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Normal pregnancy is characterized by an early expansion of regulatory T cells (Tregs), which is known to contribute to fetal tolerance. However, mechanisms and factors behind Treg expansion are not yet defined. Recently, we proposed that the pregnancy hormone human chorionic gonadotropin (hCG) efficiently attracts human Tregs to trophoblasts, favoring their accumulation locally. In this study, we hypothesized that hCG not only acts as a chemoattractant of Tregs but also plays a central role in pregnancy-induced immune tolerance. Virgin, normal pregnant, and abortion-prone female mice were treated either with 10 IU/ml hCG or PBS at days 0, 2, 4, and 6 of pregnancy. The hCG effect on Treg frequency and cytokine secretion was determined in Foxp3gfp females. hCG impact on Treg suppressive capacity was studied in vitro. In vivo, we investigated whether hCG enhances Treg suppressive capacity indirectly by modulating dendritic cell maturation in an established mouse model of disturbed fetal tolerance. Application of hCG increased Treg frequency in vivo and their suppressive activity in vitro. In females having spontaneous abortions, hCG provoked not only an augmentation of Treg numbers, but also normalized fetal abortion rates. hCG-generated Tregs were fully functional and could confer tolerance when adaptively transferred. hCG also retained dendritic cells in a tolerogenic state that is likely to contribute to both Treg expansion and prevention of abortion. Our results position hCG in a novel, so far unknown role as modulator of immune tolerance during pregnancy. The Journal of Immunology, 2013, 190: 000–000.

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Abbreviations used in this article: AP, abortion-prone; DC, dendritic cell; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; LH, luteinizing hormone; LH/CG, luteinizing hormone/human chorionic gonadotropin; NP, normal pregnant; P, progesterone; Treg, regulatory T cell.

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important modulator of Treg-mediated fetal tolerance by influencing their numbers and/or suppressive capacity. To study whether hCG-mediated effects are of direct or indirect nature, we also concentrated on the effects of hCG on the maturity state of dendritic cells (DCs) that act as APCs at the very first step of an adaptive immune response.

Materials and Methods

Animals

Wild type CBA/J female mice and BALB/c and DBA/2J male mice were purchased from Charles River (Germany), maintained in our animal facility, and treated according to the institutional guidelines with ministerial approval (Landesverwaltungsamt Sachsen-Anhalt AZ/234 and AZ/2502-2-1125UNM). The experiments were conducted by authorized persons according to the Guide for Care and Use of Animals in Agriculture Research and Teaching. DBA/2J-mated CBA/J females (AP combination) are known to spontaneously develop high abortion rates (24), whereas BALB/c-mated CBA/J females (NP combination) represented the control group. Foxp3gfp transgenic females on a C57BL6 background, kindly provided by Dr. Rudensky, were allogeneically mated to BALB/c males. In these animals, the complete coding sequence of the GFP (gfp) is inserted in the first coding exon of the Foxp3 gene, which results in a chimeric GFP-Foxp3 fusion protein (32) that enables the easy identification of Foxp3+ Tregs. Animals were pair-fed and checked twice a day for vaginal plug, whose appearance indicated day 0 of pregnancy. Virgin and pregnant animals were injected i.p. with 10 IU/ml hCG (Sigma Aldrich, Steinheim, Germany) or PBS (PAA Laboratories, Cölbe, Germany). hCG and PBS injections were performed at days 0, 2, 4, and 6 of gestation or experiment, and females were sacrificed on day 10 of pregnancy or 4 d after the last injection in nonpregnant animals.

Sample collection and flow cytometry

Blood was obtained by retroorbital puncture under anesthesia. Females were sacrificed by cervical dislocation; spleen, thymus, para-aortic lymph nodes, and uterine tissue were removed, washed in ice-cold PBS, and kept in RPMI 1640 medium at 4°C. Pregnant uteri were opened longitudinally, and the number of implantations and the abortion rate were determined. Fetoplacental units were separated from their implantation sites; decidual cells were cut in small pieces and collected in HBSS without Ca2+ and Mg2+ (Sigma, Taufkirchen, Germany). Mononuclear cells from blood, spleen, thymus, para-aortic lymph nodes, uterus, and decidua samples were isolated using our established protocol (24). Thereafter, cells were stained for extracellular and intracellular markers as described elsewhere (24) and read on a FACSDiva Flow Cytometer and Cell Sorter from Becton Dickinson. CD4+CD25+CD122+ (Foxp3gfp) cells were sorted from spleen or draining lymph nodes (para-aortic plus inguinal lymph nodes) from BALB/c-mated CBA/J females (day 12 of pregnancy) previously treated with either 10 IU/ml hCG or PBS (day 12 of pregnancy) previously treated with either 10 IU/ml hCG or PBS. The purity of the isolated Tregs was >95%. A total of 2 × 10^5 Tregs, adoptively transferred into AP animals by i.v. injection on days 0–2 of pregnancy. The used number of 2 × 10^5 Tregs, adoptively transferred into AP animals, was proved to be the minimum cell number essential to confer fetal protection in this model (24).

Suppression assay and conversion assay

To study the influence of hCG on Treg function, we isolated CD4+CD25+ responder T cells and CD4+CD25− Tregs by magnetic beads from a mixture of spleen and draining lymph nodes (para-aortic plus inguinal lymph

FIGURE 1. hCG increased Treg number and their capability to secrete suppressive cytokines. Application of 10 IU/ml hCG in BALB/c-mated Foxp3gfp females significantly increased the numbers of Foxp3-GFP+ cells (Tregs) in blood (A) [n = 7/group] and decidua (B) [n = 7–9/group] when compared with PBS treatment. Moreover, to assess cytokine secretion by Tregs obtained from either hCG- or PBS-treated pregnant Foxp3gfp females, we cultured Tregs for 24 h in the presence of ionomycin and PMA. Tregs obtained from hCG-treated females secreted increased amounts of IL-10 (C) [n = 6 per group] and TGF-β (D) [n = 3 per group] when compared with Tregs obtained from PBS-treated females. (A and B) Each square represents one single animal. Data are either presented as medians (A, B) or means ± SD (C, D), and statistical analysis was carried out by the Mann–Whitney U test or Student t test, respectively. *p < 0.05, **p < 0.01.

ELISA

The amounts of IL-10 and TGF-β were determined in the supernatants of isolated Tregs from hCG- or PBS-treated pregnant Foxp3gfp females on day 10 of pregnancy by using the BD OptEIA ELISA Set for IL-10 provided by Becton Dickinson and the Ready-Set-Go Kit for TGF-β provided by ebioscience (Frankfurt, Germany). All steps were performed according to the instructions of the manufacturer.

Magnetic cell isolation and adoptive transfer of Tregs

To investigate whether hCG induces the generation of pregnancy-protective Tregs in vivo, we isolated CD4+CD25+ cells by magnetic beads (MACS; Miltenyi Biotec, Bergish Gladbach, Germany) from a mixture of spleen and thymus from AP animals (day 12 of pregnancy) previously treated with either 10 IU/ml hCG or PBS. The purity of the isolated Tregs was >95%. A total of 2 × 10^5 Tregs, diluted in 200 μl PBS, were adoptively transferred into AP animals by i.v. injection on days 0–2 of pregnancy. The used number of 2 × 10^5 Tregs, adoptively transferred into AP animals, was proved to be the minimum cell number essential to confer fetal protection in this model (24).
Application of 10 IU/ml hCG in virgin CBA/J females increased the number of Tregs in uterus as compared with PBS treatment (Table I). Application of 10 IU/ml hCG in AP females (n = 6–9/organ) NP = 6–9/organ) significantly increased the number of CD4+Foxp3+ cells (Tregs) within the CD4+ cell population in thymus (A), para-aortic lymph nodes (B), blood (C), and decidua (D) when compared with PBS-treated AP females (n = 6–7/organ). No significant differences in the Treg number could be detected between hCG- (n = 5–6/organ) and PBS-treated (n = 5–6/organ) NP females. Each square represents one single animal, and the lines show the medians. Statistical analysis was carried out by the Mann–Whitney U test. *p < 0.05, **p < 0.01.

LH/hCG receptor detection on Tregs and DCs by flow cytometry

To confirm the presence of the LH/hCG receptor on both immune cell populations, CD4+CD25+ Tregs were isolated from a mixture of spleen and thymus, whereas CD11c+ DCs were isolated from spleen of virgin CBA/J females by magnetic beads. Isolated Tregs and DCs were stained using the polyclonal rabbit anti-mouse LH/hCG receptor Ab (Acris, Hiddenhausen, Germany) for 1 h at room temperature. Then cells were stained using an FITC-conjugated goat anti-rabbit IgG Ab for 30 min at 4°C. Afterward, cells were analyzed by flow cytometry, and data are presented as histograms using FlowJo 7.6.5 software (Tree Star).

Data analysis and statistics

Data obtained for in vitro assays and cytokine determinations by ELISA are presented as means ± SD. Statistical analysis was realized by performing the Student t test. All other data are presented as medians in graphs showing individual values for each animal. Analysis of statistical differences among all groups was performed using the nonparametric Kruskal–Wallis test. For analyzing differences between two particular groups, the Mann–Whitney U test was applied. In all cases, p < 0.05 was considered to be statistically significant.

Results

Application of hCG provoked an increase in Treg number and their capability to secrete suppressive cytokines

In this study, we investigated whether hCG influences Treg expansion and suppressive function in murine pregnancy. For this, we used transgenic Foxp3gfp female mice allogeneically mated to BALB/c males. Foxp3gfp females were treated with hCG or PBS at days 0, 2, 4, and 6 of gestation, and the frequency of Tregs was determined by flow cytometry in several organs. We observed a significant expansion of Tregs in blood and decidua of hCG-treated females when compared with PBS-treated controls (Fig. 1A, 1B). In lymph nodes, spleen, and thymus, no significant differences among the groups were found (data not shown). To further evaluate whether hCG additionally influences the cytokine secretion pattern of Tregs, we examined the amount of IL-10 and TGF-β, both known to mediate Treg suppressive capacity (33, 34). hCG treatment increased, although not significantly, the capability of Tregs to secrete IL-10 and TGF-β secretion.

hCG application restored tolerance and diminished fetal rejection by normalizing Treg frequencies

We hypothesized that hCG enables fetal survival by promoting Treg expansion. To prove this, we determined the systemic and local number of Tregs in hCG- or PBS-treated NP or AP females. hCG application at days 0, 2, 4, and 6 resulted in significantly in-

Table I. hCG increased the number of local Tregs in virgin females

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tregs in Spleen (%)</th>
<th>Tregs in Blood (%)</th>
<th>Tregs in Uterus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/J + PBS</td>
<td>4</td>
<td>4.08</td>
<td>1.63</td>
<td>12.30</td>
</tr>
<tr>
<td>CBA/J + hCG</td>
<td>4</td>
<td>4.52</td>
<td>1.06</td>
<td>17.08</td>
</tr>
</tbody>
</table>

Application of 10 IU/ml hCG in virgin CBA/J females increased the number of Tregs in uterus as compared with PBS treatment but did not change the levels of Treg in spleen and blood. Data are presented as medians. Statistical analysis was carried out by the Mann–Whitney U test. No statistical differences could be detected between hCG- and PBS-treated females.
creased Treg numbers in thymus, para-aortic lymph nodes, blood, and decidua (Fig. 2). The levels of Tregs in control pregnant mice were only slightly augmented (Fig. 2). In virgin CBA/J females, hCG application increased the number of Tregs in uterus as compared with PBS but did not change the levels of Tregs in spleen and blood (Table I). Thus, hCG seems to expand Tregs in the uterus regardless of an ongoing pregnancy, whereas systemic expansion of Tregs has been observed only in pregnant female mice.

To study the effect of hCG-mediated Treg augmentation on pregnancy, we analyzed the abortion and implantation rates after hCG or PBS treatment. Negative side effects of hCG on pregnancy were excluded after observing no differences in the pregnancy outcome when compared with PBS-treated NP females and no anomalies in any organ (Fig. 3A). hCG application could impressively prevent fetal rejection in AP females when compared with AP females treated with PBS (Fig. 3A), and this was not dependent on the number of total implantations (Fig. 3B). As shown in Fig. 3C, AP females displayed fetal resorptions together with healthy embryos, whereas hCG-treated AP females had no resorptions. These results clearly confirm a positive influence of hCG on pregnancy outcome in this model by restoring the levels of Tregs to the values observed in the normal pregnancy combination.

hCG application generated fully functional Tregs that can confer tolerance when adoptively transferred

To know whether Tregs generated after hCG injection are fully functional, we next isolated Tregs from hCG- or PBS-treated, DBA/

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** hCG prevented fetal rejection in AP females. (A) PBS-treated AP females (n = 10) showed an increased abortion rate when compared with PBS-treated NP females (n = 6). Fetal rejection in AP females could be completely prevented by application of 10 IU/ml hCG (n = 12). No differences have been observed between hCG- (n = 6) and PBS-treated NP females. (B) The number of implantations was comparable among all groups. (C) Representative pictures of whole implantation sites revealed that PBS-treated AP females showed fetal resorptions among healthy fetuses, whereas hCG-treated AP females, as well as hCG- and PBS-treated NP females, displayed only healthy implantations. Each square represents one single animal, and the lines show the medians. Statistical analysis was carried out by Kruskal–Wallis test followed by Mann–Whitney U test. ***p < 0.001.
hCG significantly decreased the total number of CD11c+ cells at the fetal-maternal interface but not in the spleen (Fig. 7A, 7B). Moreover, the number of mature DCs determined by the expression of CD11c and MHC class II was significantly reduced in decidua but not spleen after hCG treatment as compared with PBS treatment (Fig. 7C–F). In virgin CBA/J females, hCG slightly decreased numbers of CD4+Foxp3+ cells (Tregs) within the CD4+ cell population in thymus (C), para-aortic lymph nodes (D), blood (E), and decidua (F) when compared with AP females (n = 7/organ) adoptively transferred with Tregs from PBS-treated AP females (n = 6–7/organ). Each square represents one single animal, and the lines show the medians. Statistical analysis was carried out by Kruskal-Wallis test followed by Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. hCG induced fully functional Tregs that conferred tolerance and further Treg augmentation after their adoptive transfer. (A) Adoptive transfer of Tregs isolated from hCG-treated AP females (n = 12) in AP females (n = 11) rescued females from abortion. By contrast, adoptively transferred Tregs from PBS-treated AP females (n = 10) in AP females (n = 7) had no effect on the abortion rate. (B) The number of implantations was comparable among all groups. AP females (n = 6–8/organ) adoptively transferred with Tregs from hCG-treated AP females (n = 6–9/organ) showed increased numbers of CD4+Foxp3+ cells (Tregs) within the CD4+ DCs in spleen and uterus when compared with PBS, but did not alter the levels of CD11c+CD80+ DCs in both organs (Tables II, III). To test whether uterine DCs from hCG-treated animals favor the generation or expansion of Tregs, we cocultured DCs obtained from spleen or decidua of either hCG- or PBS-treated AP females with CD4+ T cells and determined the number of CD4+ cells expressing Foxp3. Indeed, we observed an upregulation of Foxp3 expression in CD4+ T cells when decidual DCs from hCG-treated AP females were present in the culture (Fig. 8A). This effect was not observed when DCs were of splenic origin (Fig. 8B). Altogether, our results confirm an effect of hCG on DC phenotype and at least in vitro, these cells contribute to augmented Treg frequency.

Discussion

Although it is well-known that both the endocrine and the immune systems substantially contribute to successful pregnancy outcome, the interplay between both systems and the major players at molecular and cellular levels are still under investigation. We had already reported that hCG produced mainly by the trophoblast is one of the factors attracting human Tregs efficiently to the fetal-maternal interface contributing to their local accumulation. This local accumulation of Tregs might also be favored 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 (38) known to attract leukocytes (39). Moreover, hCG is a potent attractor of neutrophils, monocytes, and lymphocytes at very low doses (40). In this work, we hypothesized that hCG may also be of central importance in promoting tolerance by directly influencing Treg number and functionality. Because this may also occur indirectly by interaction with, for example, DCs, we extended our study to these cells as well.

We tested our hypothesis in the murine system knowing that hCG is commonly used in superovulation protocols for genetic engineering of mouse strains and efficiently binds to the endogenous murine LH/CG receptor (41). We first took advantage of the Foxp3gfp transgenic animals that allowed us to easily isolate and characterize Foxp3+ Tregs after hormonal treatment in an allogeneic pregnancy setting. We observed that hCG significantly increased the number of Tregs in the periphery, as well as locally, suggesting an accumulation of Tregs at the fetal-maternal interface according to our observations in human pregnancy. Furthermore, hCG application contributed to Treg suppressive capacity by elevating the secretion of IL-10 and TGF-β. Having these results, we wondered whether the positive effect of hCG on Treg number and function may favor fetal survival. For this, we next used a model of disturbed tolerance, in which we previously demonstrated diminished Treg number and activity, and proved that Treg reconstitution can diminish the abortion rates to normal levels (24, 27). hCG not only restored the Treg levels to normality, but also completely prevented abortion. In particular, we proved that Treg reconstitution can diminish the abortion rates to normal levels (24, 27). hCG not only restored the Treg levels to normality, but also completely prevented abortion. In particular, we proved that Treg reconstitution can diminish the abortion rates to normal levels (24, 27).

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and knowing that hCG also fosters their migration (37), we propose that Tregs previously activated by specific paternal Ags in draining lymph nodes actively migrate to the fetal–maternal interface to support fetal survival. The positive effect of hCG on pregnancy is further underlined by a study of Mansour and colleagues (42), who lately showed that intrauterine injection of hCG before embryo transfer in patients undergoing IVF/intracytoplasmic sperm injection significantly improved implantation and pregnancy rates. Unfortunately, in that study, the authors did not analyze the number and activity of Tregs (42). However, increased pregnancy rates after IVF have been shown to be associated with increased Treg numbers in peripheral blood (26). Moreover, based on our human data showing hCG as a potent chemoattractor for Tregs (31), the Egyptian IVF-ET Center conducted a clinical trial investigating the effect of uterine injection of hCG on endometrial Tregs. Upcoming results will prove whether the intrauterine application of hCG around implantation time may increase endogenous endometrial Treg levels, and thereby favor the implantation process. This would support our assumption that normal progressing pregnancies with normal hCG levels ensure the generation and/or expansion of cells that are fundamental for pregnancy maintenance (43).

To prove the generation of functionally active, pregnancy-protective Tregs after hCG application, we adoptively transferred Tregs isolated from hCG-treated AP females (having otherwise dysfunctional Tregs) into AP mice. These cells had the capacity to completely protect from fetal rejection, whereas Tregs isolated from PBS-treated AP females did not. Our data raise the possibility that transferred Tregs induce the generation of "new" Tregs by infectious tolerance or by conversion from naive T cells, as it has been described by others (44–46). In vitro, Tregs from hCG-treated females had a significantly increased capacity to inhibit responder T cell proliferation when compared with Tregs from PBS-treated females. This confirms our in vivo data on hCG-mediated generation of pregnancy-protective Tregs. In agreement with this, Khil and colleagues (19) showed that application of hCG in a murine model for autoimmune diabetes prevented the onset of the disease. In this model, hCG application resulted in an increase of Tregs in spleen and pancreatic lymph nodes, and reduced the number of effector T cells. Moreover, hCG elevated IL-10 and TGF-β expression in splenic cells, which indicates an augmented suppressive Treg function (19). In our model, hCG also provoked Tregs to secrete more IL-10 and TGF-β. Fuchs and colleagues (18, 47) demonstrated an hCG-dependent generation of suppressor T cells, leading to inhibited mitogen-induced activation of B cells. Previous data from our group confirmed an Ag-specific Treg function in the AP model (27). Thus, we wondered whether activation of Tregs can be mediated by hCG alone or is also dependent on the presence of specific paternal Ags. We could clearly show that neither Tregs obtained from PBS- nor from hCG-treated AP females significantly suppressed proliferation of responder T cells from a third-party combination, confirming that there is a need for specific Ag stimulation of Tregs in addition to their

![FIGURE 5. hCG promoted the in vivo generation of Treg that retained their Ag-specificity in vitro.](image)

![FIGURE 6. Tregs and DCs expressed the LH/CG receptor. Ninety-seven percent of CD4+CD25+ Tregs and 69.4% of CD11c+ DCs isolated from virgin CBA/J females expressed the LH/CG receptor on their cell surface.](image)
activation by hCG. According to these data, we did not observe a significant increase in the Treg number in uterine tissue of virgin CBA/J females injected with hCG when compared with CBA/J females treated with PBS. Therefore, we assume that although hCG has a strong impact on Treg expansion and functionality, the presence of specific paternal Ags is essential to ensure complete Treg induction during pregnancy.

Next, we focused on learning whether hCG mediates Treg induction directly or indirectly via DCs. Because both Tregs and DCs expressed the LH/CG receptor, both ways are possible. According to our murine data, the presence of the LH/CG receptor has been proved on human DCs (48) and human Tregs (31). Several studies suggest an impact of tolerogenic DCs on Treg generation (49, 50). We recently demonstrated an involvement of the pregnancy-protective enzyme heme oxygenase-1 for DC-mediated Treg induction and function during murine pregnancy (37). In addition, it has been shown that P induces an immature phenotype in bone marrow–derived DCs (51) and estrogen reduces the production of inflammatory cytokines by mature DCs (52), suggesting that pregnancy hormones may also influence DC phenotype and activity. In terms of an hCG-mediated effect on DCs, in vitro data are quite controversial (48, 53, 54) and in vivo data are not available yet.

Because the maturation state of DCs is important for their capability to induce immunity or tolerance (35), we were interested to study the influence of hCG on DC phenotype and function. Blois and colleagues (55) showed that during pregnancy, the number of CD11c+ DCs in decidua strongly increases when compared with the peripheral blood. The majority of murine DCs at the fetal–maternal interface are of myeloid origin, having an immature tolerogenic phenotype, and produce high amounts of IL-10 (56). In this regard, the secretion of IL-10 seems to be an essential mechanism for DC function at the fetal–maternal interface as IL-10 directly inhibits effector T cells (57) and promotes Treg generation (36). Segerer and colleagues (54), as well as Wan and colleagues (53), nicely showed in vitro that hCG inhibits upregulation of MHC class II molecules on DCs, reduces their T cell stimulatory capacity, and induces IL-10 production. By contrast,  

![FIGURE 7](http://www.jimmunol.org/)

hCG reduced the number of DCs and retained them in an immature state. Application of 10 IU/ml hCG in AP females (n = 5–7/organ) resulted in a significant reduction of total CD11c+ DCs in decidua (B), but had no effect on the number of splenic CD11c+ DCs (A) when compared with PBS-treated females (n = 5–7/organ). The number of mature CD11c+CD80+ and CD11c+MHCII+ DCs in decidua was significantly decreased after hCG treatment (n = 7–8) as compared with PBS treatment (n = 7) (D, F). By contrast, hCG had no impact on the number of mature DCs in spleen (n = 4–5/ treatment) (C, E). Each square represents one single animal, and the lines show the medians. Statistical analysis was carried out by the Mann–Whitney U test. *p < 0.05, **p < 0.01.

### Table II. hCG decreases the number of total and CD11c+MHCII+ DCs in spleen

<table>
<thead>
<tr>
<th>Group</th>
<th>CD11c+ in Spleen (%)</th>
<th>CD11c+CD80+ in Spleen (%)</th>
<th>CD11c+MHCII+ in Spleen (%)</th>
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<tr>
<td>CBA/J + PBS</td>
<td>4</td>
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</table>

Application of 10 IU/ml hCG in virgin CBA/J females reduces the number of total CD11c+ and mature CD11c+MHCII+ DCs in spleen as compared with PBS but did not change the levels of CD11c+CD80+ DCs. Data are presented as medians. Statistical analysis was carried out by the Mann–Whitney U test. No statistical differences could be detected between hCG- and PBS-treated females.
Yoshimura and colleagues (48) revealed that hCG induces the maturation of myeloid and lymphoid DCs in conjunction with increases in the expression of adhesion/costimulatory molecules, their stimulatory activities in MLR, and cytokine secretion. In this article, we provide in vivo evidence for a strong impact of hCG that reduces the number of total and mature DCs directly at the fetal–maternal interface, but not in the periphery. Regarding the impact of a reduction of total DCs for pregnancy outcome, Plaks and colleagues (58) nicely showed that a depletion of uterine DCs by applying diphtheria toxin to CD11cDTR mice resulted in an impaired decidualization and implantation of embryos. However, DC depletion in this model took place either before pregnancy or impaired decidualization and implantation of embryos. However, DC depletion in this model took place either before pregnancy or at very early gestation days. In this study, we can only make conclusions about the number of total CD11c⁺ DCs on day 10 of pregnancy after hCG or PBS treatment. Moreover, although hCG reduced the number of total CD11c⁺ DCs, there are still plenty of DCs present at the fetal–maternal interface that can do their job. Furthermore, we could prove that hCG especially reduced the number of mature CD11c⁺ DCs within the whole CD11c⁺ DC population. Thus, we would assume that hCG favors the presence of a population consisting of immature tolerogenic DCs that have been shown to promote fetal survival by reducing alloreactive maternal T cell responses toward the fetus and supporting generation of Tregs. Coculture of decidual CD11c⁺ cells and CD4⁺ T cells further reveal that DCs from hCG-treated AP females indeed provoked the generation or expansion of Tregs by upregulating Foxp3 expression in CD4⁺ T cells. According to our observation that hCG seems to have no influence on the phenotype of splenic DCs, we could not detect Foxp3 upregulation in CD4⁺ T cells in the presence of splenic DCs from hCG-treated AP females. Thus, we suppose that hCG directly modulates DCs at the fetal–maternal interface, and this favors a local Treg expansion.

Our work provides strong evidence that hCG, the most important pregnancy hormone, secreted by the trophoblast, contributes to fetal tolerance not only by attracting Tregs to the fetal–maternal interface, but also by augmenting the number and suppressive capacity of Tregs. hCG has a direct effect on Tregs but also acts indirectly by maintaining DCs in an immature, tolerogenic state. Our observations offer a possible explanation for improved pregnancy outcomes of IVF patients treated with hCG after embryo transfer. This contributes to the basic knowledge of Treg generation and functionality during pregnancy and may help to establish therapies to prevent pregnancy failures.

Disclosures
The authors have no financial conflicts of interest.

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1. Chambers, S. P., and A. G. Clarke. 1979. Measurement of thymus weight, lumbar node weight and progesterone levels in syngeneically pregnant, alloge-


5. Chambers, S. P., and A. G. Clarke. 1979. Measurement of thymus weight, lumbar node weight and progesterone levels in syngeneically pregnant, alloge-


hCG stimulates Treg and supports pregnancy.

### Table III. hCG decreases the number of total and CD11c⁺ MHCI⁺ DCs in uterus

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CD11c⁺ in Uterus (%)</th>
<th>CD11c⁺ MHCI⁺ in Uterus (%)</th>
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<td>CBA/J + PBS</td>
<td>4</td>
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<td>0.09</td>
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<td>CBA/J + hCG</td>
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</tbody>
</table>

Application of 10 IU/ml hCG in virgin CBA/J females reduces the number of total CD11c⁺ and mature CD11c⁺ MHCI⁺ DCs in uterus as compared to PBS but did not change the levels of CD11c⁺ MHCI⁺ DCs. Data are presented as medians. Statistical analysis was carried out by the Mann–Whitney U test. No statistical differences could be detected between hCG- and PBS-treated females.


