and negative control groups including PBMCs alone with PHA; and untreated PBMCs. To identify estradiol's effect on PBMCs, E2-treated groups were compared to both PHA-stimulated PBMCs and untreated PBMCs groups. All cultures were established with 10^6 cells/mL. For T cell stimulation, PHA was used 5 μg/mL. All culture sets were incubated in a saturated humidity incubator at 37°C in the presence of 5% CO2 for 48 hours. Then suspension of PBMCs were harvested, collected by centrifugation and stored with TRizol reagent (Invitrogen, Paisley, UK) at −70°C for further analysis.

RNA Isolation and cDNA Synthesis

After lysis with TRizol reagent (Invitrogen, Paisley, UK) total RNA was prepared from the PBMCs. For cDNA synthesis, RNA was treated with DNase I (Invitrogen-Gibco, Paisley, UK) to avoid DNA contamination, then cDNA was synthesized from 5 μg of the total RNA using the First Strand cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative Real-Time RT-PCR

The abundance of IL-6, IL-17, IL-23,and TGF-β gene transcripts in treated and untreated PBMCs were determined by using the Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA, USA). GAPDH housekeeping gene expression was used as a reference for the level of target gene expression.

Table 2. Forward and reverse primers of GAPDH, IL-6, IL-17, IL-23, and TGF-β genes for real-time PCR amplification in peripheral blood mononuclear cells of patients with asthma and healthy controls. Sequences were designed by Primer3 software (Source forge, USA)

Primers	Sequence
GAPDH Forward	5’ TCGGAGTCAACGGATTTGGTC 3’
GAPDH Reverse	5’ GCCATGGGTGGAATCATATTGG 3’
IL-6 Forward	5’ CAG GTT GTT TTC TGC CAG TG 3’
IL-6 Reverse	5’ GAC CGA CAC TCA CCT CTT CA 3’
IL-17 Forward	5’ GGA CTG TGA TGG TCA ACC TG 3’
IL-17 Reverse	5’ CTC CCA GAT CAC AGA GGG AT 3’
IL-23 Forward	5’ CAA GTG GAA GTG GGC AGA G 3’
IL-23 Reverse	5’ CAG CAA CAG CAG CAT TAC AG 3’
TGF-β Forward	5’ AAC GAA CTG GCT GTC TGC 3’
TGF-β Reverse	5’ CCT CTG CTC ATT CCG CTT AG 3’

Each PCR reaction contained 0.5 μg of the cDNA product, 4.0 pmol of each primer, and 1x reaction mixture, consisting of Fast Start DNA polymerase, reaction buffer, dNTPs, and SYBR green I. Table 2 shows the forward and reverse primers for genes. Thermal cycling for all the genes was initiated with a denaturation step at 95°C for 1 minute, followed by 40 cycles (denaturation at 95°C for 10 seconds, annealing at 56°C for 30 seconds, and extension at 60°C for 60 seconds when fluorescence appeared). The qRT-PCR amplification products were analyzed by melting curve analysis.

Statistical Analysis

Data were analyzed using nonparametric Kruskal-Wallis and Mann-Whitney *U* tests by Prism 5 software (Inc; San Diego CA, USA, 2003). The relative amounts of cytokines transcripts were determined using $2^{-\Delta\Delta Ct}$ formula. Target-to-reference gene ratios were calculated with the Pfaffl method.[13] $p<0.05$ was regarded as significant in all statistical analyses.

RESULTS

Cytokine Assay in Patient and Healthy Groups

The gene expression of IL-6, IL-17, IL-23 and TGF-β in patients with asthma and normal controls were examined using qRT-PCR method. It was shown that gene expression of IL-6 among patients did not differ compared to the control group (Figure1A).However, specimens of patients group showed substantially higher levels of IL-17 ($p<0.01$) (Figure 1B), IL-23 ($p<0.0001$) (Figure 1C) and TGF-β ($p<0.05$) (Figure 1D) compared to control group.

Regulatory Effect of E2on PBMCs through the Proinflammatory and Anti-Inflammatory Cytokines

The effect of E2on the synthesis of IL-6, IL-17, IL-23, and TGF-β in PBMCsof patients and healthy controls in culture was assessed by qRT-PCR.

As shown in Figure 2A, $E210^{-8}$ M increased IL-6 expression in the PBMCs compared to PHA-stimulated PBMCs in patients' individuals. In addition, IL-6

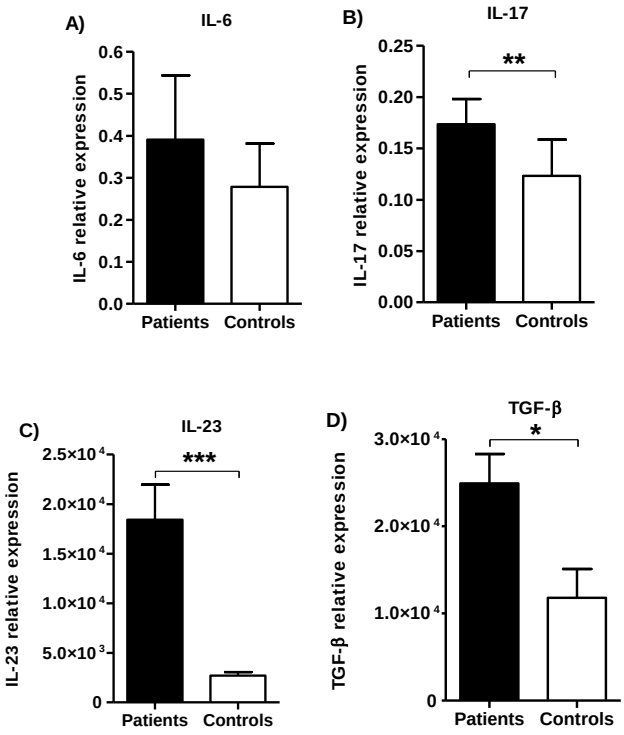

Figure 1. Gene expression of IL-6, IL-17, IL-23 and TGF-β in the peripheral blood of asthmatic patients and normal controls. Significant difference was found in expression of IL-17 (B), IL-23 (C) and TGF-β (D) in PBMCs of patients compared to healthy controls. However, there was no significant difference in level of IL-6 (A) between patients and controls. Results were analyzed with the nonparametric two-tailed Mann-Whitney *U* test. Values are the mean ± SEM; *=$p<0.05$; **=$p<0.01$; ***=$p<0.0001$.

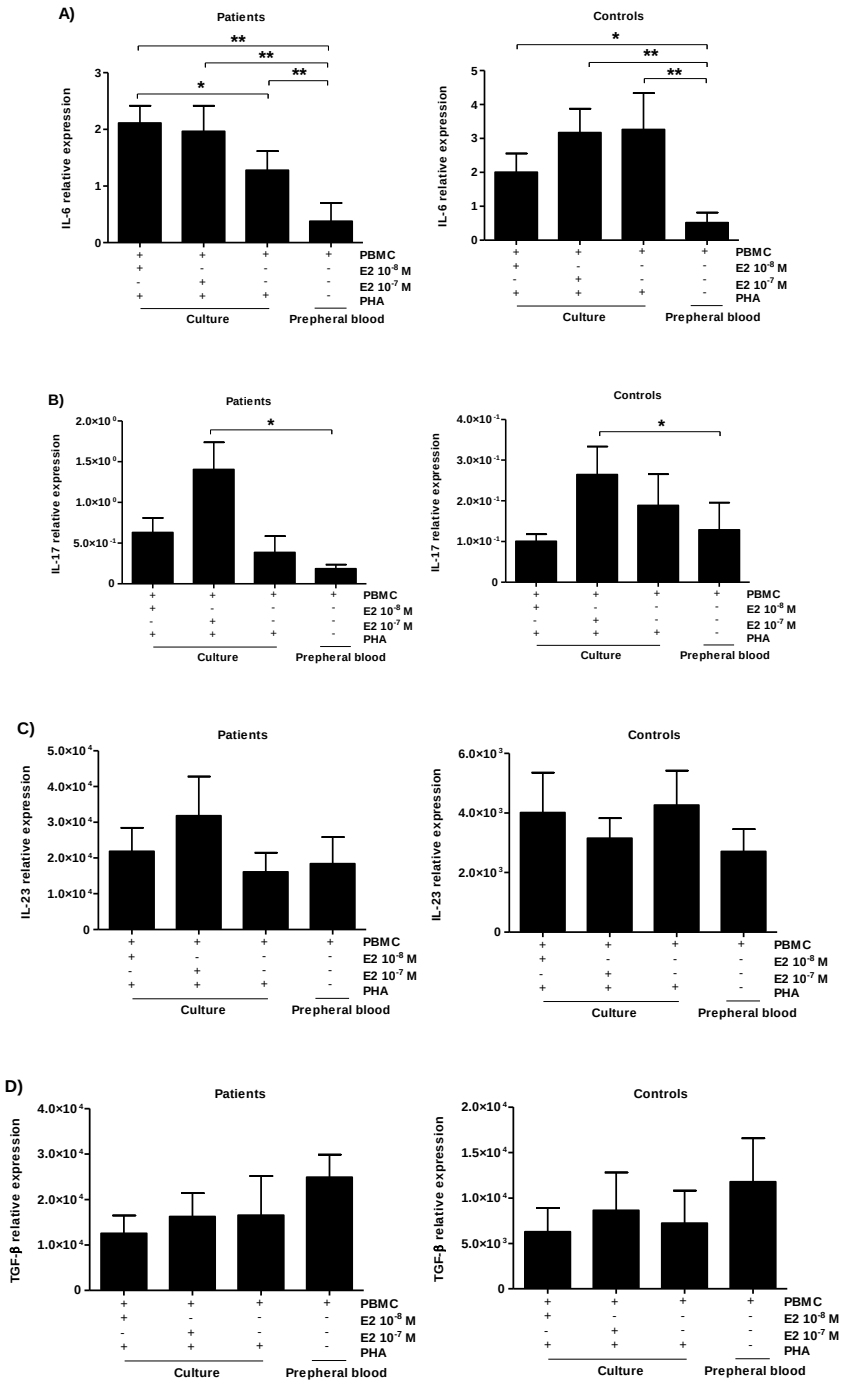

Figure 2. Effect of E2 on the expression of IL-6, IL-17, IL-23 and TGF-β in patients with asthma (n=17) and healthy individuals (n=20). PBMCs culture were established in three experimental groups and one group as untreated PBMCs that were considered for both healthy and patient individuals: involving PBMCs, E2 10^{-7} M, and PHA; PBMCs, E2 10^{-8} M and PHA; and negative control groups including PBMCs alone with PHA; and untreated PBMCs. To identify estradiol's effect on PBMCs, E2-treated groups were compared to both PHA-stimulated PBMCs and untreated PBMCs groups. Figures are showing expression of IL-6 (A), IL-17 (B), IL-23 (C), and TGF-β (D) in PBMCs under the indicated conditions. Results were analyzed with the nonparametric two-tailed Kruskal–Wallis and Mann-Whitney *U* test. Data are showing mean±SEM of values; *=*p*<0.05; **=*p*<0.01; PBMCs: Peripheral blood mononuclear cells; PHA: Phytohaemagglutinin; E2: 17-β estradiol.

expressions among PBMCs-treated with $E210^{-8}$ M, $E210^{-7}$ M and PHA-stimulated PBMCs were higher compared to untreated PBMCs in both of patient and healthy groups. Compared to untreated PBMCs group, IL-17 expression of PBMCs-treated group with $E210^{-7}$ M was higher in both patient and healthy individuals (Figure 2B). However, $E210^{-8}$ M effect was not significantly different on IL-17 production compared to untreated PBMCs and PHA-stimulated PBMCs groups.

As shown in Figure 2C, there was difference related IL-23 expression in PBMCs-treated with E2. However, it was not statistically different in both of patient and healthy groups compared to untreated PBMCs.

In order to examine the inflammatory effect of E2on the PBMCs of healthy and patient individuals, TGF-β expression was determined. TGF-β expression in both groups showed no statistically difference following E2treatment compared to untreated and PHA-stimulated PBMCs (Figure 2D).

DISCUSSION

Estrogen therapy is used as a part of the HRT for menopaused women. Although there are many links between estrogen and immune system, question in this research was focused on Th17-related immune responses, following treatment with estrogen.

In asthma, as an airway inflammatory disease, some cytokines such asIL-4, IL-5 and IL-13 play dominant pathological roles, and also Th2 cell subset is well known to mediate inflammation in the pathogenesis of the disease.[1,6] Therefore, finding new aspects of estrogen therapy which can affect immune system and asthmatic disease may lead to a promising treatment for asthma.

On the other hand Th17 immune responses are associated with autoimmune and hypersensitivity conditions including multiple sclerosis, systemic lupus erythematosus (SLE), diabetes, rheumatoid arthritis and asthma.[14] First, in this study, we evaluated the levels of IL-6 and TGF-β as the cytokines inducing Th17 cells differentiation, as well as IL-17 as hallmarks of Th17 cells and also IL-23 as the maintaining cytokine for Th17 phenotype in peripheral blood mononuclear cells of patients with asthma.

Our data demonstrated higher levelsofIL-17, IL-23 and TGF-β gene expression of PBMCs of patients compared to controls. Gene expression ofIL-6 in patient group; however, was not significant compared to control subjects. Consistent with our findings, previous studies showed increased IL-17production,[15] IL-23,[16] TGF-β,[17] and IL-6[18] in asthmatic patients. Our study suggests thatTh17-associated cytokines have key roles in severe forms of asthma.

Then, we treated patients' and controls' PBMCs with E2in culture and observed that E2 increasesIL-6 and IL-17 gene expression. However, there was no significant difference in IL-23 and TGF-β gene expressions by PBMCs between E2-treated and untreated groups. Previous studies showed the effects of estrogen on inflammatory mediators and immune system cells are complex.Some of these studies were in favor of inflammatory phenotypes[5] some others are in contrast of the inflammatory effects of estrogens on asthma and suggest a reciprocal effect of estrogens.[19] Then, in the present study, we showed effects of E2 on PBMCs of patients with asthma and healthy controls by regulating Th17 cells. We demonstrated that E2 increased T cell inflammatory responses through increase of IL-17 production at gene levels (Figure2B). This finding was in accordance with Newcomb's study which showed administration of estradiol in patients with severe asthma increased levels of pro-inflammatory cytokines including IL-23 and IL-17production.[20] Baker et al showed asthmatic women had increased $IL\text{-}17^{+}$ memory T compared to men and serum estradiol positively correlated with $IL\text{-}17^{+}$ cells.[21]

Unlike our study, the Lélu's et al findings demonstrated estrogens can protect CNS from autoimmunity through their capacity to exert anti-inflammatory and inhibitory effect on Th1 and Th17 cell priming as well as neuroprotective effects, resulting in EAE protection.[22] Baldaçaraet al reported due to action of estrogen that decreases TNF-α production, interferon-γ expression and NK cell activity, estrogen fluctuations are responsible for asthma exacerbations that occur in women.[23]

In accordance with our study, Calippe et al showed that administration of estradiol to ovariectomized mice markedly increases the expression of IL-1β, IL-6, IL-12p40, and inducible NO synthase by resident peritoneal macrophages in response to LPS ex vivo and this in vivo proinflammatory effect of estradiol was mediated through estrogen receptor α (ERα).[24]

Another study found that short-term culture in estradiol had no effect on iDC survival or the expression of cell surface markers. However, E2 treatment significantly increased the secretion of IL-6 in iDCs and also increased secretion of osteoprotegerin (OPG) by DCs. Furthermore, E2 significantly increased secretion of the inflammatory chemokines IL-8 and monocyte chemoattractant protein 1 (MCP-1) by iDCs.[25] A recent study by Siracusa et al demonstrated E2 facilitated the differentiation of BM precursor cells into functional DCs with increased expression of the costimulatory molecules CD40 and CD86. Exposure of bone marrow-derived dendritic cells to E2 also enhanced production of IL-12 in response to the TLR ligands, CpG and LPS.[26]

In contrast to our data, Rachon et al experiments showed that 17β-estradiol decreased spontaneous IL-6 production by the PBMCs of postmenopausal women. *In vivo* treatment with E2 transdermally also significantly decreased spontaneous IL-6 production by the PBMCs of postmenopausal women after 12 months of the therapy. It seems that estrogen deprivation after menopause may enhance IL-6 production by the PBMCs of postmenopausal women.[27] In addition, Dong and his collogues showed that ER1, a negative regulator of several inflammatory signaling pathways, directly affects the transcription factor signaling pathway by inhibiting NF-κB, thereby mediating changes in the secretion of several inflammatory cytokines such as IL-10, TNF, IL-6, IFN-γ and others in the NF-κB signaling pathway.[28] A previous research showed significant decreases in the spontaneous secretion of IL-6, TNF-α, IL-1ra, IL-1β, and ratio of IL-1β/IL-1ra compared with control, at physiological concentrations of E2. With E2 blocking in couture, decreasing cytokine secretion was stopped. And also decrease in cytokine secretion was not observed when the inactive form of estrogen, 17α-estradiol, was used in place of 17β-estradiol.[29]

However, the present study showed that IL-6 and IL-17 were increased in an estrogen dose-dependent manner and inflammatory effects of Th17 cells can impact on pathogenesis of the asthma after estrogen treatment. In addition, there are several evidences that IL-17 increases secretion of IL-6 from airway endothelial cells, fibroblasts and epithelial cells which lead to increased neutrophilic inflammation and increased airway reactivity in asthmatic patient.[30-32] The controversies in literature and some of our data are due to mechanisms by which estrogen act may be quite complex. Because it is known that T cells express ERα that estrogen may affect T cell function through non-genomic, ERα-independent mechanisms and that progesterone promotes the development of Th2 cells and cytokines.[33,34]

IL-23/IL-23R signaling and TGF-β are important for sustainability of the Th17cell lineage and increased IL-17.[35] Therefore, we also determined IL-23 and TGF-β expression after treatment by E2. However, there were no significant difference between groups. More likely the low sample size affected on IL-23 and TGF-β expressions after estrogen treatment. Newcomb and his colleague reported patients with severe asthma have an increased number of Th17 cells in the peripheral blood compared to healthy controls.[20] In addition, Th17cells from women had a significant increase in cell surface expression of IL-23R compared to Th17 cells from men, and IL-23R surface expression was also increased on $CD3^+$ $CD4^+$ $CCR6^+$ memory Th17 cells from women with severe asthma compared to men with severe asthma. They also found a significant increase in IL-17 protein expression in Th17 cells from ovariectomized female mice administered the combination of 17β-E2 compared to Th17 cells from ovariectomized female mice administered vehicle pellets.[20] Generally previous studies in accordance ours showed 17β-E2 increases IL-17 production from Th17 cells, providing a potential mechanism for the increased prevalence of severe asthma in women compared to men.

There were some limitations in present study including low population and not having comparison of Th17-related cytokines between postmenopausal and premenopausal women after PBMC treatment by estradiol.

In conclusion, our results indicated that estradiol can affect immune responses and an increase in E2 concentration was associated with asthma among adolescents. In addition, our results identify an impact of estradiol in the capacity of T cells to polarize towards a Th17 phenotype to produce IL-17. The manipulation of peripheral T cell plasticity in pathological systems might represent a novel strategy for future therapeutic approaches. Our data contribute to the understanding of mechanisms underlying the deterioration of asthma symptoms in women, clinically observed during fertile phase of the female reproductive cycle and after that when exogenous

compounds with estrogenic activity may exacerbate it.

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