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JEG-3 Trophoblast Cells Producing Human Chorionic Gonadotropin Promote Conversion of Human CD4⁺FOXP3⁻ T Cells into CD4⁺FOXP3⁺ Regulatory T Cells and Foster T Cell Suppressive Activity¹

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ABSTRACT

The pregnancy hormone human chorionic gonadotropin (hCG) reportedly modulates innate and adaptive immune responses and contributes thereby to fetal survival. More precisely, hCG has been shown to support human regulatory T cell (Treg cell) homing into the fetal-maternal interface and enhance the number and function of Treg cells in murine pregnancy. Here, we aimed to study whether hCG and hCG-producing human trophoblast cell lines induce Treg cells from CD4⁺FOXP3⁻ T cells and promote T cell suppressive activity. CD4⁺FOXP3⁻ T cells were isolated from peripheral blood of normal pregnant women and cultured in the presence of hCG-producing (JEG-3, HTR-8) and nonproducing (SWAN-71) cell lines. To confirm the participation of hCG in Treg cell conversion, the experiments were performed in the presence of anti-hCG and additional experiments were run with recombinant or urine-purified hCG. After culture, the number of CD4⁺FOXP3⁺ Treg cells as well as the suppressive capacity of total T cells was assessed. The hCG-producing JEG-3 cells as well as recombinant and urine-purified hCG induced CD4⁺FOXP3⁺ Treg cells from CD4⁺FOXP3⁻ T cells. Blockage of hCG impaired Treg cell induction. Moreover, hCG-producing JEG-3 cells increased suppressive activity of CD4⁺FOXP3⁻ T cells through an antigen-independent pathway. Our results propose another mechanism through which hCG modulates the female immune system during pregnancy in favor of the fetus.

fetal tolerance, human chorionic gonadotropin, pregnancy, regulatory T cells, trophoblast cells

INTRODUCTION

Despite intensive research activity during the last few decades, the survival of the semi-allogeneic fetus within the

harmful uterine environment remains a conundrum. There is a scientific consensus that an active regulation of maternal innate and adaptive immune responses is fundamental to ensure establishment and maintenance of a successful pregnancy. Moreover, pregnancy hormones such as progesterone, estrogen, and human chorionic gonadotropin (hCG) were proposed to act as key regulators of antifetal immune responses. As for hCG, several studies indicated an influence of this hormone on pregnancy outcome by affecting various immune cell populations like B cells [1, 2], dendritic cells [3], and T cells [4, 5].

Regulatory T cells (Treg cells) are a unique subpopulation of T cells with regulatory activity that are critically involved in fetal tolerance induction. The relevance of Treg cells for a successful pregnancy outcome is well documented in studies in which the occurrence of miscarriages and pre-eclampsia has been associated with insufficient Treg cell elevation and functionality [6, 7]. In human pregnancy, Treg cell augmentation takes place as pregnancy begins, although there is some discrepancy between different studies [8, 9]. In the third trimester, Treg cell numbers start to decrease [10] and further decline occur with successive stages of labor [8]. In normal pregnant mice, Treg cell augmentation starts as early as Day 2 of pregnancy, then Treg cell levels decrease around implantation time and raise again on Day 10 of pregnancy [11, 12]. Importantly, significant higher Treg cell levels were identified in normal human decidual tissue when compared to peripheral blood [13–15]. A preferential recruitment of Treg cells from the periphery into the fetal-maternal interface [16] as well as mechanisms supporting a local enrichment of Treg cells by expansion or conversion have been suggested [14, 15]. Most studies indicated a local reduction of Treg cells in human pregnancy complications [17, 18], even though contradictory observations have been reported [19]. Overall, it can be assumed that local factors and mechanisms contribute to an enrichment of Treg cells at the fetal-maternal interface. Trophoblast cells have been suggested to secrete factors that positively modulate Treg cell numbers [20, 21]. However, the participation of trophoblast-derived hCG, in the modulation of Treg cell function has not been fully explored. In previous studies, we identified hCG as an efficient attractor of Treg cells into the fetal-maternal interface and suggested that low levels of Treg cells in spontaneous abortion patients might be associated with low amounts of hCG in those patients [22]. Furthermore, we confirmed an additional function for hCG on Treg cell increment and activity in murine pregnancy [23]. In mice, significant alterations in Treg cell levels after hCG application could only be observed in pregnant animals but not in nonpregnant animals. Moreover, increased Treg cell suppression after hCG application was antigen dependent,

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suggesting that hCG enhances the number and function of antigen-preprimed Treg cells [23].

To understand whether trophoblast-derived hCG is involved in Treg cell induction during human pregnancy, we designed *in vitro* studies involving trophoblast cell lines instead of primary trophoblast cells. Trophoblast cell lines are often used because of limitations in obtaining patient samples in sufficient numbers to perform experiments and because of their infinite lifespan that facilitates the reproducibility of experiments. Here, we included one choriocarcinoma cell line (JEG-3) and two trophoblast cell lines (HTR-8, SWAN-71) [24, 25]. The method used to immortalize these cells did not affect their karyotype and phenotype [26, 27]. Both cell lines show similar characteristics of primary trophoblast cells and have already been proven to be valuable tools in *in vitro* studies [28, 29]. However, it needs to be said that differences between primary trophoblast cells and trophoblast cell lines have been identified [30, 31]. In line with this, Tilburgs and colleagues [32] confirmed differences in the transcriptome of JEG-3 cells and primary extravillous trophoblasts. Interestingly, the same authors also identified major differences in the expression pattern between primary extravillous trophoblasts and villous trophoblasts and provided evidence that extravillous trophoblasts are more capable of increasing the proportion of CD4⁺CD25^{high}FOXP3⁺CD45RA⁺ resting Treg cells [32], information that is relevant for our study. Being aware of the discrepancies between primary trophoblast cells and trophoblast cell lines, we nevertheless chose to work with cell lines for a very important reason: trophoblast cell lines consistently secrete a certain amount of hCG in the culture, a fact that could not be observed in primary cultures. Thus, we sought to evaluate whether trophoblast-derived hCG is directly involved in Treg cell induction and furthermore studied its influence in T cell-suppressive function.

MATERIALS AND METHODS

Sample Collection of Human Material and Ethical Approval

Peripheral blood samples were obtained from normal pregnant women ($n = 19$; age: 30.95 ± 5.26 ; wk of gestation: 28.47 ± 4.31). Sampling was conducted by the clinicians of the University Women's Clinic in Magdeburg, Germany. This was previously approved by the Ethics Board at the University of Magdeburg (study 28/08). All pregnant women gave their written consent after they were informed in detail about the aim of the study.

Cell Lines

Both the choriocarcinoma cell line JEG-3 and the keratinocyte cell line HaCat were cultured in Dulbecco-modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Biocrom) and 100 nM penicillin/streptomycin (Invitrogen). The immortalized human cytotrophoblast cell lines HTR-8 and SWAN-71 were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen) or Dulbecco-modified Eagle medium, respectively, supplemented with 10% fetal bovine serum, 100 nM MEM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 10 mM Hepes (Biocrom), and 100 nM penicillin/streptomycin. All cell lines were cultured as monolayers at 37°C and 5% CO₂.

Isolation and Purity of CD4⁺FOXP3⁻ T Cells

Peripheral blood mononuclear cells were obtained by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Afterward, CD4⁺CD25⁻ T cells were isolated by magnetic-activated cell sorting (MACS) using the Regulatory T Cell Isolation Kit (human) from Miltenyi Biotec. All the steps were performed under sterile conditions following the instructions of the manufacturer. After isolation, cells were analyzed for CD4, CD25, and FOXP3 expression by flow cytometry. Our analysis revealed a purity of >98% of CD4⁺CD25⁻FOXP3⁻ T cells. Accordingly, we used CD4⁺FOXP3⁻ T cells in our study (Fig. 1, A–D).

Determination of hCG Secretion in Supernatants of All Cell Lines by Enzyme-Linked Immunosorbent Assay

The amount of hCG secreted in the supernatants of all cell lines was determined using the β -hCG enzyme-linked immunosorbent assay kit from DRG Instruments. All steps were performed according to the instructions of the manufacturer. The culture plates received 5×10^4 JEG-3, HTR-8, HaCat, and SWAN-71 cells. After 24 h of culture, the supernatants were obtained and analyzed for their hCG content. While JEG-3 cells secreted high amounts of hCG, only low hCG levels were detectable in the supernatant of HTR-8 cells. No traceable hCG was present in the supernatants of SWAN-71 cells or HaCat cells (Fig. 1E).

Conversion Assays

To analyze the participation of hCG in the conversion of CD4⁺FOXP3⁻ T cells into CD4⁺FOXP3⁺ Treg cells, CD4⁺FOXP3⁻ T cells were cultured under different conditions. In the first set of experiments, 1×10^5 CD4⁺FOXP3⁻ T cells were cocultured with 5×10^4 hCG-producing (JEG-3, HTR-8) or non-hCG-producing (HaCat, SWAN-71) cells. Trophoblast cells and keratinocytes were plated 24 h before coculture to allow cell adherence to the culture plate.

In the second set of experiments, 1×10^5 CD4⁺FOXP3⁻ T cells were cocultured with 5×10^4 JEG-3 cells in the presence or absence of a neutralizing anti-hCG antibody or an immunoglobulin G (IgG) control antibody; both antibodies were diluted 1:50 and were purchased from Santa Cruz. As indicated above, JEG-3 cells were plated 24 h before starting the coculture experiment.

In the third set of experiments, 1×10^5 CD4⁺FOXP3⁻ T cells were cultured in the presence of either urine-purified hCG (uhCG) (Pregnyl; Organon) or recombinant hCG (rhCG) (Ovitrelle; Merck Serono Europe Limited). The uhCG was used in concentrations of 100, 250, and 500 IU/ml, while rhCG was used in concentrations of 50, 100, and 500 mIU/ml.

In all three sets of experiments, CD4⁺FOXP3⁻ T cells cultured alone served as controls. Coculture was performed for 72 h. T cell activation was induced by the addition of 1 μ g/ml anti-CD3 and 5 μ g/ml anti-CD28, both purchased from BD Biosciences, in the presence of 10 ng/ml IL-2 (R&D System). Conversion into Treg cells was assessed by determining the number of CD4⁺FOXP3⁺ Treg cells by flow cytometry.

Suppression Assays

To determine whether hCG-treated CD4⁺FOXP3⁻ T cells have the potential to suppress the proliferation of autologous or allogeneic T cells, the following suppression assays were performed. First, 2.5×10^5 JEG-3 or SWAN-71 cells were placed on a cell culture plate to allow adherence. After 24 h, 5×10^5 CD4⁺FOXP3⁻ T cells were added and cocultured for 72 h. In addition, 5×10^5 CD4⁺FOXP3⁻ T cells were cultured in the presence of 100 mIU/ml rhCG for 72 h. Afterward, hCG-treated and non-hCG-treated CD4⁺FOXP3⁻ T cells (including converted Treg cells), being in suspension, were separated from adherent JEG-3 or SWAN-71 cells and again cultured for 48 h with autologous or allogeneic T cells in a ratio of 1:1 (1×10^5 T_{total}: 1×10^5 T_{autologous/allologous}). Autologous/allologous T cells were either isolated from peripheral blood of the same donor who provided the CD4⁺FOXP3⁻ T cells (autologous) or from a third-party donor (allogeneic). After isolation of autologous/allologous T cells by MACS technology using the regulatory T cell isolation kit (human) (Miltenyi Biotec), T_{autologous/allologous} were stained with the green fluorescent dye CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit; Molecular Probes) to determine their proliferation. T_{autologous/allologous} cultured alone served as controls. During the whole experiment, all T cells were cultured in the presence of anti-CD3, anti-CD28, and IL-2. Analysis of T_{autologous/allologous} proliferation was performed by flow cytometry.

Flow Cytometry Analysis

To assess their purity, isolated T cells were stained for the extracellular markers CD4 and CD25 as well as for the intracellular marker FOXP3. After culture, T cells were stained for the intracellular marker FOXP3. Extra- and intracellular staining procedure was conducted using our established staining protocols [33]. The following antibodies were used: PE-labeled CD4 (dilution 1:10; clone RPA-T4), APC-labeled CD25 (dilution 1:5; clone M-A251), AF488-labeled FOXP3 (dilution 1:5; clone 259D/C7), PE-labeled IgG isotype control (dilution 1:10; clone MOPC-21), APC-labeled IgG isotype control (dilution 1:5; clone MOPC-21), and AF488-labeled IgG isotype control (dilution 1:5; clone MOPC-21). All the antibodies were purchased from BD Pharmingen. T cells were analyzed on a FACSCalibur from BD Biosciences.

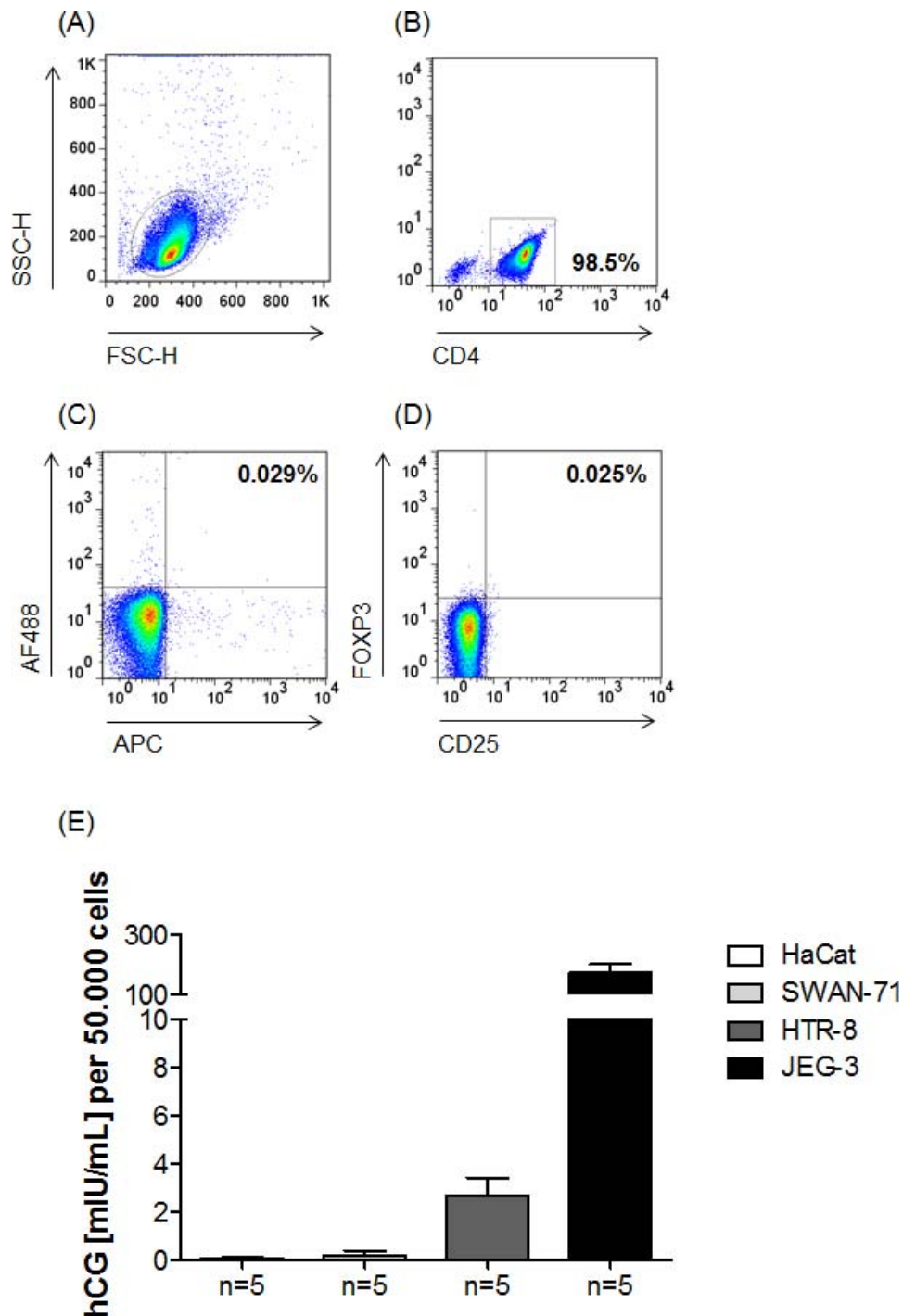


FIG. 1. Number of resting CD4⁺CD25⁻FOXP3⁺ T cells after magnetic-activated cell sorting (MACS) and hCG secretion by different cell lines. **A–D**) After isolation of CD4⁺CD25⁻ T cells by MACS from peripheral blood mononuclear cells of pregnant women, the number of resting CD4⁺CD25⁻FOXP3⁺ T cells was assessed by flow cytometry. A total of 98.5% of all lymphocytes (**A**) were stained positive for CD4 (**B**). Within the total CD4⁺ T cell population, only 0.025% cells were CD25⁻ and FOXP3 double positive (**D**). Isotype control is shown in **C**. **E**) To assess hCG secretion by different cell lines, 5×10^4 JEG-3, HTR-8, HaCat, and SWAN-71 cells were placed into culture plates. After 24 h of culture, the supernatants were obtained and analyzed for their hCG content by enzyme-linked immunosorbent assay. While JEG-3 cells secreted high amounts of hCG, only low hCG levels were detectable in the supernatant of HTR-8 cells (**E**). Moreover, no hCG was present in the supernatants of SWAN-71 cells and HaCat cells (**E**).

Data Analysis and Statistics

All the conversion assays were performed three to seven times in duplicates. Suppression assays were conducted three times in duplicates. Data presentation and analysis were performed with GraphPad Prism 5.0 software (Statcon). Each data set was tested for normal distribution. If the data were normally distributed, they were presented as means plus the standard error of

the mean. Statistical analysis was performed using one-way ANOVA followed by Bonferroni correction for multiple comparisons. For not normally distributed data, the nonparametric Friedman test followed by Dunn posttest was applied, and medians plus interquartile range are displayed.

RESULTS

JEG-3 Trophoblast Cells Induced the Conversion of CD4⁺FOXP3⁻ T Cells into CD4⁺FOXP3⁺ Treg Cells

Following the hypothesis that hCG, produced by trophoblast cells, might be involved in the local conversion of CD4⁺FOXP3⁻ T cells into Treg cells, we cocultured CD4⁺FOXP3⁻ T cells with either hCG-producing HTR-8 and JEG-3 trophoblast cells or non-hCG-producing SWAN-71 trophoblast cells (Fig. 1E). We included the keratinocyte cell line HaCat, lacking hCG expression, in our analysis (Fig. 1E). We decided to analyze pregnancy-primed CD4⁺FOXP3⁻ T cells from pregnant women because our murine data had indicated an effect of paternal antigens on hCG-mediated Treg cell induction [23]. To evaluate Treg cell levels, we determined the number of CD4⁺FOXP3⁺ cells because FOXP3 is a common marker to identify and characterize Treg cells. We found a significant increase in the number of CD4⁺FOXP3⁺ Treg cells after coculture with hCG-producing JEG-3 cells (Fig. 2). In contrast, the non-hCG-producing HaCat cells were not able to enhance CD4⁺FOXP3⁺ Treg cell number (Fig. 2). Interestingly, HTR-8 and SWAN-71 trophoblast cells, although secreting only little or no hCG, respectively, tended to augment the number of CD4⁺FOXP3⁺ Treg cells (Fig. 2). This suggests that in addition to hCG, other factors are involved in Treg cell conversion.

Blockage of hCG Abrogated JEG-3 Cell-Induced Conversion of CD4⁺FOXP3⁻ T Cells in CD4⁺FOXP3⁺ Treg Cells

We next aimed to investigate to what extent hCG is responsible for Treg cell augmentation. For this, we blocked hCG in the supernatant of JEG-3 cells and analyzed the Treg cell number. We confirmed an increase in the number of CD4⁺FOXP3⁺ Treg cells when CD4⁺FOXP3⁻ T cells were cultured in the presence of JEG-3 cells. This effect was absent in the presence of an hCG-neutralizing antibody (Fig. 3). Hence, although the participation of other factors cannot be excluded, JEG-3 trophoblast cell-derived hCG is an important modulator of Treg cell number because its blockage hindered Treg cell augmentation.

Addition of rhCG and uhCG to CD4⁺FOXP3⁻ T Cell Cultures Provoked Their Conversion into CD4⁺FOXP3⁺ Treg Cells

To unequivocally confirm the direct effect of hCG on Treg cell generation, we cultured CD4⁺FOXP3⁻ T cells in the presence of either rhCG or uhCG. In addition, we analyzed whether various concentrations of both hCG forms would differently affect CD4⁺FOXP3⁻ T cells. Regardless of the concentration used, both rhCG (Fig. 4) and uhCG (Fig. 5) significantly increased the number of CD4⁺FOXP3⁺ Treg cells. Thus, hCG has the ability to stimulate the conversion of T cells into Treg cells.

Pretreatment with hCG Augmented the Suppressive Capacity of CD4⁺FOXP3⁻ T Cells

Finally, we wondered whether CD4⁺FOXP3⁻ T cells that were pretreated with hCG possess an increased suppressive capacity. We hypothesized that an increased suppressive activity of the T cell pool is due to the shown augmentation of Treg cells within the pool. Unfortunately, due to the low number of Treg cells, it was technically impossible to re-isolate

Treg cells from the whole T cell pool and to analyze their suppressive capacity separately. We therefore decided to determine the suppressive activity of all hCG-treated CD4⁺FOXP3⁻ T cells (cocultured with JEG-3 cells or 100 mIU/ml rhCG). We included non-hCG-treated CD4⁺FOXP3⁻ T cells (cocultured with SWAN-71 cells) in our analysis because SWAN-71 cells also augmented Treg cell number. Additionally, we were interested in studying whether prepriming by autoantigens or alloantigens might play a role in T cell function. Thus, the capacity of total T cells that have been previously cultured in the presence or absence of hCG to suppress proliferation was tested on both autologous and allogeneic T cells. We found a significantly elevated suppressive activity of CD4⁺FOXP3⁻ T cells previously cocultured with hCG-producing JEG-3 cells and rhCG. Coculture with SWAN-71 cells increased, although not significantly, the potential of CD4⁺FOXP3⁻ T cells to suppress responder T cells (Fig. 6, A and B). Moreover, hCG-treated CD4⁺FOXP3⁻ T cells suppressed autologous and allogeneic T cells to the same extent (Fig. 6, A and B). Hence, the contact of T cells with hCG-producing trophoblast cells fosters T cell suppressive function.

DISCUSSION

Normal pregnancy is characterized by strong hormonal changes, and there is accumulating evidence that pregnancy-associated hormones are positive modulators of the immune system during pregnancy. Several studies indicated that hormones such as progesterone, estradiol, and hCG are able to modulate both innate and adaptive immune responses (reviewed in [34]) and thereby contribute to fetal tolerance. We identified hCG as a key factor for human Treg cell homing into the fetal-maternal interface [22], and we proved a positive influence of hCG on the number and function of murine Treg cells [23]. Here, we investigated whether hCG is involved in trophoblast cell-induced conversion of human CD4⁺FOXP3⁻ T cells into CD4⁺FOXP3⁺ Treg cells and whether hCG-producing JEG-3 trophoblast cells affect T cell suppressive capacity. We found a significant increase in the number of Treg cells after coculture with JEG-3 cells that produces high amounts of hCG. Keratinocytes do not produce hCG and had no effect on Treg cell number. The trophoblast cell lines HTR-8 and SWAN-71, which in our hands only produce low or no detectable amounts of hCG, respectively, also augmented the number of Treg cells. In line with this finding, Ramhorst and colleagues [20] showed that cell culture supernatants from SWAN-71 and HTR-8 cells contributed to the differentiation of inducible Treg cells from maternal naïve T cells by significantly augmenting FOXP3 expression. The authors revealed a high expression of TGF-β1 and TGF-β2 by HTR-8 and SWAN-71 cells and proposed TGF-β as a factor involved in local Treg cell differentiation at the fetal-maternal interface. More recently, the same group showed that the anti-inflammatory molecule vasoactive intestinal peptide secreted by SWAN-71 cells may also play a role in local Treg cell induction [35]. Additionally, Ramhorst and colleagues [36] proposed galectin-1 as a factor provoking Treg cell increment. These findings proposed trophoblast cell-associated factors involved in Treg cell generation besides hCG. Here and to confirm the important participation of hCG as an additional factor in trophoblast cell-mediated Treg cell conversion, we performed blocking experiments. We observed that hCG blockage abrogated JEG-3-induced Treg cell augmentation, which highlights the fact that hCG represents one of the major factors involved in JEG-3-mediated Treg cell induction.

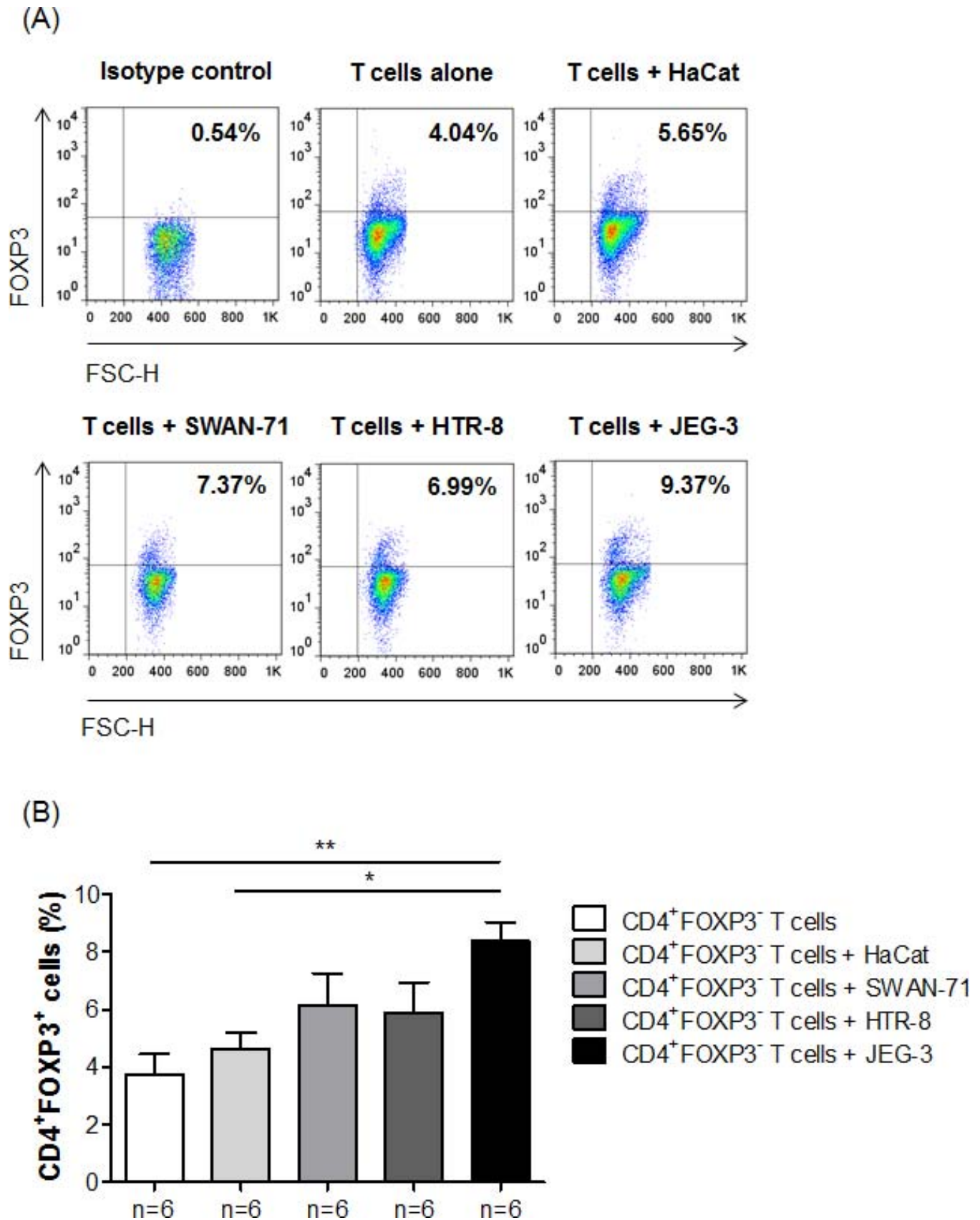


FIG. 2. JEG-3 trophoblast cells induced CD4⁺FOXP3⁺ Treg cells. **A–B**) CD4⁺FOXP3⁻ T cells were cocultured with hCG-producing (JEG-3, HTR-8) or non-hCG-producing (SWAN-71, HaCat) cell lines. CD4⁺FOXP3⁻ T cells cultured alone served as controls. After 72 h of culture, the number of CD4⁺FOXP3⁺ Treg cells was determined by flow cytometry. Representative dot plots of each coculture are shown in **A**, and all cocultures are displayed as graphic in **B**. Results are representative of six independent experiments with six donors individually analyzed. Data are presented as means plus SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni correction for multiple comparisons (* $P < 0.05$; ** $P < 0.01$).

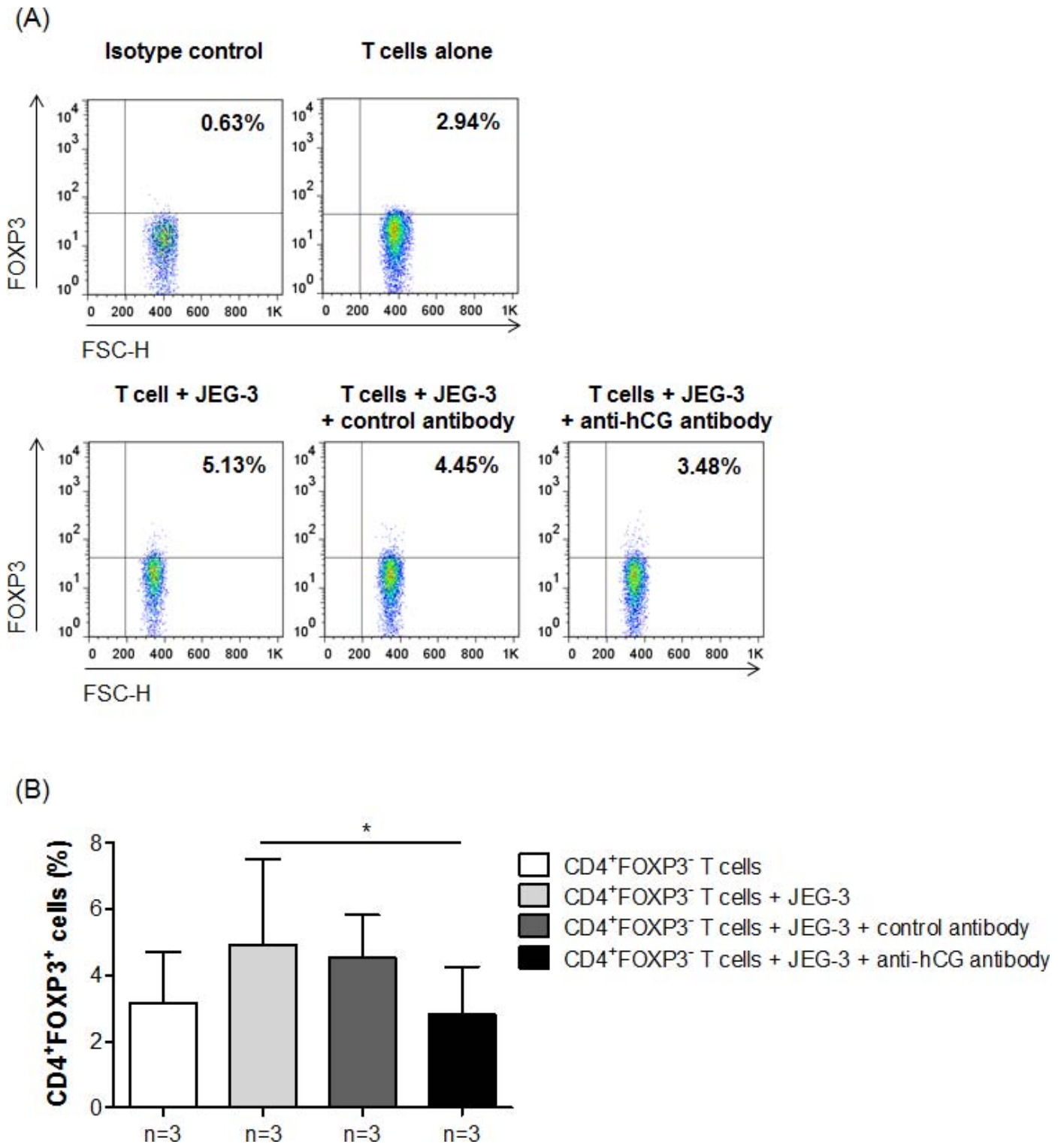


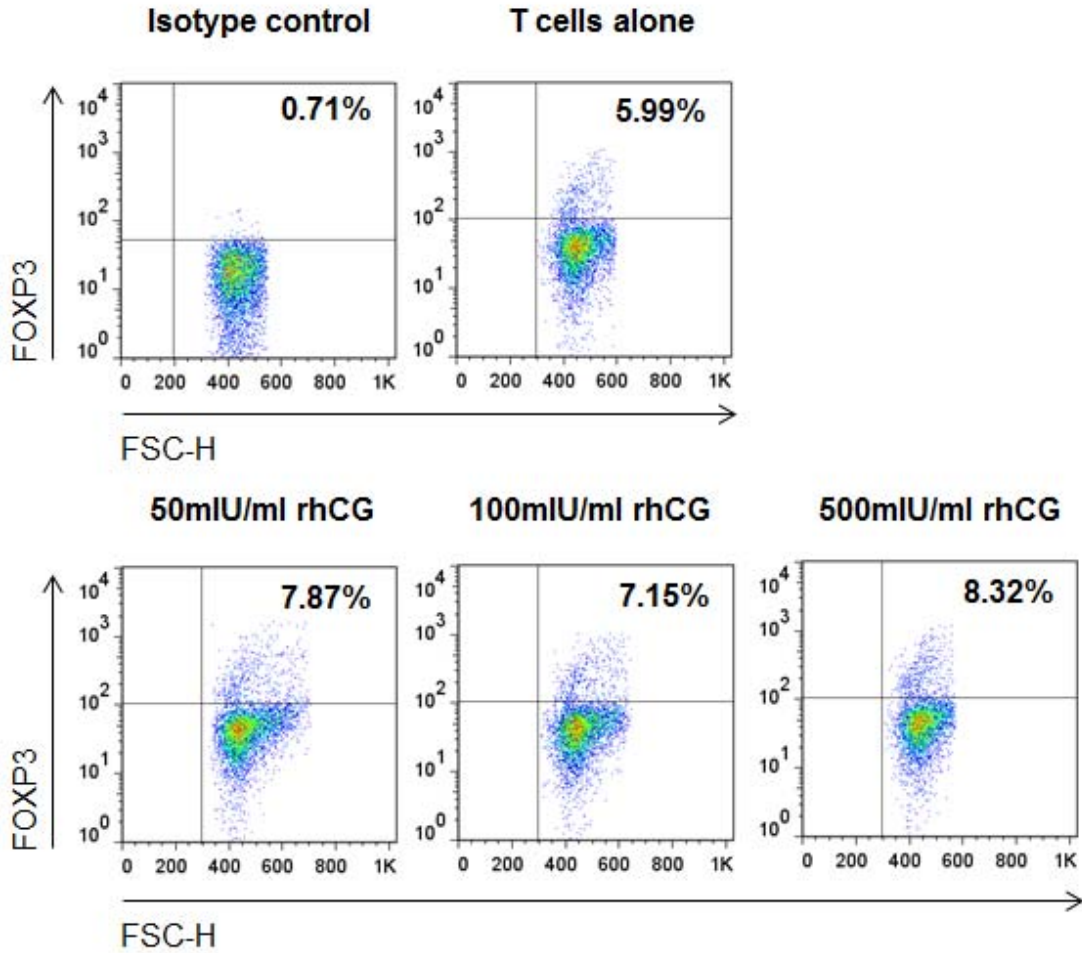
FIG. 3. Blockage of hCG impaired JEG3-mediated CD4⁺FOXP3⁺ Treg cell induction. **A–B**) CD4⁺FOXP3⁻ T cells were cocultured with hCG-producing JEG-3 cells in the presence or absence of a neutralizing anti-hCG antibody. After 72 h of culture, the number of CD4⁺FOXP3⁺ Treg cells was determined by flow cytometry. Representative dot plots of each coculture are shown in **A**, and all cocultures are displayed as graphic in **B**. Three different patient samples were analyzed in duplicates. Data are presented as medians plus interquartile range. Statistical analysis was performed using the Friedman test followed by Dunn posttest (**P* < 0.05).

Furthermore, our observation that both rhCG and uhCG significantly augmented Treg cell number confirmed the potential of hCG to induce Treg cells from CD4⁺ T cells.

In the next set of experiments, we showed that CD4⁺FOXP3⁻ T cells that have been cultured in the presence

of hCG-producing JEG-3 cells or rhCG possessed an increased suppressive capacity. Unfortunately, due to technical difficulties and the high number of Treg cells that would be required for performing further experiments, we were not able to re-isolate Treg cells from the total T cell pool in enough numbers

(A)



(B)

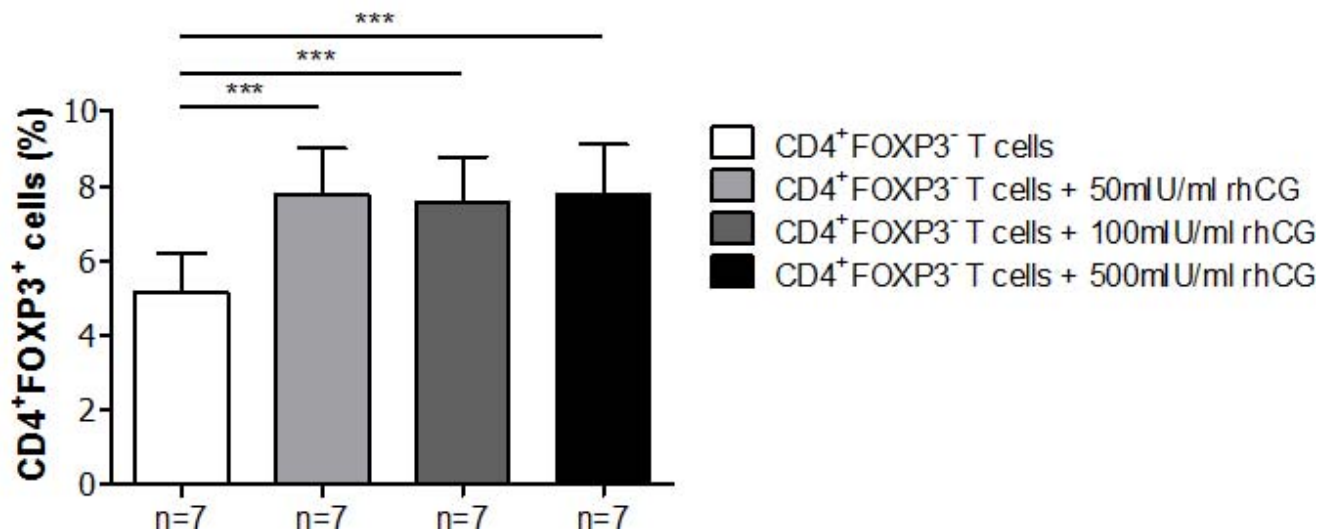


FIG. 4. CD4⁺FOXP3⁺ Treg cells induced by rhCG. **A–B**) CD4⁺FOXP3⁻ T cells were cocultured in the presence of different concentrations of rhCG. After 72 h of culture, the number of CD4⁺FOXP3⁺ Treg cells was determined by flow cytometry. Representative dot plots of each hCG concentration are shown in **A**, and all concentrations are displayed as graphic in **B**. Seven different patient samples were analyzed in duplicates. Data are presented as means plus SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni correction for multiple comparisons (***P* < 0.001).

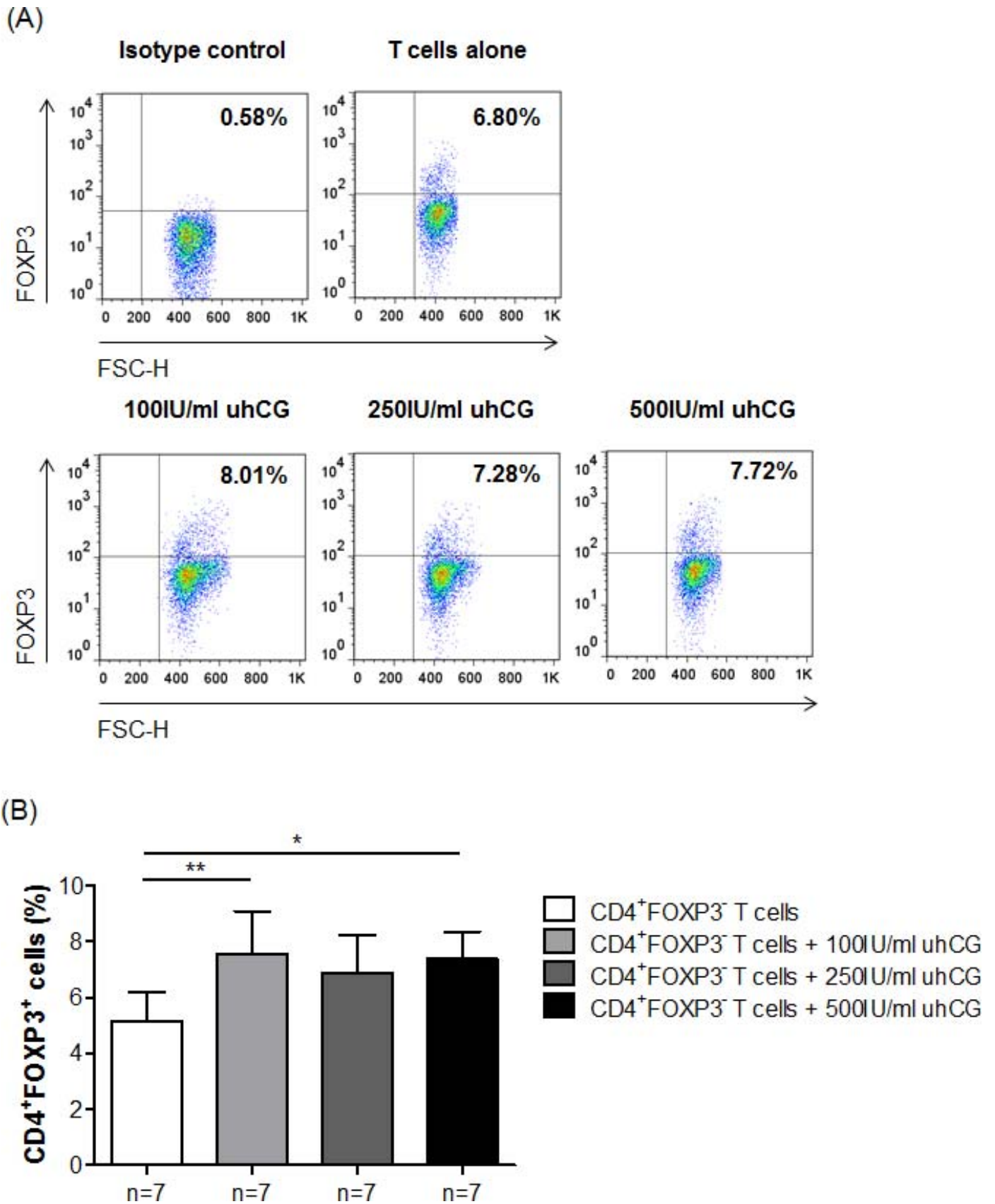


FIG. 5. CD4⁺FOXP3⁺ Treg cells induced by uhCG. **A–B** CD4⁺FOXP3⁻ T cells were cocultured in the presence of different concentrations of uhCG. After 72 h of culture, the number of CD4⁺FOXP3⁺ Treg cells was determined by flow cytometry. Representative dot plots of each hCG concentration are shown in **A**, and all concentrations are displayed as graphic in **B**. Seven different patient samples were analyzed in duplicates. Data are presented as means plus SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni correction for multiple comparisons (* $P < 0.05$; ** $P < 0.001$).

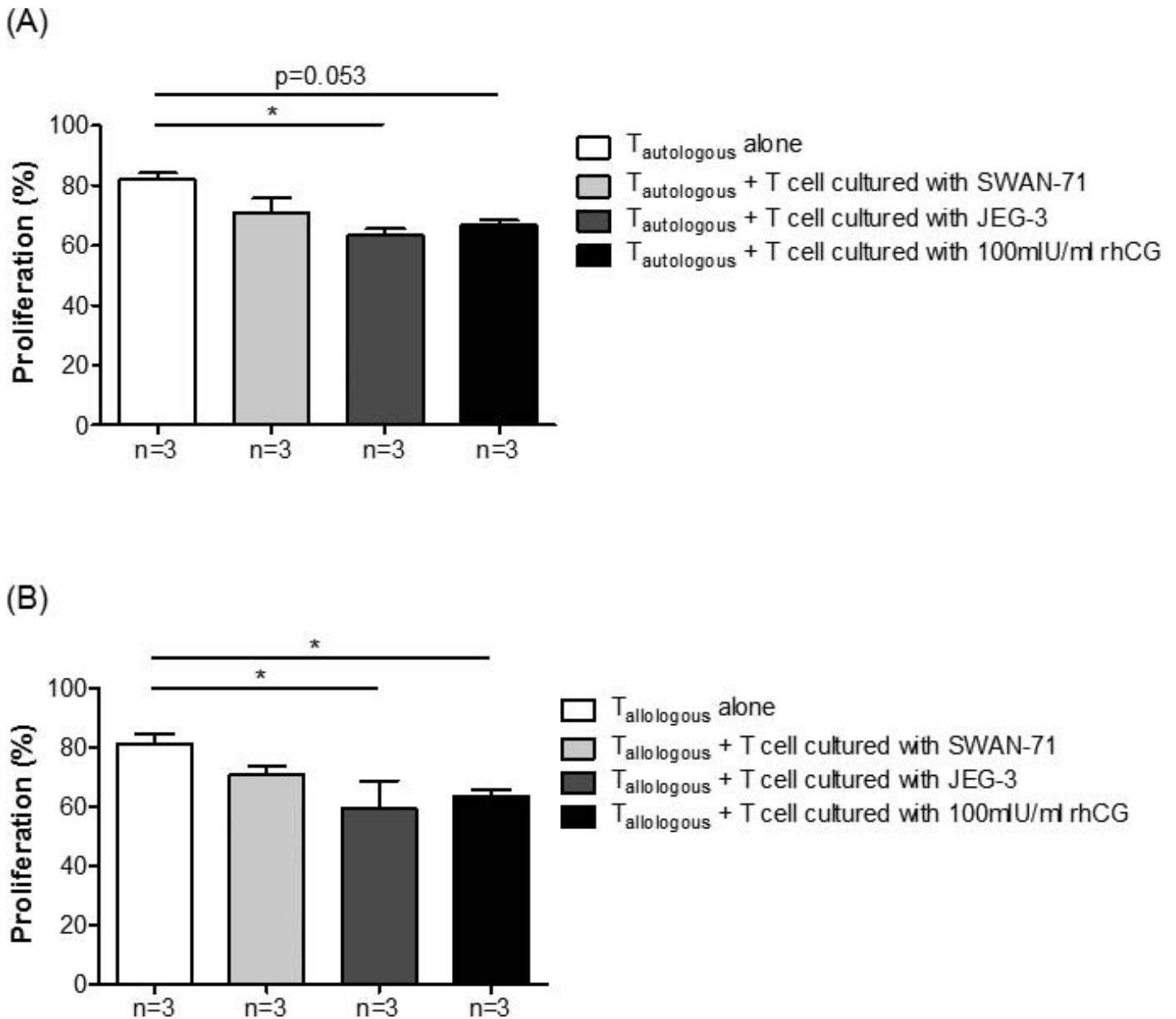


FIG. 6. The hCG-producing JEG-3 trophoblast cells increased T cell suppressive activity. **A–B** $CD4^+FOXP3^-$ T cells were cultured in the presence of hCG-producing JEG-3 cells, non-hCG-producing SWAN-71 cells, or 100 mIU/ml rhCG for 72 h. Afterward, hCG-treated and non-hCG-treated $CD4^+FOXP3^-$ T cells were separated from JEG-3 cells, SWAN-71 cells, or rhCG and cultured either with autologous (obtained from the same donor) (A) or allogous (obtained from a third-party donor) (B) responder T cells for 48 h. The suppressive capacity of hCG-treated and non-hCG-treated $CD4^+FOXP3^-$ T cells was determined by their potential to inhibit proliferation of responder T cells via flow cytometry. Three different patient samples of autologous or allogous donors were analyzed in duplicates. Data are presented as medians plus interquartile range. Statistical analysis was performed using the Friedman test followed by Dunn posttest ($*P < 0.05$).

to separately analyze their suppressive activity. Therefore, we proceeded to compare the suppressive capacity of hCG-treated and non-hCG-treated total T cells on responder T cells. Because SWAN-71 cells provoked an hCG-independent elevation in the number of Treg cells, we wondered whether this trophoblast cell line would also affect the T cell-suppressive capacity. We also studied whether prepriming by self- or alloantigens may play a role in this process as it does in the mouse model. We proved a significant enhanced suppressive capacity of total T cells after they have been cultured with hCG-producing JEG-3 cells or rhCG. Coculture with SWAN-71 cells resulted in a stronger potential of total T cells to suppress responder T cells. However, this effect did not reach statistical significance, which might be due to the lower

number of converted Treg cells. The hCG-mediated effect was antigen independent because autologous and allogous responder T cells were suppressed to the same extent. This is in contrast to our mouse data where hCG-treated Treg cells suppressed responder T cells in an antigen-specific fashion [23]. However, in the murine system, we were able to directly study suppressive activity of Treg cells after hCG treatment. In the present study, it can be speculated that increased T cell suppressive activity is due to the augmentation of Treg cells within the total T cell pool, but the presence of other T cells may mask the effect. In line with this, an older study also showed that hCG is able to suppress T cell proliferation [37]. Hence, hCG may alter the function of conventional T cells in addition to provoking Treg cell induction.

In summary, here we suggest that hCG produced by JEG-3 trophoblast cells is, apart from other trophoblast cell-associated factors, involved in human Treg cell generation from CD4⁺ T cells. We proved that hCG-producing JEG-3 trophoblast cells enhance T cell suppressive activity in an antigen-independent way. Our data uncovers another two important mechanisms through which hCG contributes to fetal tolerance during human pregnancy.

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