Immunomodulatory effects of human amniotic epithelial cells on naive CD4$^+$ T cells from women with unexplained recurrent spontaneous abortion

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ABSTRACT

Introduction: Immune imbalance at the maternal-fetal interface plays a fundamental role in the pathogenesis of unexplained recurrent spontaneous abortion (URSA). Human amniotic epithelial cells (hAECs) possess pregnancy-friendly immunomodulatory effects. Here, we investigated how function of naive CD4$^+$ T cells from URSA patients is affected by hAECs.

Methods: Phenotypic characteristics of hAECs were determined by flow cytometry and their effect on proliferation of allogeneic peripheral blood mononuclear cells (PBMCs) was evaluated by a BrdU cell proliferation assay. Naive CD4$^+$ T cells were isolated from 25 URSA patients and 5 healthy women and co-cultured with hAECs. Immunomodulatory effects of hAECs on cytokines profile, proliferation of stimulated CD4$^+$ T cells and induction of regulatory T cells (Tregs) were assessed by ELISA and flow cytometry, respectively. Functional competency of Tregs was evaluated in an allogeneic mixed lymphocyte reaction (MLR) system.

Results: hAECs did not elicit allogeneic proliferative responses of PBMCs, inhibited proliferation of naive CD4$^+$ T cells, induced production of Th2 and suppressed production of Th1 and Th17 cytokines. hAECs showed the ability to induce differentiation of Tregs and production of transforming growth factor-beta1 (TGF-β1) and interleukin-10 (IL-10). This ability was found to be superior in control subjects compared to URSA patients. Indeed, Tregs generated in the presence of hAECs expressed higher levels of CTLA-4 compared to Tregs generated in their absence and restrained the proliferation of autologus PBMCs in MLR system.

Conclusion: Based on these findings, hAECs can be considered as one potential candidate in immunotherapy of patients with URSA.

1. Introduction

Recurrent spontaneous abortion (RSA) is a common health problem in women of reproductive age [1]. It is defined by the loss of three or more consecutive pregnancies before the 20th week of gestation [2,3]. Although a variety of factors such as infections and abnormalities including genetic, chromosomal, anatomic, immunologic, and endocrine have been reported for their attribution into the disease, no identifiable etiology was diagnosed in about 40–60% of patients [1,4,5]. This condition is classified as unexplained recurrent spontaneous abortion (URSA) [4]. It is associated with the failure of feto–maternal immunologic tolerance [1]. Several studies established a number of immunological defects in URSA women, including reduced number and impaired function of regulatory T cells (Tregs) [6], Th1 versus Th2 dominancy [7], and increase in the number of Th17 cells and natural killer (NK) cells in the peripheral blood and decidua [8,9].

Human amniotic epithelial cells (hAECs) are a potential source of stem cells and are isolated from the amniotic membrane, the closest layer to the fetus and in contact with the amniotic fluid [10–12]. In addition to their capability to differentiate into several cells originating from the ectoderm, endoderm, and mesoderm, such as myocytes, cardiomyocytes, adipocytes, hepatocytes, pancreatic cells, and neural cells

[11,13–15], several studies reported that hAECs exert immunomodulatory effects on innate and adaptive immune cells. It is demonstrated that hAECs are able to extremely suppress B cell proliferation and to inhibit macrophages and neutrophil migration [16,17]. Additionally, these cells can inhibit CD4$^+$ T cell activation and decrease proinflammatory cytokine production of CD4$^+$ T cells [11,16]. Previous studies have provided evidence that immunoregulatory effects of hAECs are mainly mediated through the production of immunosuppressive compounds such as transforming growth factor-beta (TGF-β) and prostaglandin E2 (PGE2) [18,19].

Regarding the fact that hAECs exert immunosuppressive effects on activation, proliferation, and cytokine production of different cells from the immune system [11,20], we investigated whether hAECs can affect the function of naive CD4$^+$ T cells from URSA patients. Moreover, the allogeneic effects of these cells were assessed to detect potential immune-rejection reactions in case of clinical application.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (protocol number: 394205) and performed in accordance with the Declaration of Helsinki. All participants provided informed consent before entering the study.

2.2. Study population

The study population comprised 25 URSA women (mean age of 28.33 years, range: 22–37 years) who were referred to a cell therapy center in Isfahan, Iran, and 5 volunteer control women (mean age of 26.89, range: 20–31 years) without history of abortion who had at least one live birth. The sampling from URSA patients was performed 1 week before any immunotherapeutic modalities including immunotherapy with paternal lymphocytes. The diagnosis of URSA was made after excluding any verifiable causes including the detection of any uterus or cervix abnormalities and chromosomal abnormality; infections due to *Chlamydia* and *Ureaplasma urealyticum*; endocrine diseases such as hyperprolactinemia, hypothyroidism, hyperthyroidism, diabetes, and hyperandrogenemia; and diagnoses of congenital thrombophilia, antiphospholipid syndrome, and systemic lupus erythematosus. The URSA patients whose partners had abnormal semen status were also excluded according to World Health Organization criteria [21].

2.3. Isolation of hAECs

Full-term human placenta was obtained from 25 healthy women with a normal singleton pregnancy undergoing uncomplicated elective cesarean section. hAECs were isolated using a method as previously described [22]. Briefly, amnion membrane was manually stripped from the chorion and washed several times with 0.15 M phosphate-buffered saline (PBS) to remove the blood and cellular debris. The amnion was digested with 0.05% trypsin (Gibco, USA) for 20 min at 37 °C. The cells from the first digestion were discarded to exclude the damaged cells. The enzymatic digestion was followed twice for 30 min at 37 °C. The cells from the second and third digests were pooled and washed with Hank's balanced salt solution (HBSS). The purity of cells obtained from 16 donors was determined by flow cytometry using the epithelial marker cytokeratin (BioLegend, USA). Cells with a purity of ≥96% were considered as hAECs.

2.4. hAEC culture

The isolated hAECs were cultured in 75-cm^2 tissue culture flasks at a density of 2.5×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). After 24 h of incubation at 37 °C, adhered hAECs were dissociated by trypsin and plated at different numbers on 24-well plates for the next experiments.

2.5. Characterization of hAECs

Phenotypic characteristics of freshly isolated hAECs were determined using flow cytometry. The cells ($4–8 \times 10^5$ cells/ml) were stained with different antibodies (Table 1) or matched to isotype control IgG for 25 min at 4 °C. The matched isotype control antibodies were used as negative controls. Further, the samples were subjected to cytokeratin staining as an intracellular epithelial marker after fixation and permeabilization of the cells according to the manufacturer's guidlines (eBiosciences, USA). Data were acquired by a FACSCalibur system (Becton Dickinson, San Jose, CA) and were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

2.6. Assessment of the allogeneic effects of hAECs

To assess the possible allogeneic effects of hAECs, the isolated cells were co-cultured with peripheral blood mononuclear cells (PBMCs) and proliferative response was measured. The freshly isolated hAECs from

Table 1
Antibodies used for the determination of phenotypic characteristics of hAECs by Flow cytometry.

Fluorochrome/Antibody	Isotype (Clone)	Company (All from USA)
Alexa Fluor® 488 anti-Cytokeratin (pan reactive)	Mouse IgG1, κ (C-11)	BioLegend
FITC anti-human CD105	Mouse IgG1, κ (43A3)	BioLegend
FITC anti-human CD90	Mouse IgG1, κ (5E10)	BioLegend
FITC anti-human CD45	Mouse IgG1, κ (2D1)	BioLegend
FITC anti-human CD14	Mouse IgG1, κ (63D3)	BioLegend
FITC anti-human CD4	Mouse IgG1, κ (A161A1)	BioLegend
FITC anti-human CD8a	Mouse IgG1, κ (HIT8a)	BioLegend
FITC anti-human CD38	Mouse IgG1, κ (HB-7)	BioLegend
FITC anti-human CD3	Mouse IgG1, κ (HIT3a)	BioLegend
PE anti-human SSEA-4	Mouse IgG3, κ (MC-813-70)	BioLegend
PE anti-human CD133	Mouse IgG$_1$, κ (Clone 7)	BioLegend
FITC anti-human HLA-DR	Mouse IgG$_{2a}$, κ (G46-6)	BD Biosciences
FITC anti-human CD34	Mouse IgG$_1$, κ (8G12)	BD Biosciences
PE anti-human CD56	Mouse IgG$_1$, κ (B159)	BD Biosciences
PE anti-human CD44	Mouse IgG$_{2b}$, κ (515)	BD Biosciences
PE anti-human CD9	Mouse IgG$_1$, κ (M-L13)	BD Biosciences
PE anti-human CD29	Mouse IgG$_{2a}$, κ (HUTS-21)	BD Biosciences
PE anti-human CD73	Mouse IgG$_1$, κ (AD2)	BD Biosciences

the three amnion membranes were mixed and considered as a single hAECs sample. In this experiment, four pools of hAECs obtained from healthy pregnant women were prepared. PBMCs from four healthy donors were isolated by Ficoll-Paque centrifugation. Afterwards, 2.5×10^4 PBMCs from each donor were co-cultured with a single hAEC sample at different ratios (1:1, 1:2, 1:3) in 96-well, flat-bottomed microtiter plates, at 37 °C with 5% CO_2. All assays were performed in duplicate. PBMCs stimulated with antihuman CD3/anti-CD28 antibodies (1 μg/ml; Mabtech, Sweden) were used as a positive control, and unstimulated PBMCs without any hAECs, as well as hAECs alone, were used as negative controls. After 3 days, PBMC proliferation was assessed using a bromodeoxyuridine (BrdU) cellular enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Abnova, USA).

2.7. Isolation of naive CD4⁺ T cells

PBMCs were obtained from heparinized whole blood of URSA and control women by Ficoll-Paque centrifugation. Naive CD4⁺ T cells were isolated from PBMCs by negative selection using magnetic beads (Miltenyi Biotec, Germany). Subsequently, the isolated cells were stained with picoerythrin/cyanin5 (PE/Cy5) antihuman CD4 and fluorescein isothiocyanate (FITC) anti-human CD45RA antibodies. The relevant isotype control antibodies served as the negative control. Cell purity was analyzed using a FACSCalibur flow cytometer. The CD4+CDRA + T cells were considered as naive CD4⁺ T cells. Cell samples with > 90% purity were used in the following experiments. All primary and matched isotype control antibodies were purchased from BioLegend (USA).

2.8. T cell proliferation assay

Naive T cells were labeled with carboxyfluorescein succinimidyl ester dye (CFSE) (BioLegend, USA) according to the manufacturer's protocol. Briefly, naive CD4⁺ T cells (1×10^7) from URSA women were suspended in 1 ml of PBS, and 1 μl of CFSE (5 mM/ml) was then added to make a final concentration of 5 μM. After 15 min of incubation in the dark at 37 °C, 2 ml of ice-cold complete Roswell Park Memorial Institute (RPMI) medium (Gibco, USA) was added to quench the staining. Thereafter, different numbers of hAECs ($4–40 \times 10^4$ cells/ml) were co-cultured at different ratios (1:1, 1:2, 1:5, 1:10) with the CFSE-labeled naive T cells (4×10^5 cells/ml) in 24-well plates and incubated at 37 °C with 5% CO_2, one set incubated for 3 days and another set for 6 days. Naive T cells were stimulated with anti-CD3/anti-CD28 antibodies (1.5 μg/ml). Naive T cells stimulated with anti-CD3/anti-CD28 antibodies without any hAECs were used as the positive control, and unstimulated naive T cells without any hAECs were used as the negative control. Proliferation of naive T cells was assessed through fluorecent intensity measurement of CSFE dye using a FACSCalibur flow cytometer.

2.9. Cytokine assay

To investigate the effects of hAECs on the cytokine profile of naive CD4⁺ T cells, hAECs ($4–40 \times 10^4$ cells/ml) were co-cultured at different ratios (1:1, 1:2, 1:5, 1:10) with naive T cells (4×10^5) from URSA women in 24-well plates and stimulated with anti-CD3/anti-CD28 (1.5 μg/ml) antibodies. The cells were then incubated at 37 °C with 5% CO_2. The co-cultured supernatants were collected after 3 and 6 days, and quantitative analysis of cytokines was performed using an ELISA kit (Mabtech, Sweden). The following cytokines were measured: IFN-γ, TNF-α, IL-4, and IL-17 A. The hAECs cultured without any naive T cells and unstimulated naive T cells without any hAECs were considered as negative controls, and naive T cells stimulated with anti-CD3/anti-CD28 antibodies served as the positive control. The limit of detection of ELISA kits was 2 pg/ml for IFN-γ, TNF-α, and IL-17 A and 1 pg/ml for IL-4.

2.10. Differentiation of naive CD4⁺ T cells into tregs

Different numbers of hAECs ($4–80 \times 10^4$ cells/ml) were seeded in 24-well plates. Naive CD4⁺ T cells (4×10^5 cells/ml) from control and URSA women were then added to the wells to prepare hAEC-to-naive T cell ratios of 2:1, 1:1, 1:2, 1:5, and 1:10. Naive T cells were stimulated with anti-CD3/anti-CD28 antibodies (1.5 μg/ml) and incubated at 37 °C with 5% CO_2. Naive T cells cultured alone and stimulated with anti-CD3/anti-CD28 antibodies served as control. After 6 days, T cells were stained with PE antihuman CD25 and PE/Cy5 antihuman CD4 antibodies for 20 min at 4 °C. Fixation and permebilization were done using True-Nuclear™ Transcription Factor Buffer Set and then subjected to intracellular staining using Alexa Fluor 488 anti-human Foxp3 antibody or the relevant isotype control antibody according to the manufacturer's instructions (BioLegend, USA). To further evaluate the phenotypic characteristics of Tregs differentiated in the presence of hAECs, five samples from URSA women were simultaneously stained with PE anti-CD25, Alexa Fluor 488 anti-FoxP3, and peridinin chlorophyll protein/cyanin5.5(PerCp/Cy5.5) anti-human CD152 (CTLA-4) antibodies for 30 min at 4 °C. All antibodies and buffers were purchased from BioLegend (USA). Data were acquired using a FACSCalibur flow cytometer and analyzed using CellQuest software. In addition, the levels of TGF-β1 and IL-10 in the supernatant of the co-cultures were measured using an ELISA kit (Mabtech, Sweden) according to the manufacturer's guidelines.

2.11. Functional characterization of tregs

To investigate the functional activity of Tregs generated in the presence of hAECs, their effect on the suppression of allogeneic mixed lymphocyte reaction (MLR) was assessed. Naive CD4⁺ T cells from five URSA patients were cultured in the presence (hAEC-to-naive T cell ratio of 2:1) or absence (control) of hAECs for 6 days as described above and used as a source of Tregs in the allogeneic MLR system. To set the MLR system up, allogeneic stimulator PBMCs were treated with Mitomycin C (5 μg/ml, Sigma-Aldrich, USA) and were cultured (1×10^5 cells/well) in round-bottomed 96-well plates as stimulator cells. Responder PBMCs were obtained from the same URSA patients, labeled with CFSE, and added (1×10^5 cells/well) to the wells containing stimulator cells. Afterwards, different numbers of cells from Treg source ($2–5 \times 10^4$) were added to the wells to prepare the Tregs-to-autologous PBMC ratios of 1:2 and 1:5. Responder PBMCs co-cultured with allogeneic stimulator PBMCs served as the positive control, while responder PBMCs cultured alone were considered as the negative control. All assays were performed in duplicate. The cells were incubated at 37 °C with 5% CO_2 for 3 days. The proliferation of responder PBMCs was then evaluated by flow cytometry.

2.12. Statistical analysis

Data are represented as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism 6 (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) and unpaired t-tests were used to compare the groups with normal distribution and Mann–Whitney and Kruskal–Wallis tests in the case of non-normal distribution. P value < 0.05 was considered statistically significant.

3. Results

3.1. hAECs show some variations in their phenotypic characteristics

To determine the phenotypic characteristics of hAECs, we assessed the presence of some reported markers of these cells isolated from four distinct amnion donors using flow cytometry. Our results showed that the isolated hAECs from four amnion donors extensively expressed

Table 2
Phenotypic characterization of hAECs.

Marker	Donor 1	Donor 2	Donor 3	Donor 4
Pan Cytokeratin	+ + + +	+ + + +	+ + + +	+ + + +
CD73	+ + +	+ + +	+ + +	+ + +
SSEA-4	+ + +	+ + +	+ + +	+ + +
CD133	–	–	–	–
CD90	–	–	–	–
CD105	–	–	–	–
CD34	–	–	–	–
CD45	–	–	–	–
CD9	+ +	–	+	–
CD38	–	–	–	–
CD29	+	+	+	+
CD44	–	+	+	+ +
HLA-DR	–	–	–	–
CD56	– / +	– / +	– / +	– / +
CD14	+	+ +	–	–
CD3	–	–	–	–
CD4	–	–	–	–
CD8	–	–	–	–

-: No expression, − / +: very low expression (< 10%), +: low expression (10–30%).
+ +: intermediate expression (30–60%), + + +: high expression (60–90%).
+ + + +: very high expression (> 90%).

cytokeratin (an epithelial marker) and SSEA-4 (an embryonic stem cell marker), whereas the cells did not express hematopoietic markers including CD34, CD133, and CD45 and some of the mesenchymal stem cell (MSC) markers (CD90, CD105), as expected (Table 2). In contrast to some studies [11,19], hAECs from four donors expressed high level of CD73, an MSC marker (Table 2). Regardless of donor, all cells were negative for HLA-DR, CD38, CD3, CD4, and CD8 markers and expressed very low level of CD56 marker (Table 2). It was also observed that all hAECs expressed low level of integrin-β_2 (CD29), an adhesion molecule (Table 2). As shown in Table 2, hAECs isolated from different donors showed a great variation in hyaluronic acid receptor (CD44), CD14, and CD9 expression.

### 3.2. hAECs do not induce alloreactive responses

To evaluate allogeneic properties of hAECs, allogeneic PBMCs were co-cultured with hAECs at different ratios and the proliferative response of PBMCs was measured. As shown in Fig. 1, there was no statistically significant difference in the proliferation of PBMCs co-cultured with hAECs at different ratios and those cultured alone. As a positive control, PBMCs stimulated with anti-CD3/anti-CD28 antibodies showed high level of proliferation (Fig. 1).

3.3. hAECs suppress the proliferation of naive CD4$^+$ T cells from URSA patients

To assess the possible suppressive effects of hAECs on the proliferation of naive T cells, the proliferation of naive T cells stimulated with anti-CD3/anti-CD28 antibodies in the presence or absence of hAECs was measured. After 3 days, except for the 1:10 ratio, the proliferation of activated T cells were significantly suppressed compared to activated T cells cultured alone (p < 0.05–0.01, Fig. 2A and C). The same trend was also observed for 6-day culture except that the proliferation of naïve T cells co-cultured with hAECs at the 1:10 ratio was also inhibited (p < 0.05–0.001, Fig. 2B and D).

3.4. hAECs exert immunomodulatory effects on the cytokine profile of naive CD4$^+$ T cells from URSA patients

Having considered that cytokines play an important role in pregnancy outcome, we evaluated immunomodulatory effects of hAECs on
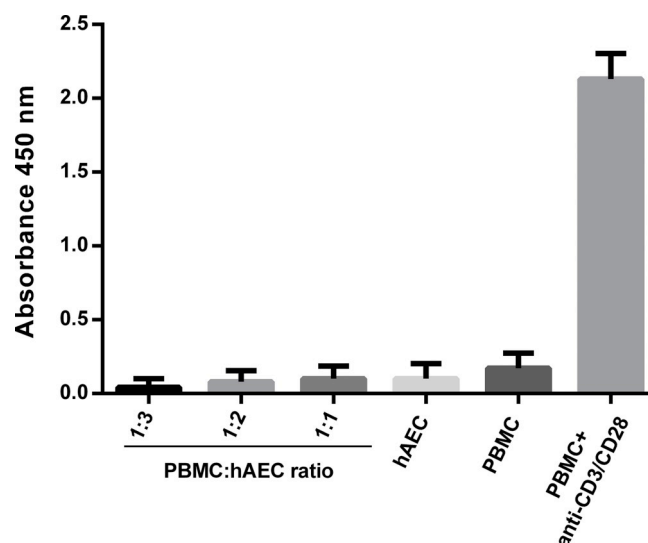
Fig. 1. Allogeneic effects of hAECs on PBMCs from control subjects. PBMCs were co-cultured with different numbers of hAECs for 3 days. The alloreactive response of PBMCs was measured using a BrdU cellular ELISA kit (n = 4). PBMCs stimulated with anti-CD3/anti-CD28 antibodies were used as the positive control. All data are represented as mean ± SEM.

cytokine profile of naive T cells. As shown in Fig. 3A–D, a statistically significant reduction in the levels of IFN-γ and IL-17 A was observed at all hAEC-to-naive T cell ratios after 3 and 6 days of co-culture (p < 0.05–0.0001). Moreover, the level of IL-4 was significantly higher at all hAEC-to-naive T cell ratios after 3 days (p < 0.01–0.0001, Fig. 3E). This significant increase in IL-4 level was not observed on day 6 (Fig. 3F). Despite the changes in the levels of IFN-γ, IL-4, and IL-17 A, no statistically significant difference was observed in the TNF-α level (Fig. 3G and H).

3.5. hAECs induce differentiation of naive CD4$^+$ T cells into CD25 + Foxp3 + CTLA-4 + Tregs

As Tregs favor pregnancy success, we evaluated whether hAECs can induce the production of Tregs from naive CD4$^+$ T cells of URSA and control women. Our results showed that in both URSA patients (p < 0.0001) and normal controls (p < 0.001–0.0001), hAECs significantly induced the production of Tregs at all ratios (Fig. 4A and B). Interestingly, hAECs induced the production of significantly more Tregs in control subjects compared to URSA patients at all ratios tested, with the exception of the 1:10 ratio (p < 0.01–0.05, Fig. 4B). Our data also revealed that Tregs differentiated in the presence of hAECs expressed higher levels of CTLA-4 compared to Tregs differentiated in the absence of hAECs (p < 0.0001, Fig. 5A, B and C).

The levels of TGF-β1 and IL-10, the main cytokines for Treg function, in the co-culture supernatants were also measured. As shown in Fig. 5D and E, there was a statistically significant increase in the levels of TGF-β1 and IL-10 in the co-culture system compared to control wells (p < 0.0001). Although there was no statistically significant difference between the levels of TGF-β1 in URSA patients and control women (Fig. 5D), the level of IL-10 in control women was significantly higher than that in URSA women (p < 0.01, Fig. 5E).

3.6. Tregs differentiated in the presence of hAECs suppress allogeneic MLR

The functional activity of Tregs differentiated in the presence of hAECs was assessed in an allogeneic MLR system. As shown in Fig. 6A and C, Tregs differentiated in the presence of hAECs inhibited MLR assay when added to the wells at all ratios (p < 0.05–0.01), whereas such an inhibitory effect was not observed for Tregs differentiated in
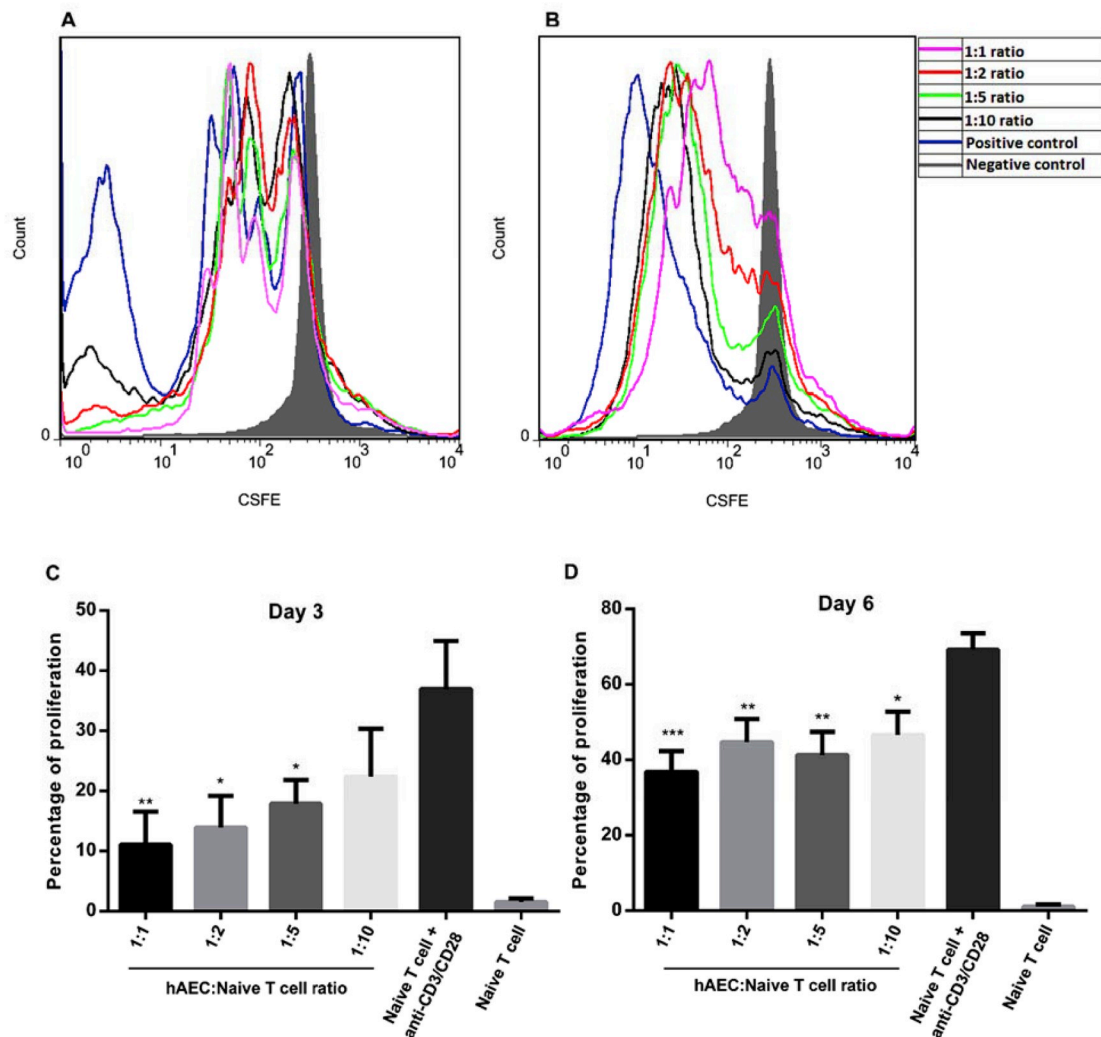
Fig. 2. Effect of hAECs on the proliferation of naive CD4$^+$ T cells from URSA patients. Naive T cells were isolated from URSA patients and then co-cultured at different ratios with hAECs; they were also stimulated with anti-CD3/anti-CD28 antibodies (n = 10). Unstimulated T cells and T cell stimulated with anti-CD3/anti-CD28 antibodies cultured alone were considered as the positive control and negative control, respectively. The proliferation of the CSFE-labeled T cells was monitored by flow cytometry after 3 days (A) and 6 days (B) and later analyzed (C and D). Each bar in C and D shows mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the absence of hAECs (Fig. 6B and C). We also found that the proliferation of responder cells in the wells containing Tregs obtained from hAECs co-culture was significantly lower than that of cells containing Tregs differentiated in the absence of hAECs (Fig. 6C, $p < 0.05$).

4. Discussion

hAECs are a type of stem cells that can be isolated from the amniotic membrane [23]. Previous studies revealed that hAECs have potent immunomodulatory characteristics [11,18]. In this study, we evaluated the immunomodulatory effects of hAECs on naive CD4$^+$ T cells from women with URSA in which multiple defects in maternal immunological tolerance to the fetus have been proposed to be responsible for miscarriage [1]. Our results showed that hAECs suppressed CD4$^+$ T cells proliferation, induced cytokines profile of Th2, and promoted the differentiation of naive CD4$^+$ T cells into Tregs.

URSA is one of the most frustrating problems in reproductive medicine [24,25]. It is reported that in a large proportion of URSA patients, abnormalities in cell-mediated immunity, such as increased Th1-to-Th2 ratio, Tregs deficiency, and increased Th17 cell number, were observed, which may lead to miscarriage [6–8]. Several therapeutic approaches for URSA patients have been proposed to modulate

abnormal maternal immune responses such as paternal lymphocyte immunotherapy and intravenous immunoglobulin (IVIG) therapy [26]. Although the results of some studies indicated that immunotherapy with paternal lymphocytes and IVIG may be helpful in treatment of URSA patients, the evidence is insufficient to support the efficacy of these therapeutic approaches [26]. In addition, there are a number of concerns regarding the side effects of these approaches such as transfusion-related reactions, autoimmunity, and cancer [27,28]. hAECs, which are cells with immunoregulatory effects, may be considered as a potential candidate for immunotherapy of URSA women. Some unique characteristics of these cells make them a suitable cell source in cell therapy of URSA patients, such as a very low level of HLA-class I antigen expression, absence of HLA-class II antigen, co-stimulatory molecules on their surface, and the possibility of their isolation in large quantities without the need to expand them *ex vivo* [11,23,29]. In line with this notion, our results demonstrated that hAECs did not express HLA-DR and did not elicit allogeneic proliferative responses when co-cultured with PMBCs. Accordingly, Liu et al. reported that these cells did not stimulate xenogeneic proliferation of mouse splenocytes [18]. Indeed, hAECs do not express telomerase and might not be tumorigenic upon transplantation [29]. These features alleviate such concerns associated with the application of other stem cell sources as
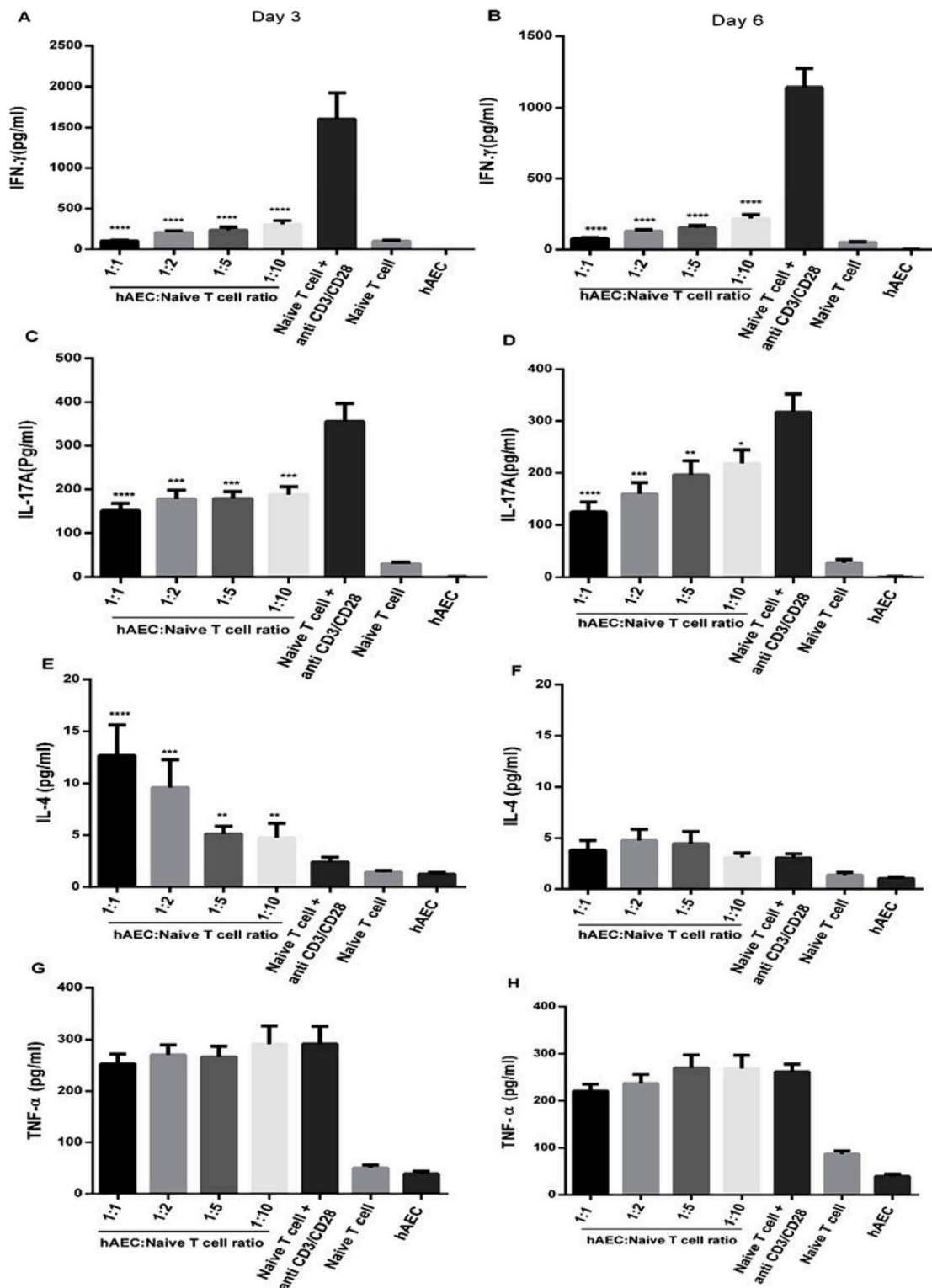
Fig. 3. Cytokine profile of naive CD4$^+$ T cells from URSA patients after co-culture with hAECs in different ratios. Naive T cells from URSA patients were stimulated with anti-CD3/anti-CD28 antibodies in the presence or absence of hAECs at different ratios for 3 and 6 days (n = 18). T cells cultured alone and stimulated with anti-CD3/anti-CD28 antibodies were considered as the positive control. Unstimulated T cells cultured alone and hAECs cultured alone served as negative controls. The levels of IFN-γ (A and B), IL-17 A (C and D), IL-4 (E and F), and TNF-α (G and H) were measured by an ELISA assay. All data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
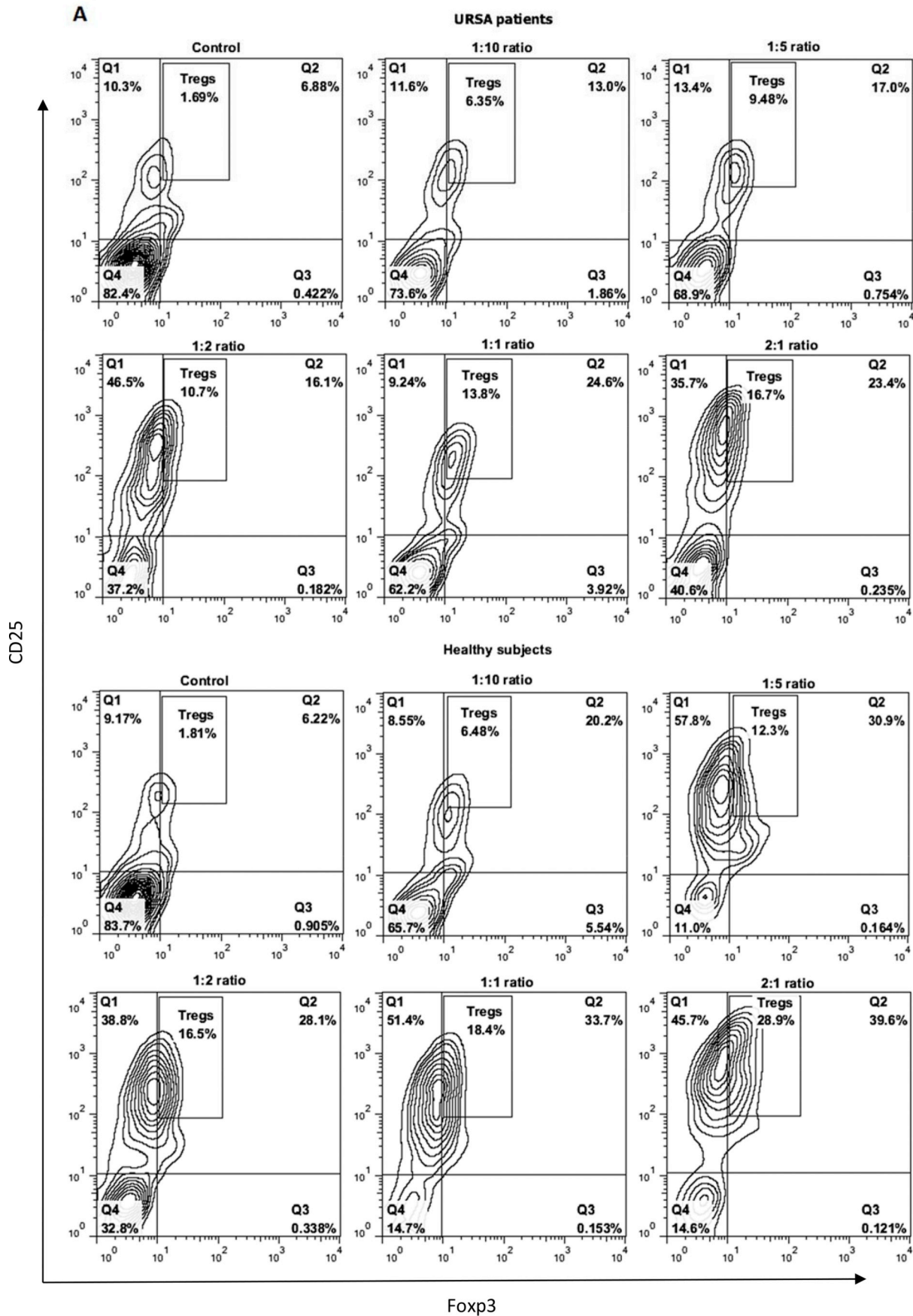
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Fig. 4. Effect of hAECs on Tregs differentiation from naive CD4$^+$ T cells. Different numbers of hAECs were co-cultured with naive CD4$^+$ T cells from URSA women (n = 15) and control women (n = 5). Naive T cells were stimulated with anti-CD3/anti-CD28 antibodies for 6 days. T cells stimulated with anti-CD3/anti-CD28 antibodies without any hAECs were used as control. The percentage of Tregs was monitored by flow cytometry on day 6 (A) and then analyzed (B). Each bar in B shows mean ± SEM. ***p < 0.001, ****p < 0.0001.
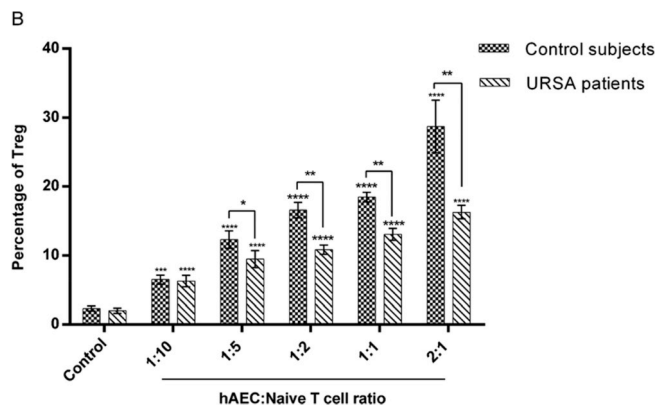

Fig. 4. (*continued*)

immunological rejection and tumor formation after transplantation [29,30].

To assess potential variation in immunophenotype of hAECs from different donors, which may potentially affect their therapeutic efficacy, the expression of a wide range of surface and intracellular markers was evaluated. Our results showed that hAECs from different donors had almost a similar phenotype in terms of the expression pattern of markers attributed to epithelial cells (cytokeratin), embryonic stem cells (SSEA-4), MSCs (CD105, CD73, and CD90), hematopoietic stem cells (CD34), immune cells (HLA-DR, CD56, CD3, CD4, and CD8), and adhesion molecules (CD29). Despite this considerable homogeneity between different hAECs, there were differences in the levels of CD9, CD14, and CD44. There is a noticeable inconsistency in the literature regarding the expression of MSC markers and some other markers on hAECs. Although there are reports pointing to the lack of CD73, CD90, and CD105 expression on hAECs [19,31], there are reports indicating the expression of these markers [11,32]. In this study, we observed that
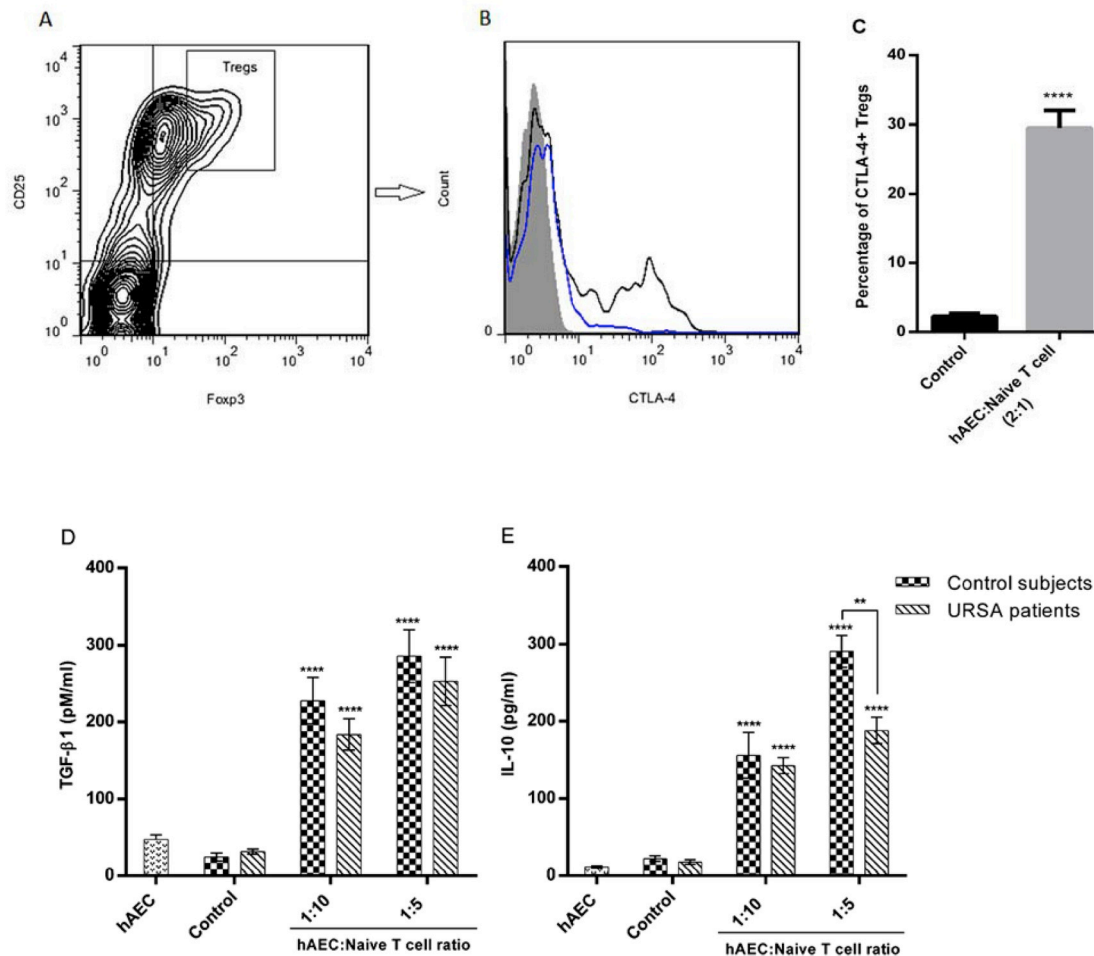
Fig. 5. Phenotypic characterization and cytokine profile of Tregs differentiated in the presence or absence of hAECs. Tregs differentiated in the presence (hAEC-to-naive T cell ratio of 2:1) or absence (control) of hAECs were stained with anti-CD25, anti-Foxp3, and anti-CTLA-4 antibodies (n = 5). The percentage of CTLA-4 + Tregs was monitored by flow cytometry on day 6 (A and B) and then analyzed (C). Gray-shaded histogram: Tregs differentiated in the presence or absence of hAECs were stained with matched isotype control antibodies as negative controls. Black line: Tregs differentiated in the presence of hAECs were stained with anti-CD25, anti-Foxp3, and anti-CTLA-4 antibodies. Blue line: Tregs differentiated in the absence of hAECs were stained with anti-CD25, anti-Foxp3, and anti-CTLA-4 antibodies. The levels of TGF-β1 and IL-10 in co-culture supernatants of hAECs with naive T cells from URSA women (n = 15) and control women (n = 5) were also measured by ELISA on day 6 (D, E). Each bar in C, D and E shows mean ± SEM. **p < 0.01, ****p < 0.0001.
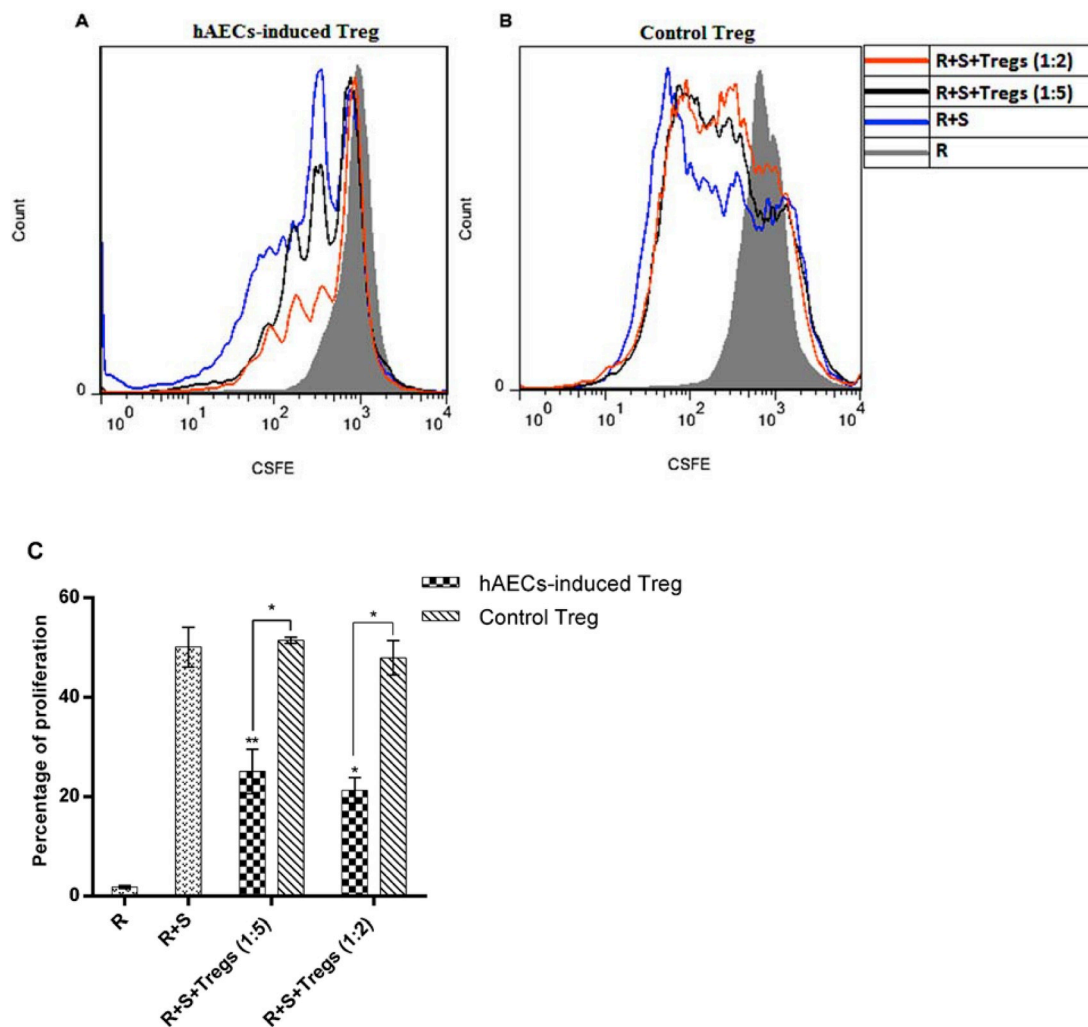
Fig. 6. Immunosuppressive effects of Tregs differentiated in the presence or absence of hAECs on autologous PBMCs from URSA patients. Autologous CSFE-labeled PBMCs were co-cultured with heterologous PBMCs treated with Mitomycin C in an MLR assay. Different numbers of autologous Tregs differentiated in the presence (hAEC-to-naive T cell ratio of 2:1) or absence (control) of hAECs were added to the MLR system (n = 5). Autologous PBMCs and heterologous PBMCs were considered as responder (R) and stimulator (S) cells, respectively. The responder and stimulator cells co-cultured without autologous Tregs served as the positive control (R + S), whereas the responder PBMCs cultured alone were considered as the negative control (R). The proliferation of the responder PBMCs was monitored by flow cytometry on day 3 (A and B) and then analyzed (C). Each bar in C shows mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$.

hAECs isolated from different sources were negative for CD90 and CD105, whereas they expressed a significant level of CD73. This discrepancy could be attributed to cell isolation procedure, culture condition, number of cell passage, and the time of delivery in which the placenta samples were obtained [22,32–34]. For instance, the level of HLA-G expression in preterm hAECs is less than that in term hAECs [34].

In the next step, we evaluated immunomodulatory effects of hAECs on the proliferation and differentiation of naive CD4$^+$ T cells from URSA patients. Our results showed that hAECs restrained naive CD4$^+$ T cells from cell division in a dose-dependent manner. Several lines of evidence suggest that hAECs suppress the proliferation of T cells through TGF-β1 and PGE2 production [18,19]. We also observed that hAECs were capable of producing TGF-β1. In an effort to assess the immunoregulatory effects of hAECs on Th1/Th2 cytokine profile, we found a significant reduction in IFN-γ level at all tested hAECs to naive T cells co-culture ratios after 3 and 6 days, which is consistent with *in vitro* studies conducted on CD4$^+$ T cells from healthy individuals [11]. An increase in the level of IL-4 was also observed after 3 days of co-culture, but not after 6 days, which may be due to the instability and quick degradation of this cytokine. Our results support previous studies and indicate that immunotherapy with hAECs led to a shift from Th1-

type responses to Th2-type responses in an animal model of multiple sclerosis and also to an improvement in disease course [11,18].

As it has been suggested that Th17 cells are harmful to the successful pregnancy and the number of Th17 cells is higher in the peripheral blood of URSA patients compared to the normal controls [8,35], we next evaluated the impact of hAECs on the production of IL-17 from naive CD4$^+$ T cells in URSA women. Interestingly, hAECs caused a significant decrease in IL-17 level after 3 and 6 days co-culture. The same effect has also been reported after intraperitoneal injection of hAECs to animals with experimental autoimmune encephalomyelitis (EAE) [11].

Tregs play an important role in the maintenance of self-tolerance and establishment of immunologic tolerance at the feto–maternal interface [6,36,37]. Previous studies indicated that the percentage of decidual Tregs was decreased in patients with URSA [6,38]. In the current study, we showed that hAECs had the ability to induce differentiation of naive CD4$^+$ T cells toward Tregs in both URSA patients and control women. Interestingly, our data revealed that differentiation of naive CD4$^+$ T cells into Tregs in the presence of hAECs occurred more efficiently in control subjects compared to URSA women. This finding was in line with our finding showing that supernatant of naive T cells/ hAECs co-cultures from control women contained significantly more

TGF-β1, which is needed for Treg differentiation. Indeed, the levels of IL-10 and TGF-β1 were dramatically increased when naive T cells were co-cultured with hAECs, thus indicating the supportive role of hAECs for Treg induction. The ability of hAECs in inducing Tregs is consistent with the finding from previous studies in which an increase in the number of Tregs was observed in animals with EAE and fibrotic lung injury following immunotherapy with hAECs [11,39]. In an effort to determine the functionality of Tregs differentiated in the presence of hAECs, we found the result contrary to that of control Tregs; these cells were able to suppress the proliferation of autologous PBMCs from URSA patients in an MLR assay. This finding may be due to altered immunophenotype of Tregs generated in the presence of hAECs, as we showed that these cells expressed significantly higher level of CTLA-4 compared to that of the control Tregs. Notably, CTLA-4 was reported to be responsible for all three characteristic Treg functions of suppression, TCR hyposignaling, and anergy [40]. Indeed, a significant increase in the number of Tregs differentiated in the presence of hAECs compared to those differentiated in the absence of hAECs might also be responsible for their differential suppressive effect in the MLR system.

Overall, the results of this study provide evidence to show that hAECs possess potent immunomodulatory effects on naive CD4$^+$ T cells from URSA patients, including suppressing cell proliferation, promoting functional Tregs differentiation, skewing cytokine profile to Th2 pattern, and inhibiting Th1 and Th17 cytokine production. Based on these findings and also their partial inertness in eliciting alloreactive immune responses, hAECs can be considered as one of the potential candidates for the immunotherapy of patients with URSA. Further studies and more information are required to determine whether hAECs can be considered for clinical application in URSA.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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