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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 adoptively 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.

Fetal survival within the maternal uterus is ensured by strong hormonal changes and the regulation of maternal immune responses toward the foreign fetal Ags. The interplay between hormonal and immunological factors contributes substantially to fetal tolerance. Pregnancy-associated hormones like the human chorionic gonadotropin (hCG), estrogen, and progesterone (P) increase during pregnancy and are essential for successful pregnancy outcome (1, 2). hCG is a primate-specific hormone; that is, it is only produced by humans (3) and primates (4), and cannot be detected in rodents (5). However, rodents produce the highly homologous luteinizing hormone (LH) binding to the same receptor as hCG (namely, the LH/CG receptor) (6). Because of this circumstance, hCG effects can be easily studied in the murine system. Immediately after fertilization, hCG is produced by the blastocyst and later by the syncytiotrophoblast (7, 8). After reaching its maximum level between the 9th and 12th weeks of pregnancy, hCG concentrations decline until birth (9). In addition to its main function in stimulating P production by the corpus luteum, hCG has been described to facilitate trophoblast invasion (10–12), support angiogenesis, and ensure nourishment of the fetus (13–15). In the 1970s and 1980s, several studies suggested hCG as an important factor modulating T and B cell responses (16, 17) via the induction of the at that time called suppressor T cells (18). This concept was no longer followed until more recently when Khil and colleagues (19) showed an hCG-mediated inhibitory effect on Th1 responses that was associated with increased numbers of CD4+CD25+ regulatory T cells (Tregs) in a murine model of autoimmune diabetes. During normal human and murine pregnancy, Tregs expand systemically and locally at the fetal–maternal interface (20–22). Disturbed pregnancies are associated with a diminished Treg number and activity (23–25). Zhou and colleagues (26) recently confirmed the positive association of Treg expansion with pregnancy establishment as they demonstrated that an increase of Tregs in peripheral blood was a requisite for a successful in vitro fertilization (IVF). We first introduced Tregs as a useful tool to control disturbed tolerance during pregnancy in an abortion-prone (AP) model. We observed that the transfer of Ag-specific Tregs at early pregnancy stages restored tolerance and thereby prevented fetal rejection (24, 27), which is in agreement with recent findings by Yin and colleagues (28). Treg transfer induced a tolerant microenvironment directly at the fetal–maternal interface (29), and Treg protective effects on pregnancy were mediated at least in the studied model by IL-10 and programmed death-1, whereas TGF-β and CTLA-4 rather played a secondary role (27, 30). In humans, we found a correlation between hCG and Treg levels, which are both diminished in patients suffering from spontaneous abortions or extrauterine pregnancies when compared with normal pregnant (NP) women (31). We also found hCG to attract Tregs, which express the LH/CG receptor, into the fetal–maternal interface (31). Thus, hCG acts as a chemoattractant to Tregs at early pregnancy stages. This gives rise to a concept in which the very first hormone produced by the trophoblast, hCG, contributes to the recruitment of immune cells to the fetal–maternal interface that are pivotal for later tolerating the conceptus. In this work, we followed the hypothesis that hCG would not only be a chemoattractant, but also an im-
hCG STIMULATES TREG AND SUPPORTS PREGNANCY

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 AZ2/868 and AZ42502-2-1125UniMD). 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. Treg numbers were increased in BALB/c-mated CBA/J females on a C57BL/6 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, Co¨lbe, 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, uterus, and decidua samples were isolated from pregnant animals. (DCs) that act as APCs at the very first step of an adaptive immune response.

To study the influence of hCG on Treg function, we isolated CD4+CD25+ cells by magnetic beads (MACS; Miltenyi Biotec, Bergisch 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 assessed by flow cytometry.

To investigate whether hCG induces the generation of pregnancy-protective Tregs in vivo, we isolated CD4+CD25+ cells by magnetic beads (MACS; Miltenyi Biotec, Bergisch 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 assessed by flow cytometry.

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 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 nodes from BALB/c-mated Foxp3gfp transgenic females (treated with either PBS or hCG), washed in ice-cold PBS, and kept in RPMI 1640 medium supplemented with 10% PBS (Biochrom, Berlin, Germany) and 100 ng/ml penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 4 °C. Mononuclear cells were isolated by disaggregating the tissue, filtering it through a 100-μm cell strainer (Becton Dickinson), and lysing the erythrocytes with an NHCl/NaCl solution. After lysis, cells were stained with PerCP-Cy5.5-conjugated anti-mouse CD4 (clone RM4-5) from Becton Dickinson. CD4+GFP+/Foxp3+ Tregs were sorted by FACS using a FACSDiva Flow Cytometer and CellSorter from Becton Dickinson. Sorted Tregs were cultured for 24 h in RPMI medium supplemented with PBS and penicillin/streptomycin. To induce cytokine secretion, we added 1 μg/ml ionomycin (Sigma-Aldrich, Steinheim, Germany) and 50 ng/ml PMA (Sigma-Aldrich) to the culture. After 24 h, supernatants were taken for analysis of IL-10 and TGF-β by ELISA.

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 isolated 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.
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 females 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 (Fig. 1C, 1D) when compared with PBS. Thus, hCG provokes an expansion of Tregs and fosters their 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 increased Treg numbers in hCG-treated females 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 (Fig. 1C, 1D) when compared with PBS. Thus, hCG provokes an expansion of Tregs and fosters their IL-10 and TGF-β secretion.

| Table 1. | hCG increased the number of local Tregs in virgin females |
|---|---|---|---|
| Group | n | Tregs in Spleen (%) | Tregs in Blood (%) | Tregs in Uterus (%) |
| CBA/J + PBS | 4 | 4.08 | 1.63 | 12.30 |
| CBA/J + hCG | 4 | 4.52 | 1.06 | 17.08 |

*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.*

FIGURE 2. hCG application normalized Treg frequencies in AP females. Application of 10 IU/ml hCG in AP females (n = 6–9/organ) significantly increased the number of CD44Foxp3+ 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.
increased 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 (Fig. 2A), 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 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.

To know whether Tregs generated after hCG injection are fully functional, we next isolated Tregs from hCG- or PBS-treated, DBA/2J-mated AP CBA/J females and adoptively transferred them into AP animals at days 0–2 of pregnancy. As expected, Tregs obtained from PBS-treated, DBA/2J-mated CBA/J females were not able to confer fetal protection to AP females because of their impaired functionality (Fig. 4A). By contrast, Tregs obtained from hCG-treated, DBA/2J-mated CBA/J females completely rescued fetuses from immune rejection after adoptive transfer in AP females (Fig. 4A). This was not dependent on the number of implantations because they were all comparable among the groups (Fig. 4B). The adoptive transfer of Tregs from hCG-treated females resulted in a significant increase in systemic and local Treg levels when compared with the transfer of Tregs from PBS-treated females (Fig. 4C–F).

Tregs generated in vivo after hCG application retained their Ag specificity toward paternal Ags

We next wondered whether Tregs isolated from hCG- or PBS-treated AP females would suppress proliferation of autologous responder T cells in an MLR in vitro and whether this effect is Ag specific. As expected, Tregs from both hCG- and PBS-treated females had a strong suppressive impact on the proliferation rate of responder T cells when compared with proliferation of responder T cells without Tregs (Fig. 5A). Interestingly, we found that Tregs from hCG-treated female mice had a significantly increased suppressive capacity compared with Tregs obtained from PBS-treated females (Fig. 5A), which confirms that hCG contributes to Treg suppressive capacity. To further examine whether the effect of hCG on Treg function is specific for paternal Ags, we determined the proliferation rate of responder T cells obtained from a “third-party” combination (CBA/J×C57BL/6) in the presence of Tregs from hCG- or PBS-treated AP females. Tregs from both sources had no effect on the proliferation rate of responder T cells when compared with their proliferation without Tregs (Fig. 5B). These results reveal the important influence of hCG on Treg functionality: hCG applied to pregnant animals fosters the expansion of Tregs that are specific against paternal Ags and play, therefore, an important role in tolerating these Ags.

Tregs and DCs expressed the LH/CG receptor

After we proved an influence of hCG on Treg expansion and functionality, we wondered whether this effect is mediated directly by hCG binding to the LH/CG receptor on Treg cell surface or indirectly by modulation of APCs, that is, DCs, which then may induce Treg generation. To test both possibilities, we checked for the presence of the LH/CG receptor on both murine Tregs and DCs. Interestingly, 97% of Tregs and 69.4% of DCs isolated from virgin CBA/J females expressed the LH/CG receptor (Fig. 6) as expected. DCs from hCG-treated female mice had a significantly increased suppressive capacity compared with Tregs obtained from PBS-treated females (Fig. 5A), which confirms that hCG contributes to Treg suppressive capacity. To further examine whether the effect of hCG on Treg function is specific for paternal Ags, we determined the proliferation rate of responder T cells obtained from a “third-party” combination (CBA/J×C57BL/6) in the presence of Tregs from hCG- or PBS-treated AP females. Tregs from both sources had no effect on the proliferation rate of responder T cells when compared with their proliferation without Tregs (Fig. 5B). These results reveal the important influence of hCG on Treg functionality: hCG applied to pregnant animals fosters the expansion of Tregs that are specific against paternal Ags and play, therefore, an important role in tolerating these Ags.

hCG retained DCs in an immature state, and in vitro these cells favor Treg generation/expansion

To evaluate the possibility that the influence of hCG on Treg number and function is mediated indirectly via altering the phenotype and activity of other immune cells, especially cells of the innate immune system presenting Ags, we investigated the influence of hCG on the phenotype of DCs. According to their maturation state and compared with their proliferation without Tregs (Fig. 5B). These results reveal the important influence of hCG on Treg functionality: hCG applied to pregnant animals fosters the expansion of Tregs that are specific against paternal Ags and play, therefore, an important role in tolerating these Ags.

hCG stimulated Tregs and supports pregnancy

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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 increased the number of mature DCs determined by the expression in CD80 and MHC class II was significantly reduced in decidua but not spleen after hCG treatment as 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).

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 Foxp3<sup>gfp</sup> transgenic animals that allowed us to easily isolate and characterize Foxp3<sup>+</sup> Tregs after hormonal treatment in an alloimmune 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).

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.

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<sup>+</sup>Foxp3<sup>+</sup> cells (Tregs) within the CD4<sup>+</sup> 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.
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. (A) Both, Treg isolated from hCG- and PBS-treated AP females significantly suppressed proliferation of autologous responder T cells (Tresp). Interestingly, Treg from hCG-treated females showed a significant increased suppressive capacity when compared with Treg from PBS-treated females. (B) Neither Tregs from hCG- nor PBS-treated AP females were able to suppress the proliferation of allogeneic responder T cells from a third-party combination (Tthird party). In addition, no differences could be observed between the suppressive capacities from hCG- or PBS-treated females. Suppression assays were performed in duplicates and repeated at least three times to ensure reproducibility. Data are presented as means ± SD. Statistical analysis between two different groups was performed using the Student t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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 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. Negative controls are included to exclude unspecific binding of the second Ab. Data are presented as histograms using FlowJo software.
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 MHCII+ molecules on DCs, reduces their T cell stimulatory capacity, and induces IL-10 production. By contrast,

**FIGURE 7.** 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>n</th>
<th>CD11c+ in Spleen (%)</th>
<th>CD11c+CD80+ in Spleen (%)</th>
<th>CD11c+MHCII+ in Spleen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/J + PBS</td>
<td>4</td>
<td>3.38</td>
<td>0.97</td>
<td>1.69</td>
</tr>
<tr>
<td>CBA/J + hCG</td>
<td>4</td>
<td>2.81</td>
<td>1.13</td>
<td>1.18</td>
</tr>
</tbody>
</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.
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.

References


