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Human Chorionic Gonadotropin Attracts Regulatory T Cells into the Fetal-Maternal Interface during Early Human Pregnancy

Anne Schumacher,* Nadja Brachwitz,* Sindy Sohr,† Kurt Engeland,‡ Stefanie Langwisch,* Maria Dolaptchieva,‡ Tobias Alexander,§ Andrei Taran,** Sara Fill Malfertheiner,** Serban-Dan Costa,** Gerolf Zimmermann,† Cindy Nitschke,‖ Hans-Dieter Volk,‡ Henry Alexander,‡ Matthias Gunzer,‖ and Ana Claudia Zenclussen*‡

Regulatory T cells (Treg) expand during pregnancy and are present at the fetal-maternal interface at very early stages in pregnancy. The migration mechanisms of Treg to the pregnant uterus are still unclear. Human chorionic gonadotropin (hCG) is secreted by the blastocyst immediately after fertilization and has chemoattractant properties. Therefore, we sought to analyze whether hCG secreted by early trophoblasts attracts Treg to the uterus and hence contributes to maternal tolerance toward the fetus. Decidua and placenta tissue samples from patients having spontaneous abortions or ectopic pregnancies were employed to evaluate Treg and hCG levels. Age-matched samples from normal pregnant women served as controls. We further performed in vitro studies with primary first trimester trophoblast cells and a choriocarcinoma cell line (JEG-3) aiming to evaluate the ability of secreted hCG to attract Treg. Patients having miscarriages or ectopic pregnancy presented significantly decreased hCG mRNA and protein levels associated with decreased Foxp3, neuropilin-1, IL-10, and TGF-β mRNA levels as compared with normal pregnant women. Using migration assays we demonstrated that Treg were attracted by hCG-producing trophoblasts or choriocarcinoma cells. Treg migration toward cells transfected with hCG expression vectors confirmed the chemoattractant ability of hCG. Our data clearly show that hCG produced by trophoblasts attracts Treg to the fetal-maternal interface. High hCG levels at very early pregnancy stages ensure Treg to migrate to the site of contact between paternal Ags and maternal immune cells and to orchestrate immune tolerance toward the fetus. The Journal of Immunology, 2009, 182: 5488–5497.

Pregnancy is a fascinating phenomenon if one keeps in mind that it comprises the tolerance of the fetus, bearing foreign Ags of paternal origin, by the maternal immune system. The transient tolerance during gestation is thought to be achieved, at least partially, via the presence of CD4+CD25+Foxp3+ regulatory T cells (Treg). This unique subpopulation, which plays a central role in tolerance and prevention of autoimmunity (1–3), is known to play an important role in the survival of allogeneic organ grafts (4–8). Regarding pregnancy, it has been found that the number of Treg increases very early after conception, peaking during the second trimester to a decline postpartum (9). Treg have been shown to suppress proliferative responses of autologous CD4+CD25+ T cells to allogeneic dendritic cells and therefore to facilitate fetal survival (9, 10). The suppressive function of Treg is mediated either via cell-cell contact (11) or by secretion of immunosuppressive cytokines such as IL-10 and TGF-β (12–16). Additionally, it has been demonstrated that the proportion of decidual Treg was significantly lower in specimens from spontaneous abortion compared with those from induced abortions (10). Furthermore, infertile women have lower expression of Foxp3 in their endometrium when compared with fertile women (17).

Human chorionic gonadotropin (hCG) is a heterodimeric placental glycoprotein hormone required to maintain pregnancy. The α-subunit is identical to that of other glycoprotein hormones such as thyroid-stimulating hormone, follicle-stimulating hormone, and luteinizing hormone, whereas the β-subunit is unique to hCG (18). β-hCG is encoded by six different but very similar genes located in a gene cluster on chromosome 19 together with the β-luteinizing hormone gene (19). β-hCG1 and β-hCG2 were described as pseudogenes (20, 21). In pregnancy and in germ cell tumors mainly β-hCG3, β-hCG5, and β-hCG8 are expressed, which code for an identical protein. In contrast, β-hCG7 encodes a protein differing in three amino acids and is expressed at low levels in normal nonmalignant tissues (20, 22, 23). During pregnancy hCG is initially produced by the blastocyst 6–8 days after fertilization (24, 25) and later by the syncytiotrophoblast (26). The level of hCG increases during the first trimester of pregnancy and decreases to 10% of the peak value during the second and third trimesters (27). It is widely known that hCG has immunoregulatory properties, for example, it is able to suppress mitogen-induced responses of T (28,
29, 30) and B lymphocytes (31) via induction of suppressor T cells (32). It has also been shown that hCG facilitates trophoblast differentiation (33) and invasion by up-regulating molecules supporting the implantation process such as leukemia inhibitory factor (34, 35). hCG also positively influences angiogenesis by inducing the expression of vascular endothelial growth factor and matrix metalloproteinase 9 (35). Moreover, hCG has a direct influence on the decidualization process (36, 37). In the clinical practice, hCG application has been found to support a successful pregnancy outcome in women undergoing in vitro fertilization (IVF). Several studies demonstrated that hCG administration for luteal support in IVF cycles benefits corpus luteum function and thereby increases pregnancy rates compared with placebo-treated women (38, 39, 40).

Taking into account this background, it seemed interesting to us to investigate whether hCG may attract Treg to the fetal-maternal interface. As no IL-2 is present at the human fetal-maternal interface (41), Treg are likely to migrate from the periphery immediately after being generated. Therefore, the main aim of the present study was to investigate whether Treg are attracted by hCG, produced by the trophoblast, during early pregnancy.

Materials and Methods

Sample collection

For analyzing the levels of Treg and hCG in tissue, snap-frozen and paraffin-embedded decidua and placenta tissue were obtained from the Department of Gynecology and Obstetrics, University of Leipzig. Tissue samples were obtained from pregnant women undergoing selective termination of pregnancy, suffering from spontaneous abortion, or having extrauterine pregnancies (Table I). The tissue sampling was approved by the University of pregnancy rates compared with placebo-treated women (38, 39, 40).

Table I. Numbers and characteristics (mean ± SEM) of normal pregnant (NP), spontaneous abortion (SA), and extraterine pregnant patients (EU)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Age (years)</th>
<th>Week of Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP patients (n = 8,</td>
<td>27.25 ± 6.08</td>
<td>16.25 ± 2.76</td>
</tr>
<tr>
<td>and second trimester)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP patients (n = 4,</td>
<td>29 ± 6.08</td>
<td>30.25 ± 0.50</td>
</tr>
<tr>
<td>third trimester)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP patients (n = 19)</td>
<td>29.47 ± 6.90</td>
<td>10.11 ± 2.84</td>
</tr>
<tr>
<td>SA patients (n = 21)</td>
<td>33.69 ± 6.21</td>
<td>8.46 ± 2.45</td>
</tr>
<tr>
<td>EU patients (n = 15)</td>
<td>30.33 ± 6.41</td>
<td>4.26 ± 1.82</td>
</tr>
</tbody>
</table>

Transfection of HCT116 cells with hCG plasmid DNA

For transfection of HCT116 cells, plasmid DNA was used for migration assays. The purity of the isolated Treg was determined by flow cytometry, with CD25-PE (provided with the kit), CD4-FITC (BD Pharmingen), and real-time PCR performed as described elsewhere (43). For Foxp3, IL-10, and TGF-β, real-time PCR was conducted using the ABI PRISM 7700 sequence detection system (PerkinElmer/Applied Biosystems) with primers and fluorescent probes, whereas for hCG and neuropilin-1 (Nrp-1) real-time PCR analysis was performed on the iCycler (Bio-Rad) using SYBR Green (Applied Biosystems) for the detection of PCR products. Primer and probe sequences are available upon request.

Immunohistochemistry and immunofluorescence

Cytokeratin staining was used to differentiate between decidua basalis and decidua parietalis, which helped analyzing the distribution of hCG-positive cells. For cytokeratin and hCG staining, paraffin-embedded tissue sections containing decidua and placental tissue were dewaxed and washed twice with PBS (pH 7.4). For Ag retrieval, tissue sections were cooked in citrate buffer for 10 min for cytokeratin and 5 min in EDTA buffer (pH 9.0) for hCG staining. After washing in TBS, tissues were treated with 3% hydrogen peroxide in methanol for 20 min at room temperature (RT) to block the endogenous peroxidase activity. Tissues were then blocked with 5% BSA (Sigma-Aldrich) in TBS for 20 min at RT, and then, HRP-conjugated solution (Dako) was added for 30 min at RT. Finally, the samples were developed with 3-amino-9-ethylcarbazole (Dako), counterstained with hematoxylin (Sigma-Aldrich), and mounted with mounting medium (Dako). Negative controls were performed by replacing the primary Ab with 5% BSA in TBS or by using diluted mouse or rabbit serum.

For detection of luteinizing hormone/chorionic gonadotropin (LH/CG) receptor, Treg were isolated from human peripheral blood of pregnant or nonpregnant women and cocultured with JEG-3 cells for 4 h. After coculture, Treg were washed with PBS containing 1% BSA and incubated with a polyclonal rabbit anti-LH/CG Ab (dilution 1:200 in PBS plus 1% BSA) (Acris Antibodies) for 1 h at RT. After washing, Treg were stained with FITC-conjugated goat anti-rabbit IgG (H+L) (1:100; Vector Laboratories) for 1 h at RT. Then, HRP-conjugated solution (Dako) was added for 30 min at RT. Finally, the samples were developed with 3-amino-9-ethylcarbazole (Dako), counterstained with hematoxylin (Sigma-Aldrich), and mounted with mounting medium (Dako). Negative controls were performed by replacing the primary Ab with 5% BSA in TBS or by using diluted mouse or rabbit serum. Slides were analyzed using a fluorescence microscope (Zeiss Axio) and images of LH/CG receptor-positive Treg were taken in a magnification of 400 (×40 objective and ×10 ocular).

ELISA for hCG determination

Supernatants from cell cultures were analyzed for their hCG content using the β-hCG ELISA kit from DRG Instruments. All individual steps were performed according to the manufacturer’s instructions.

Treg isolation

For isolation of Treg, CD4<sup>+</sup>CD25<sup>+</sup> Treg were isolated from peripheral blood samples from normal pregnant women from the first and second trimester (n = 8) as well as from the third trimester (n = 4) of pregnancy. The Treg population was obtained using magnetic beads from Miltenyi Biotec, following the instructions of the manufacturer. After isolation, Treg were counted and diluted at 4 × 10<sup>6</sup> cells/ml in Opti-MEM medium and used for migration assays. The purity of the isolated Treg was determined before using the cells for migration assays by staining an aliquot of the cells with CD25-PE (provided with the kit), CD4-FITC (BD Pharmingen),
Migration assay using a two-chamber system and flow cytometry analysis

Both cell lines, JEG-3 and HaCat, were trypsinized and plated overnight at 1 × 10^5 cells/well in their respective growth media (Invitrogen) in 24-well plates. For migration assays the medium of either JEG-3, HaCat, or transduced HCT116 cells was changed to Opti-MEM medium (1 ml/well). Cell inserts (8 µm; BD Falcon) were placed in the wells. Then, 1 ml of Opti-MEM medium containing isolated Treg (4 × 10^4/well for JEG-3 and HaCat; 1–2 × 10^4/well for HCT116) was filled in each insert, which separates the Treg in the upper chamber from the adherent cells in the lower chamber. After 4, 8, 24, or 48 h, inserts were removed and supernatants from the lower chamber were taken. Cells were washed with PBS and fixed overnight with 1% paraformaldehyde solution (Carl Roth). After washing, the absolute number of migrated Treg was determined by flow cytometry (FACSCalibur; BD Biosciences). In addition to fluorescence microscopy, the expression of LH/CG receptor in Treg was determined by using flow cytometry analysis.

Migration assay using a three-dimensional collagen matrix and computer-assisted cell tracking

The three-dimensional collagen matrix assay was employed to visualize Treg migration to JEG-3 cells using video microscopy. Three-dimensional collagen matrices were prepared as described elsewhere (44, 45). Briefly, 100,000 Treg and 100,000 JEG-3 cells were resuspended in 33 µl of RPMI 1640 medium (Invitrogen) and embedded within a total of 100 µl of collagen (33 µl of cells and 66 µl of collagen (dermal bovine collagen; Collagen Corp.)) at a final collagen concentration of 1.7 mg/ml. This solution was filled into a small chamber built by a hollowed coverslip on a glass slide and allowed to polymerize for 20 min (37°C, 5% CO_2). The remaining space in the chamber was filled with RPMI 1640 medium. The chamber was then sealed with wax. For video documentation, cells incorporated within collagen lattices were visualized on a conventional upright microscope (Olympus CellIR). Time-lapse video microscopy was used to record the movement of 5–10 cells visible within one optical field over a period of up to 16 h with a rate of three frames per minute. Subsequently, the path of one single Treg was reconstructed from the recorded film by computer-assisted cell tracking. Therefore, time-lapse video movie was displayed on a computer screen. From the first frame of the time-lapse sequence, three cells (one Treg and two JEG-3 cells) were selected, giving a nonbiased, representative sample of the mixed cell population. Then, the movement of the Treg was individually followed by a trackball over a specific time period.

Data analysis and statistics

The patterns and intensities of the immunohistochemical staining were evaluated by two independent observers using a light microscope (Zeiss Axiophot) in a ×200 magnification (×20 objective and ×10 ocular). The degree of staining was graduated semiquantitatively from 0 (negative) to 5 (intense). Data from immunohistochemistry are shown as scatter plots accompanied by representative images of decidual tissue.
A statistical difference between all groups was performed using the Kruskal-Wallis test, while differences between two groups were calculated using the nonparametric Mann-Whitney U test. The nonparametric Spearman correlation was used to analyze the correlation of RT-PCR data. Parametric data (e.g., migration assays) were analyzed using a Student’s t test to compare differences between two groups, and two-way ANOVAs were used to compare three or more groups.

Results

Decreased hCG mRNA and protein levels in patients suffering from miscarriages or ectopic pregnancies as compared with normal pregnant women

hCG has been shown to be indispensable for a successful pregnancy outcome (46, 47). Here, the expression of hCG in tissue containing decidua and placenta at early pregnancy stages was determined at the mRNA and protein levels in samples from patients suffering from spontaneous abortion or ectopic pregnancies and compared with samples from women having normal pregnancies that were interrupted for social reasons. We observed significantly decreased hCG mRNA levels in tissue samples from patients with spontaneous abortions or ectopic pregnancies when compared with normal pregnant women (Fig. 1A). These observations were confirmed at the protein level when analyzing the samples stained for hCG (Fig. 1B). Most of the patients having spontaneous abortions or ectopic pregnancies had nondetectable or very low hCG protein expression. In contrast, normal pregnant women showed a very high expression of hCG in decidua cells of decidua basalis (Fig. 1B). The implantation of the fertilized egg occurs in the decidua basalis, and therefore essential tolerance mechanisms allowing the acceptance of the fetus are expected to be in this area. Interestingly, we observed a significant difference regarding hCG expression in decidua basalis from patients suffering from spontaneous abortion or ectopic pregnancies as compared with normal pregnancy. This difference was not so strong when analyzing decidua parietalis (Fig. 1B). Representative images of decidua parietalis and basalis tissue sections from all three groups are shown in Fig. 2. Taken together, our data lend additional support to the already known important role of hCG for a successful pregnancy outcome.

Miscarriages and ectopic pregnancies are associated with diminished levels of Foxp3, Nrp-1, IL-10, and TGF-β mRNA at the fetal-maternal interface

To first analyze whether high hCG levels indicate high numbers of Treg or their associated molecules, we next quantified the levels of Foxp3, Nrp-1, IL-10, and TGF-β mRNA in tissues whose hCG had been analyzed before. Foxp3 was described as an essential transcription factor for CD4+/CD25high Treg responsible for the development and function of these cells (48). Recently it has been discussed whether Foxp3 is as important in humans as it is in mice for Treg function (49). In addition to Foxp3, Nrp-1 was described as a marker for Treg (50). Regarding Nrp-1, we were able to show that abortion-prone mice express...
diminished levels of this molecule as compared with normal pregnant mice (51). It has been found that the secretion of IL-10 and TGF-β by Treg is associated with their protective function (12, 16). Here, we clearly show that patients suffering from spontaneous abortion or ectopic pregnancies had significantly lower levels of Foxp3 as compared with normal pregnant women (Fig. 3A). Moreover, Nrp-1 mRNA levels were significantly reduced in patients having spontaneous abortions when compared with normal pregnant women (Fig. 3B). Patients suffering from spontaneous abortions and ectopic pregnancies showed significantly diminished IL-10 mRNA levels and slightly diminished TGF-β mRNA levels as compared with the normal pregnant group (Fig. 3C and D). Although not conclusive, data on IL-10 and TGF-β are in agreement with lower Treg levels. Correlation analysis of Foxp3 mRNA and hCG mRNA levels revealed a significant positive correlation (Spearman = 0.5954; p < 0.05) between both (Fig. 3E), emphasizing the possibility that low hCG levels are accompanied by low Treg occurrence.

Treg are susceptible to hCG chemoattraction by expressing the LH/CG receptor

After confirming the presence of both hCG and Treg at the fetal-maternal interface from normally progressing pregnancies and their correlation, we hypothesized that the presence of Treg at the fetal-maternal interface is associated with a normal human pregnancy outcome (10, 17), and we confirmed that low hCG levels are accompanied by low Treg occurrence.
hCG produced by JEG-3 cells (Fig. 4B). This observation is in accordance with other studies in which 2–50% of Treg were found to be positive for molecules (e.g., CCR2, CCR4, and CD62L) associated with the migration of Treg (52, 53, 54).

Additionally, we found LH/CG receptors on Treg at the single-cell level (Fig. 4F–H). We also investigated the LH/CG receptor expression on Treg from age-matched nonpregnant women. We found that, as expected, Treg from nonpregnant women express the LH/CG receptor after exposure to hCG, suggesting that the presence of hCG is sufficient to stimulate the up-regulation of the receptor (Fig. 4C–E). These data clearly demonstrate that Treg have the ability to sense the attraction of hCG, supporting our hypothesis that Treg migrate from the periphery to the fetal-maternal interface along a hCG gradient generated by the trophoblast cells.

hCG-producing primary first trimester trophoblast cells as well as the choriocarcinoma cell line JEG-3 attract Treg

After confirming the presence of LH/CG receptors on the surface of Treg, we investigated whether Treg might be attracted by hCG-producing primary first trimester trophoblast cells as well as by choriocarcinoma cell line JEG-3. For this, we used a two-chamber transwell system to determine the active migration of Treg to the trophoblast cells. We could clearly show that Treg obtained from normal pregnant women from the second trimester (n = 4; different patients tested in independent experiments) were able to migrate efficiently to JEG-3 cells (Fig. 5A), which actively produced hCG, as we could observe by ELISA (25 IU/L per 10,000 cells).

To investigate to which extent this reflected the physiological situation and to exclude experimental artifacts by using a carcinoma cell line, we repeated the assay using primary trophoblast cells obtained from women undergoing selective termination of pregnancy (20 IU/L per 10,000 cells). Comparable results were obtained (Fig. 5B). We further observed that Treg obtained from nonpregnant women from the third trimester (n = 4) were attracted in the same way by trophoblasts (data not shown). To analyze whether and how Treg contacted trophoblasts directly ex vivo, we proceeded to coinoculate JEG-3 cells and isolated Treg together in three-dimensional collagen matrices, which express many features of true extracellular matrix (45). In fact, Treg actively migrated toward JEG-3 cells and contacted them several times (Fig. 6).

Overexpression of hCG in HCT116 cells line benefits the Treg migration. HCT116 cells transfected with both subunits (presenting whole hCG molecule) attracted Treg significantly more than HCT116 cells transfected with GFP/LacZ (A). Moreover, Treg are attracted in the same way when HCT116 cells were transfected with the subunits (α or β) alone (B). Data were analyzed using two-way ANOVA to compare two or more groups at different time points. *, p < 0.05 and **, p < 0.01.
cinoma cell line, hCG was highly effective in attracting Treg. Our results clearly demonstrate that among all factors A (Fig. 7 comparison to control-transfected cells that did not produce hCG attracted by HCT116 cells producing recombinant hCG in HCT116 cells, we demonstrated that Treg were significantly more attracted by them as they also produce several chemokines that potentially attract lymphocytes (55, 56, 57, 58). As shown in Fig. 8A, no hCG was detectable in the culture supernatant of HaCat cells as compared with JEG-3 cells. By performing migration assays, we could clearly demonstrate that Treg were exclusively attracted by the hCG-producing JEG-3 cells but not by the hCG nonproducing HaCat cells (Fig. 8B). Our data strongly suggest that hCG secreted by trophoblast cells is necessary to attract Treg to the fetal-maternal interface. This may explain the low numbers of Treg observed in patients suffering from spontaneous abortion associated with low hCG levels.

**Discussion**

Although it is well known that the maternal immune system tolerates the foreign paternal Ags expressed in the fetus during pregnancy, the mechanisms underlying this phenomenon are still under investigation. Treg were described to play an important role in the maintenance of the tolerant state during pregnancy. This unique T cell subpopulation is known to have immunoregulatory properties preventing autoimmune diseases (1–3) and allowing the acceptance of allografts (4–8). It has been found that Treg increase during the very early stages of pregnancy and that this expansion is essential for a successful pregnancy outcome, as women with impaired augmentation in their Treg level suffer from spontaneous abortion and infertility (10, 17, 59). Moreover, it has been demonstrated that Treg from women with recurrent spontaneous abortion were functionally deficient, as higher numbers were required to exert a similar magnitude of suppression as compared with Treg from fertile women (60). In mice, Treg are generated immediately after fertilization and expand at the periphery, being specific for paternal Ags (61). Accordingly, paternal lymphocyte immunization used in the clinical practice to improve pregnancy outcome in women with previous recurrent abortions (62) is able to augment the number of male-specific suppressor T cells (63).

The pregnancy hormone hCG has been shown to be indispensable for the establishment of a successful pregnancy (46, 47) and has been described to have immunoregulatory properties supporting the implantation process of the fetus in the maternal endometrium (34–37). Treatment of PBMCs with hCG and their further transfer 2 days after oocyte retrieval was shown to increase the implantation rates of blastocysts in women suffering from repeated IVF failure (64). Thus, it seemed interesting to us to investigate whether hCG produced by trophoblasts may attract Treg to the fetal-maternal interface.

We first performed a descriptive study in which we searched for a coincidence between high hCG levels and high Treg numbers. We confirmed high hCG levels in normally progressing pregnancies. In sharp contrast, significantly lower mRNA and protein levels of hCG were found in samples from women suffering from

\[ \text{hCG concentration (IU/L)} \]

- **A**
- **B**

**FIGURE 8.** Treg are not attracted by a non-hCG-producing cell line (HaCat). The keratinocyte cell line HaCat did not produce hCG (A). In contrast, JEG-3 cells produce significant higher levels of hCG (A). The two-chamber transwell system was used to determine the migration of Treg (isolated from peripheral blood of 30 wk pregnant women; \( n = 4 \)) to both the JEG-3 cells and the HaCat cells (B). Nearly no migration could be observed to the non-hCG-producing HaCat cells when compared with the hCG-producing JEG-3 cells (B). Data from A were analyzed using Student’s t test to compare differences between two groups. Data from B were analyzed using two-way ANOVA to compare two or more groups at different time points. \( * \), \( p < 0.05 \); \( ** \), \( p < 0.01 \); \( *** \), \( p < 0.001 \).

Overexpression of hCG in HCT116 cells benefits the migration of Treg

After confirming that Treg migrate to choriocarcinoma cells or primary trophoblasts, both known to secrete hCG, we next sought to confirm that hCG is responsible for Treg migration. Therefore, we investigated whether transient overexpression of hCG in HCT116 cells would attract Treg to these cells normally not producing hCG or attracting Treg. To this end, HCT116 cells were cotransfected with expression vectors for the \( \alpha \)-subunit of hCG together with expression vectors for \( \beta \)-hCG3 or \( \beta \)-hCG7. First, production of recombinant hCG by transfected HCT116 cells was confirmed. Upon transfection, 500-2800 IU/L of hCG could be detected in the supernatant depending on the experiment. In contrast, control-transfected HCT116 cells were not able to produce hCG. By performing migration assays using the transfected HCT116 cells, we demonstrated that Treg were significantly more attracted by HCT116 cells producing recombinant hCG in comparison to control-transfected cells that did not produce hCG (Fig. 7A). Our results clearly demonstrate that among all factors secreted by primary first trimester trophoblast cells or a choriocarcinoma cell line, hCG was highly effective in attracting Treg. Moreover, we found that both the \( \alpha \)- and \( \beta \)-subunits of hCG alone were able to attract Treg efficiently, showing no significant difference between the \( \beta \)-hCG isoforms 3 and 7 (Fig. 7B), which might be explained by the supraphysiological levels of expression upon transfection or by the fact that they can dimerize. Even though the concentration of hCG produced by transfected cells is much higher than those produced naturally by trophoblasts or by JEG-3 cells, the migration rates were comparable, which could be interpreted as a determined amount of hCG needed to attract Treg, which once reached is sufficient for their attraction. More hCG would not mean migration of more Treg, as we did not observe differences in the migration of Treg to different hCG concentrations toward HCT116 cells upon transfection.

Lack of Treg migration to the hCG nonproducing HaCat cell line

As a further validation of hCG-dependent attraction of Treg into the fetal-maternal interface, we used the hCG nonproducing keratinocyte cell line HaCat to determine whether Treg were attracted by them as they also produce several chemokines that potentially attract lymphocytes (55, 56, 57, 58). As shown in Fig. 8A, no hCG was detectable in the culture supernatant of HaCat cells as compared with JEG-3 cells. By performing migration assays, we could clearly demonstrate that Treg were exclusively attracted by the hCG-producing JEG-3 cells but not by the hCG nonproducing HaCat cells (Fig. 8B). Our data strongly suggest that hCG secreted by trophoblast cells is necessary to attract Treg to the fetal-maternal interface.

*The online version of this article contains supplemental material.*
spontaneous abortion and extraterine pregnancies. To analyze whether low hCG levels correlate with decreased number and function of Treg, we measured Foxp3, Nrp-1, IL-10, and TGF-β expression. Foxp3 and Nrp-1, described as specific markers for Treg (48, 50), were expressed significantly lower in patients having miscarriages as compared with normal pregnant women. Furthermore, hCG mRNA levels correlated significantly with Foxp3 mRNA levels, emphasizing that low hCG levels are associated with low Treg number and vice versa. These data are in agreement with those from other research groups (10, 17). Moreover, IL-10 levels were significantly diminished and TGF-β levels were slightly diminished in pathological pregnancies compared with normal pregnancies, further suggesting low Treg numbers in pathological pregnancies, since Treg are known to affect immune responses by secreting antiinflammatory cytokines at the fetal-maternal interface (14, 61, 67). Thus, our data on low levels of hCG coinciding with low levels of Treg-associated molecules suggest that hCG may be chiefly responsible for Treg attraction to the fetal-maternal interface.

To test whether migration toward hCG is possible, we first analyzed whether Treg express the LH/CG receptor, necessary to respond to hCG-mediated signals and hCG attraction. We were able to show that at least 25% of Treg from human peripheral blood of pregnant donors expressed the LH/CG receptor, with individual cells having several LH/CG receptor molecules on their surface. We also found Treg from nonpregnant women expressing the LH/CG receptor after coculture with JEG-3 cells producing hCG, suggesting that the presence of hCG is sufficient to up-regulate the LH/CG receptor on the surface of Treg from nonpregnant women. In agreement with our observation, an older study demonstrated the presence of the LH/CG receptor on human suppressor T cells (68). However, at the time of this mentioned study it was not possible to define the nature of these cells by analyzing, for example, CD25 and Foxp3 expression. Having confirmed that Treg were principally susceptible to attraction by hCG by expressing the receptor, we went ahead studying whether in fact Treg were attracted by hCG using a two-chamber transwell system. We analyzed the attraction of Treg by primary first trimester trophoblast cells and the choriocarcinoma cell line JEG-3, which is confirmed to secrete hCG. Treg from the second trimester and from the third trimester migrated efficiently to primary trophoblast cells and to JEG-3 cells. This supports our hypothesis of an active migration of Treg into the fetal-maternal interface and suggests that hCG may be involved in this migration. However, at this stage we could not exclude that Treg are attracted by chemokines generally produced in great extent by trophoblasts (69–72). We next conducted additional experiments to determine to which extent hCG directed Treg migration toward trophoblasts. We observed no Treg migration in the direction of HaCat cells, which are unable to produce hCG as compared with the hCG-producing JEG-3 cells. To finally prove that hCG is responsible for Treg migration, two isoforms of the hCG β-chain, β-hCG3 and β-hCG7, alone or together with the α-subunit, were recombinantly expressed in the hCG nonproducing HCT116 cells. The synthesis of recombinant hCG (with or without the α-subunit) by these cells supported the migration of Treg when compared with control-transfected HCT116 cells with no difference between the different β-hCG isoforms used. Hence, Treg migration does not discriminate between the β-hCG isoforms 3 and 7, although in normal pregnancy mainly β-hCG3, β-hCG5, and β-hCG8 are expressed (20). These results clearly show that the β-subunit, which is unique to hCG, is already sufficient to attract the Treg. However, the observed migration of Treg to the α-subunit alone might indicate that also luteinizing hormone, follicle-stimulating hormone, and thyroid stimulating hormone, which share this subunit with hCG, are able to attract Treg. This was not analyzed in the context of this study and may further open new possibilities in studying Treg migration toward hormones. The observed high hCG levels after transfection of HCT116 cells might favor the formation of hCG homodimers (hCGα/α or hCGβ/β). In this regard several publications describe the natural occurrence of hCG homodimers. Both the hCGα/α and hCGβ/β homodimer are biological stable and activate the LH/CG receptor specifically. Additionally, the homodimers can stimulate the signal transduction to the same extent as hCG heterodimers (73–76). Thus, we assume that Treg migration observed to the α- or β-subunit alone might be a result of the homodimerization of these subunits followed by an activation of the LH/CG receptor on the Treg. As we found that the percentage of Treg migration achieved using trophoblast cells was higher than the one achieved using hCG-transfected HCT116 cells, it needs to be clarified further which other factors produced by the trophoblast cells contribute to the overall chemoattractant effect, albeit to a much lower degree than hCG. To conclusively confirm that hCG and not other factors orchestrate the main Treg migration, we attempted to knock down hCG by means of siRNA. Unfortunately, the best percentage of inhibition that could be obtained among all the sequences of hCG-specific siRNA was 40% (data not shown). Although this was not statistically significant, we observed that with this modest reduction in hCG secretion Treg migration was reduced 30% as compared with the controls. Unfortunately, no commercially available blocking Abs for hCG exist, and the Abs we checked were shown not to have blocking activity in vitro.

In an in vivo study it has been shown that hCG administration was able to prevent autoimmune diabetes resulting from destruction of pancreatic β-cells in NOD mice (77). hCG injection was associated here with a significant increase of Treg in spleen and pancreatic lymph node, accompanied by an increase in IL-10 and TGF-β levels. This study suggests that Treg are attracted to the site of inflammation by hCG to allow suppression of T effector cells responsible for the onset of the disease (77). A similar hypothetical scenario can be proposed for early implantation. Our observations support Treg migration to the site in which implantation is taking place, known to be a site of inflammatory conditions (78–80).

In 1985, suppressor T cells were shown to be present in the human decidua (81). Recently, the presence of so-called CD^4^ CD25^+^Foxp3^+^ Treg in decidual tissue was confirmed, further supporting the important role of Treg at the site of fetal-maternal contact (82–85). Knowing that no IL-2 is available at the fetal-maternal interface in mice and humans (41, 65, 66) and IL-2 is necessary for Treg to survive and proliferate in vivo, we hypothesized that Treg need to migrate to the decidua after generation or expansion. Data from mice support this hypothesis (61, 86). Moreover, it was postulated that after conception macrophages and lymphocytes, which produce hCG, infiltrate the endometrium, contributing to its function (87–89). Taking this into account, Treg might be attracted not only by hCG secreted by the trophoblast but also by other immune cells secreting hCG at the fetal-maternal interface. In this context, it was already demonstrated that stimulation of monocytes with hCG augmented the production of IL-8 (90) known to attract leukocytes (91). In another in vitro study it was shown that hCG is a potent attractor of neutrophils, monocytes, and lymphocytes at very low doses (92). Moreover, it was found that the type of migration is dependent on positive concentration gradients of hCG (92). An alternative explanation for the presence of Treg at the fetal-maternal interface may be that they are de novo-generated cells with regulatory activity, as, for example, by TGF-β or IDO, both present in the decidua.
In conclusion, our data suggest a possible mechanism as to how Treg could be attracted into the fetal-maternal interface following conception immediately after being generated. Treg may be attracted to the fetal-maternal interface by high hCG production in pregnancy subjects and spontaneous abortion cases. 5496 HCG ATTRACTS Treg TO THE UTERUS

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Disclosures
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