

# Human Chorionic Gonadotropin Has Anti-Inflammatory Effects at the Maternal-Fetal Interface and Prevents Endotoxin-Induced Preterm Birth, but Causes Dystocia and Fetal Compromise in Mice<sup>1</sup>

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## ABSTRACT

Human chorionic gonadotropin (hCG) is implicated in the maintenance of uterine quiescence by down-regulating myometrial gap junctions during pregnancy, and it was considered as a strategy to prevent preterm birth after the occurrence of preterm labor. However, the effect of hCG on innate and adaptive immune cells implicated in parturition is poorly understood. Herein, we investigated the immune effects of hCG at the maternal-fetal interface during late gestation, and whether this hormone can safely prevent endotoxin-induced preterm birth. Using immunophenotyping, we demonstrated that hCG has immune effects at the maternal-fetal interface (decidual tissues) by: 1) increasing the proportion of regulatory T cells; 2) reducing the proportion of macrophages and neutrophils; 3) inducing an M1 → M2 macrophage polarization; and 4) increasing the proportion of T helper 17 cells. Next, ELISAs were used to determine whether the local immune changes were associated with systemic concentrations of progesterone, estradiol, and/or cytokines (IFN $\gamma$ , IL1 $\beta$ , IL2, IL4, IL5, IL6, IL10, IL12p70, KC/GRO, and TNF $\alpha$ ). Plasma concentrations of IL1 $\beta$ , but not progesterone, estradiol, or any other cytokine, were increased following hCG administration. Pre-

treatment with hCG prevented endotoxin-induced preterm birth by 44%, proving the effectiveness of this hormone as an anti-inflammatory agent. However, hCG administration alone caused dystocia and fetal compromise, as proven by Doppler ultrasound. These results provide insight into the mechanisms whereby hCG induces an anti-inflammatory microenvironment at the maternal-fetal interface during late gestation, and demonstrate its effectiveness in preventing preterm labor/birth. However, the deleterious effects of this hormone on mothers and fetuses warrant caution.

*decidua, estradiol, hCG, interleukin-1 $\beta$ , M1 macrophages, M2 macrophages, mouse, neutrophils, parturition, pregnancy, preterm labor, progesterone, regulatory T cells, Th17 cells*

## INTRODUCTION

Pregnancy maintenance involves endocrine interactions between the mother and fetus. The first hormone to participate in this interaction is human chorionic gonadotropin (hCG) [1–3]. Human CG is a complex glycoprotein composed of  $\alpha$  and  $\beta$  glycosylated subunits [4], and is considered a superagonist of luteinizing hormone receptors [5]. Human CG is produced by the syncytiotrophoblast [6–8] and, to a lesser extent, by the invasive extravillous cytotrophoblast [8, 9]. Human CG receptors are expressed in reproductive tissues [2, 10, 11], fetal-placental tissues [12–14], and immune cells [15–17], indicating that this hormone has multiple targets, and is not only relevant to pregnancy initiation [18]. In line with this concept, hCG is involved in: 1) preserving the progesterone-producing corpus luteum [19]; 2) promoting angiogenesis [20, 21], trophoblast differentiation [22, 23], and maternal-fetal tolerance [24–28]; and 3) maintaining myometrial quiescence [29–35].

Maternal-fetal tolerance involves the function of regulatory T cells (Tregs) [36], dendritic cells (DCs) [37–41], B cells [42–44], and M2 or immunosuppressive macrophages [45–47]. During early pregnancy, hCG contributes to this tolerance by: 1) increasing the migration of Tregs into the maternal-fetal interface [26, 28]; 2) enhancing Treg frequencies in the circulation, lymphatic organs, and decidual tissues of abortion-prone mice (DBA/2-mated CBA females) [28]; 3) inducing Treg suppressive activity in vivo [28]; 4) stimulating IL10 production by circulating B cells [43]; and 5) driving a tolerogenic phenotype in bone marrow-derived DCs [48].

<sup>1</sup>This research was supported by the Perinatology Research Branch, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services, and by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health. This study was partially presented at the 34th Annual Meeting of the American Society for Reproductive Immunology, Long Beach, New York, 2–5 June, 2014, and the 62nd Annual Scientific Meeting of the Society for Reproductive Investigation, San Francisco, California, 25–28 March, 2015.

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Received: 8 February 2016.

First decision: 13 March 2016.

Accepted: 2 May 2016.

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eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

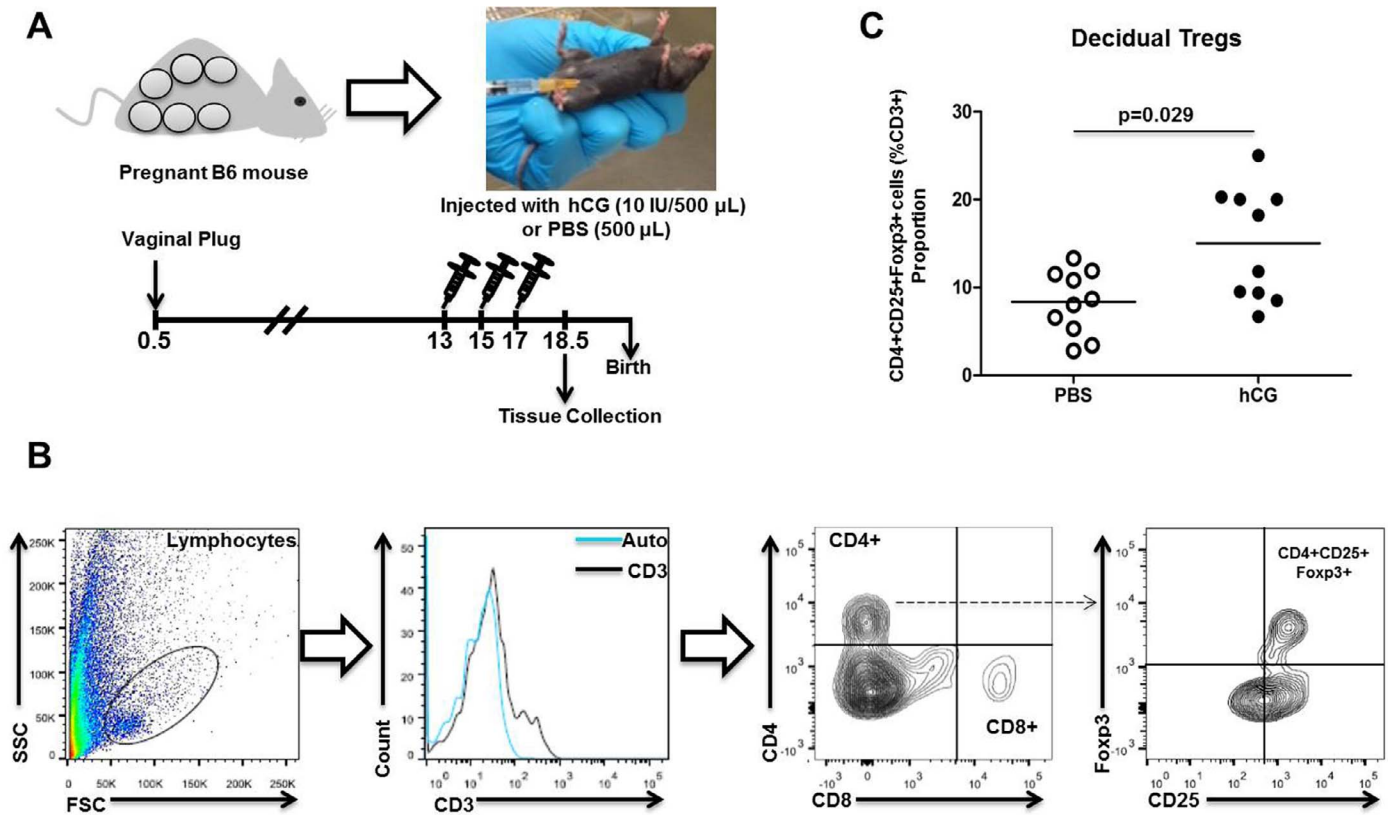


FIG. 1. Animal model and identification of Tregs at the maternal-fetal interface. **A)** hCG administration scheme. **B)** Gating strategy used to identify Tregs ( $CD4^+CD25^+Foxp3^+$  T cells) in decidual tissues.  $CD3^+$  T cells were gated within the total lymphocyte gate (FSC vs. SSC). The blue line represents the autofluorescence control. Tregs were then gated within the  $CD4^+$  T cell gate. **C)** Proportions of Tregs in decidual tissues from mice injected with PBS or hCG.  $n = 10$  each. Data are represented as dot plots (median).

However, the effect of hCG on these immune cells during late gestation is poorly understood.

Human CG was considered a vestigial hormone in late gestation [49]. This is due to the fact that systemic concentrations of hCG increase in the first trimester and reduce thereafter [50]. Furthermore, systemic concentrations of hCG significantly decrease 2–3 wk before labor [51], suggesting that decreasing concentrations of this hormone participate in the onset of parturition. We therefore hypothesize that the systemic administration of hCG will delay the onset of parturition.

Elucidating the role of pregnancy-related hormones in late gestation is relevant, since maternal-fetal tolerance, and therefore immune cells activity, is still required during this period. For example, the diminished suppressive function of Tregs is associated with spontaneous preterm labor/birth [52], and mice deficient in T and B cells (*Rag1*<sup>-/-</sup>) are more susceptible to endotoxin-induced preterm birth than wild-type mice [53]. In addition, a reduction of Tregs in the myometrial tissues is observed in wild-type mice prior to endotoxin-induced preterm birth [54]. Therefore, a breakdown of maternal-fetal tolerance during late gestation is considered a mechanism of disease for preterm labor [55, 56], which leads to preterm birth in 70% of cases [57]. Prematurity is the leading cause of perinatal morbidity and mortality worldwide [58], and represents a substantial burden for society and the healthcare system [59]. Hence, the prevention of preterm birth is a health care priority. Herein, we hypothesize that pretreatment with a natural pregnancy hormone, hCG, fosters maternal-fetal tolerance during late gestation by favoring an anti-inflammatory micro-environment at the maternal-fetal interface, thereby preventing preterm birth.

The main aim of this study was to determine the effects of hCG on Tregs and other T cell subsets, macrophages, neutrophils, natural killer (NK) cells, as well as M1-like and M2-like macrophages in decidual tissues (maternal-fetal interface). The effect of hCG on the concentrations of progesterone, estradiol, and IL1 $\beta$  in maternal plasma was also determined. The effectiveness of hCG in preventing preterm birth was tested in mice injected with an endotoxin, and the safety of hCG was evaluated using in vivo imaging via ultrasound.

## MATERIALS AND METHODS

### Animals

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University (Detroit, MI). All mice were kept under a circadian cycle (12L:12D). Females (8–12 wk old) were mated with males of the same phenotype. Female mice were checked daily between 0800 and 0900 h for the appearance of a vaginal plug, which indicated 0.5 days postcoitum (dpc). Females were then separated from males, their weight was monitored, and a gain of  $\geq 2$  g by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (protocol nos. A09-08-12 and A07-03-15).

### Human CG Administration

Pregnant B6 females were intraperitoneally injected on 13.0, 15.0, and 17.0 dpc with 10 IU of hCG (Sigma-Aldrich, St Louis, MO) dissolved in 500  $\mu$ l of 1 $\times$  sterile PBS (Fisher Scientific Bioreagents, Fair Lawn, NJ) or 500  $\mu$ l of PBS alone as a control, as previously described [28]. Prior to delivery (18.5 dpc), mice were euthanized, and the peripheral blood, lymphoid organs (the uterine-draining lymph nodes, thymus, and spleen), and decidual tissues were harvested (Fig. 1A). Leukocyte isolation was immediately performed on

lymphoid organs and decidual tissues, and plasma was separated for hormone and cytokine determination.

### Leukocyte Isolation

Leukocytes were isolated from decidual tissues as previously described [60]. Briefly, decidual tissues were immersed in a cell-dissociating reagent (StemPro Accutase Cell Dissociation Reagent; Life Technologies, Grand Island, NY) and homogenized with a pair of small surgical scissors for 1–2 min. The homogenized tissue was incubated for 35 min at 37°C with gentle shaking (MaxQ 4450 Benchtop Orbital Shaker; Thermo Fisher Scientific, Marietta, OH). The cell suspension was washed with FACS buffer (bovine serum albumin 0.1% [Sigma-Aldrich], sodium azide 0.05% [Fisher Scientific Chemicals, Fair Lawn, NJ], and 1× PBS) and filtered using a 100 µm cell strainer (Fisher Scientific, Hanover Park, IL). Simultaneously, leukocytes were isolated from the uterine-draining lymph nodes, thymus, and spleen by gentle dissociation using two double-frosted glass micro slides (Corning Inc., Corning, NY). Both decidual and lymphoid cell suspensions were then centrifuged at 1300 × g at 4°C for 10 min, and the resulting cell pellet was used for immunophenotyping.

### Immunophenotyping

Decidual and lymphoid cell suspensions were incubated with a mouse monoclonal CD16/CD32 antibody (FcγIII/II Receptor; BD Biosciences, San Jose, CA) at 4°C for 10 min. Next, cell suspensions were incubated at 4°C with extracellular and/or intracellular conjugated antibodies (Supplemental Table S1; available online at www.biolreprod.org) for 30 min in the dark. Two groups of mice were used for immunophenotyping. In the first group, mice were injected with PBS or hCG (n = 8–10 each), and proportions of Tregs (CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells) were determined in the decidua, spleen, thymus, and uterine-draining lymph nodes. Macrophages (CD45<sup>+</sup>F4/80<sup>+</sup> cells), neutrophils (CD45<sup>+</sup>Ly6G<sup>+</sup> cells), and NK cells (CD45<sup>+</sup>CD49b<sup>+</sup> cells) were also determined in decidual tissues. In the second group, mice were injected with PBS or hCG (n = 10 each), and proportions and numbers of M1-like (CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup>IFNγ<sup>+</sup> and/or iNOS<sup>+</sup> cells) and M2-like (CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup>Arg1<sup>+</sup> and/or IL10<sup>+</sup> cells) macrophages, T helper (Th) 1 (CD3<sup>+</sup>CD4<sup>+</sup>IFNγ<sup>+</sup> cells), Th2 (CD3<sup>+</sup>CD4<sup>+</sup>IL10<sup>+</sup>), Th9 (CD3<sup>+</sup>CD4<sup>+</sup>IL9<sup>+</sup> cells), and Th17 (CD3<sup>+</sup>CD4<sup>+</sup>IL17A<sup>+</sup> cells) cells were determined in decidual tissues.

Nuclear staining of Foxp3 was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA), and intracellular cytokine staining was performed using the Cytofix/Cytoperm Fixation and Permeabilization Buffer Kit (BD Biosciences), according to the manufacturers' protocols. Finally, cell suspensions were acquired using the LSRFortessa Flow Cytometer (BD Biosciences) and analyzed using BD FACSDiva Software Version 8.0 (BD Biosciences). The figures were prepared using FlowJo Software Version 10 (FlowJo, LLC, Ashland, OR).

### Enzyme-Linked Immune Assays

Plasma concentrations of progesterone and estradiol were determined using the PROG-EASIA ELISA kit (GenWay Biotech, Inc., San Diego, CA) and the Calbiotech Mouse/Rat Estradiol ELISA kit (Calbiotech Inc., Spring Valley, CA), according to the manufacturers' instructions. Plasma concentrations of IFNγ, IL1β, IL2, IL4, IL5, IL6, IL10, IL12p70, KC/GRO, and TNFα were assessed using sensitive and specific immunoassays according to the manufacturer's instructions (V-PLEX Proinflammatory Panel 1 Kit; Meso Scale Discovery, Gaithersburg, MD). The sensitivities of the assays were: 0.04 pg/ml (IFNγ), 0.11 pg/ml (IL1β), 0.22 pg/ml (IL2), 0.14 pg/ml (IL4), 0.07 pg/ml (IL5), 0.61 pg/ml (IL6), 0.95 pg/ml (IL10), 9.95 pg/ml (IL12p70), 0.24 pg/ml (KC/GRO), and 0.13 pg/ml (TNFα); and the interassay and intra-assay coefficients of variation were below 15% and 7%, respectively.

### Endotoxin-Induced Preterm Birth in Mice Pretreated with hCG

Pregnant B6 females were injected as follows: 1) PBS alone (n = 10); 2) hCG alone (n = 7); 3) hCG + endotoxin (n = 11); and 4) endotoxin alone (n = 10). Groups 1, 2, and 3 were injected on 13.0, 15.0, and 17.0 dpc. On 16.5 dpc, groups 3 and 4 were intraperitoneally injected with 10 µg of an endotoxin (*Escherichia coli*, 055:B5; Sigma-Aldrich) in 200 µl of PBS. Between 16.5 and 17.0 dpc, mice were placed for video recording in order to determine gestational age, duration of labor, and the rates of stillbirth and preterm birth. Gestational age was defined as the time elapsed from the sighting of the vaginal plug (0.5 dpc) through the delivery of the first pup. Duration of labor was

defined as the time elapsed from the delivery of the first pup through the delivery of the last pup. Rate of stillbirth was defined as the percentage of pups found dead among the total litter size. Preterm birth was defined as all deliveries occurring prior to 18.0 dpc, and its rate was represented by the percentage of females delivering preterm among those delivering at term (19.5 ± 0.5 dpc). Dystocia was defined as disturbed progression of labor (duration of labor > 6 h), and its rate was represented by the percentage of females in dystocia among those delivering successfully.

### In Vivo Imaging by Ultrasound

Pregnant B6 mice were injected with PBS or hCG on 13.0, 15.0, and 17.0 dpc, as previously described (n = 4–5 each) [60]. On 18.5–19.5 dpc, pregnant B6 mice were anesthetized by inhalation of 2%–3% isoflurane (Aerrane; Baxter Healthcare Corp., Deerfield, IL) and 1–2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5%–2% isoflurane and 1.5–2 L/min of oxygen. Dams were positioned on a heating pad and stabilized with adhesive tape. Fur was removed from the abdomen and thorax after Nair cream (Church & Dwight Co., Inc., Ewing, NJ) was applied to the area. Body temperature was maintained in the range of 37 ± 1°C and detected with a rectal probe. Respiratory rate and heart rate were monitored by electrodes embedded in the heating pad. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved toward the abdomen. Fetal heart rate and umbilical artery hemodynamic parameters were examined with the 55 MHz linear ultrasound probe (VisualSonics Inc., Toronto, ON, Canada). Ultrasound signals were processed, displayed, and stored using the Vevo Imaging Station (VisualSonics Inc.). Following ultrasound, dams were placed under a heat lamp for recovery, which occurred 10–20 min after heating.

### Statistical Analysis

Statistical analyses were performed using SPSS, Version 19.0 (IBM Corp., Armonk, NY). Mann-Whitney *U*-tests were performed to analyze observational data (gestational age, duration of labor, and rate of stillbirth), flow cytometry proportions and numbers, and hormone and cytokine plasma concentrations. The *t*-tests were performed to analyze fetal heart rates and umbilical artery pulsatility indexes. Fisher exact tests were used to compare the rates of preterm birth and dystocia between groups. A *P* value of ≤0.05 was considered statistically significant.

## RESULTS

### Administration of hCG Increases the Proportion of Decidual Tregs

First, we determined whether hCG administration increased the proportion of Tregs, a T cell subset that participates in maternal-fetal tolerance [25, 36], in decidual tissues and lymphatic organs. Figure 1B shows the gating strategy used to identify Tregs in decidual tissues. Pregnant mice injected with hCG had higher proportions of decidual Tregs than control mice injected with PBS (Fig. 1C). In contrast, hCG administration did not alter the proportion of Tregs in the thymus, spleen, or uterine-draining lymph nodes when compared to control mice (Fig. 2). These results demonstrate that hCG administration from mid- to late gestation increases Tregs at the maternal-fetal interface.

### Administration of hCG Decreases the Proportion of Macrophages and Neutrophils in Decidual Tissues

Next, we investigated whether hCG administration altered the proportion of macrophages, neutrophils, and NK cells in decidual tissues, all of which participate throughout pregnancy and/or parturition [56, 62, 63]. The gating strategy used to identify macrophages, neutrophils, and NK cells is shown in Figure 3A. Pregnant mice injected with hCG had lower proportions of decidual macrophages (Fig. 3B) and neutrophils (Fig. 3C) than control mice. However, hCG administration did not alter the proportions of NK cells in decidual tissues (data not shown). These data show that hCG administration from

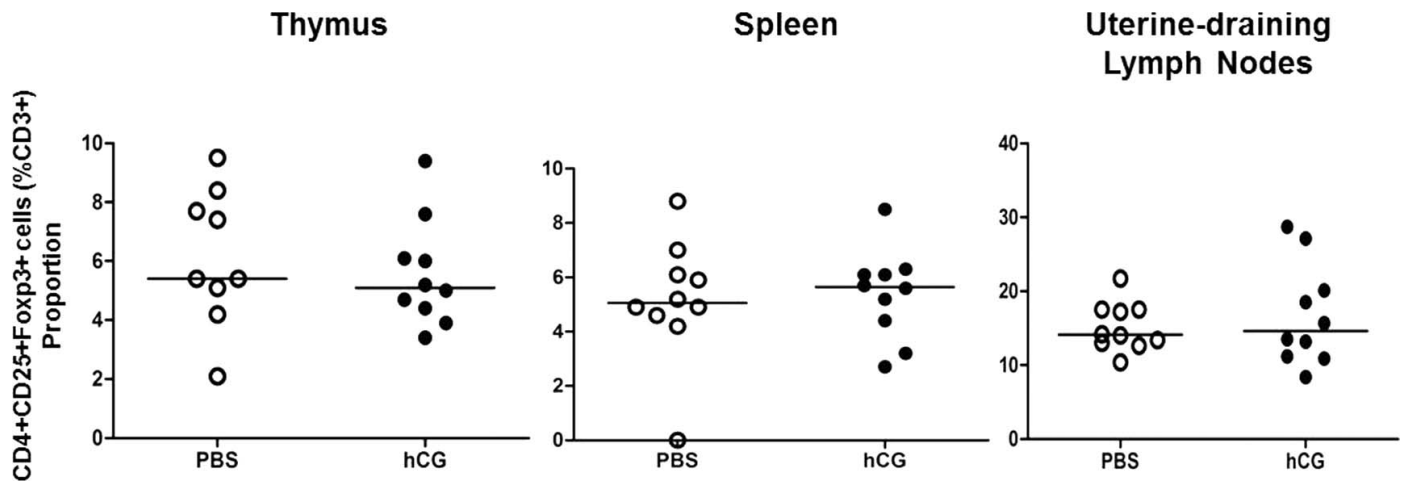


FIG. 2. Proportions of Tregs in lymphoid organs. Proportions of Tregs in the thymus, spleen, and uterine-draining lymph nodes from mice injected with PBS or hCG.  $n = 9-10$  each. Data are represented as dot plots (median).

mid- to late gestation reduces the proportion of innate immune cells, which are known to have proinflammatory roles at the maternal-fetal interface.

#### *Administration of hCG Induces an M1 $\rightarrow$ M2 Macrophage Polarization in Decidual Tissues*

The current hypothesis states that decidual M2-polarized macrophages are implicated in supporting maternal-fetal tolerance throughout pregnancy [46, 47], and that during parturition, these cells shift to a proinflammatory phenotype [64]. Here, we investigated whether hCG induces an M1  $\rightarrow$  M2 macrophage polarization in decidual tissues. The gating strategy used to characterize M1-like and M2-like macrophages in decidual tissues is shown in Figure 4A. Pregnant mice injected with hCG had lower numbers of  $\text{IFN}\gamma^+$  M1-like macrophages (Fig. 4B), but higher numbers of  $\text{Arg1}^+$  M2-like macrophages (Fig. 4C) when compared to control mice. No differences between groups were observed in  $\text{iNOS}^+$  M1-like macrophages or  $\text{IL10}^+$  M2-like macrophages (data not shown). These results show that hCG can induce an anti-inflammatory microenvironment by inducing an M1  $\rightarrow$  M2 macrophage polarization at the maternal-fetal interface.

#### *Administration of hCG Increases the Proportion of Th17 Cells in Decidual Tissues*

Since hCG administration induced macrophage polarization, we next investigated whether this hormone would induce Th cell differentiation in decidual tissues. The gating strategy used to determine Th1, Th2, Th9, and Th17 cells in decidual tissues is shown in Figure 5A. Pregnant mice injected with hCG had higher proportions of decidual Th17 cells than control mice (Fig. 5B). No changes were observed in Th1, Th2, or Th9 cells in decidual tissues upon hCG administration (data not shown). These data demonstrate that hCG administration from mid to late gestation induces a Th17 cell polarization at the maternal-fetal interface, but does not alter other Th cell subsets.

#### *Administration of hCG Does Not Change the Plasma Concentrations of Progesterone and Estradiol*

In vivo and in vitro studies have demonstrated that hCG stimulates the production of progesterone and estrogen [65, 66]. Therefore, we investigated whether in vivo administration

of hCG from mid- to late gestation would change the systemic concentrations of progesterone and/or estradiol. Human CG did not change the plasma concentrations of progesterone or estradiol (Fig. 6, A and B). These results show that the local anti-inflammatory effect of hCG at the maternal-fetal interface is not associated with systemic concentrations of progesterone and estradiol.

#### *Administration of hCG Increases the Plasma Concentration of IL1 $\beta$*

Next, we investigated whether hCG administration would change the systemic concentrations of pro- and anti-inflammatory cytokines. Human CG administration from mid- to late gestation increased the plasma concentration of  $\text{IL1}\beta$  (Fig. 6C). However, no differences were observed in the systemic concentrations of  $\text{IFN}\gamma$ , IL2, IL5, IL6, KC/GRO, IL10, or  $\text{TNF}\alpha$  between mice injected with hCG and those injected with PBS (data not shown). Concentrations of IL4 and IL12p70 were undetectable in plasma samples. Our data suggest that, although hCG has anti-inflammatory effects at the maternal-fetal interface, this hormone may have proinflammatory effects in maternal circulation.

#### *Pretreatment with hCG Reduces the Rate of Endotoxin-Induced Preterm Birth*

To evaluate the effectiveness of hCG in the prevention of endotoxin-induced preterm birth, pregnant mice were pretreated with hCG and then injected with an endotoxin. Mice pretreated with hCG had a reduced rate of endotoxin-induced preterm birth when compared to control mice injected with endotoxin alone ( $36.3 \pm 28.4\%$  [4/11] vs.  $80 \pm 24.7\%$  [8/10];  $P = 0.04$ ; Table 1). However, pretreatment with hCG did not reduce the rate of stillbirth in mice injected with an endotoxin ( $85.2 \pm 8.9\%$  [52/61] vs.  $100\%$  [70/70]; Table 1). Human CG administration to endotoxin-injected mice led to an increased rate of dystocia when compared to PBS controls ( $36.3 \pm 28.4\%$  [4/11] vs.  $0\%$ ;  $P = 0.03$ ; Table 1). This was likely due to hCG, since its administration alone delayed parturition and caused dystocia in all cases without inducing preterm birth (Table 1). These results demonstrate that pretreatment with hCG from mid- to late gestation reduced the rate of endotoxin-induced preterm birth, yet caused dystocia.

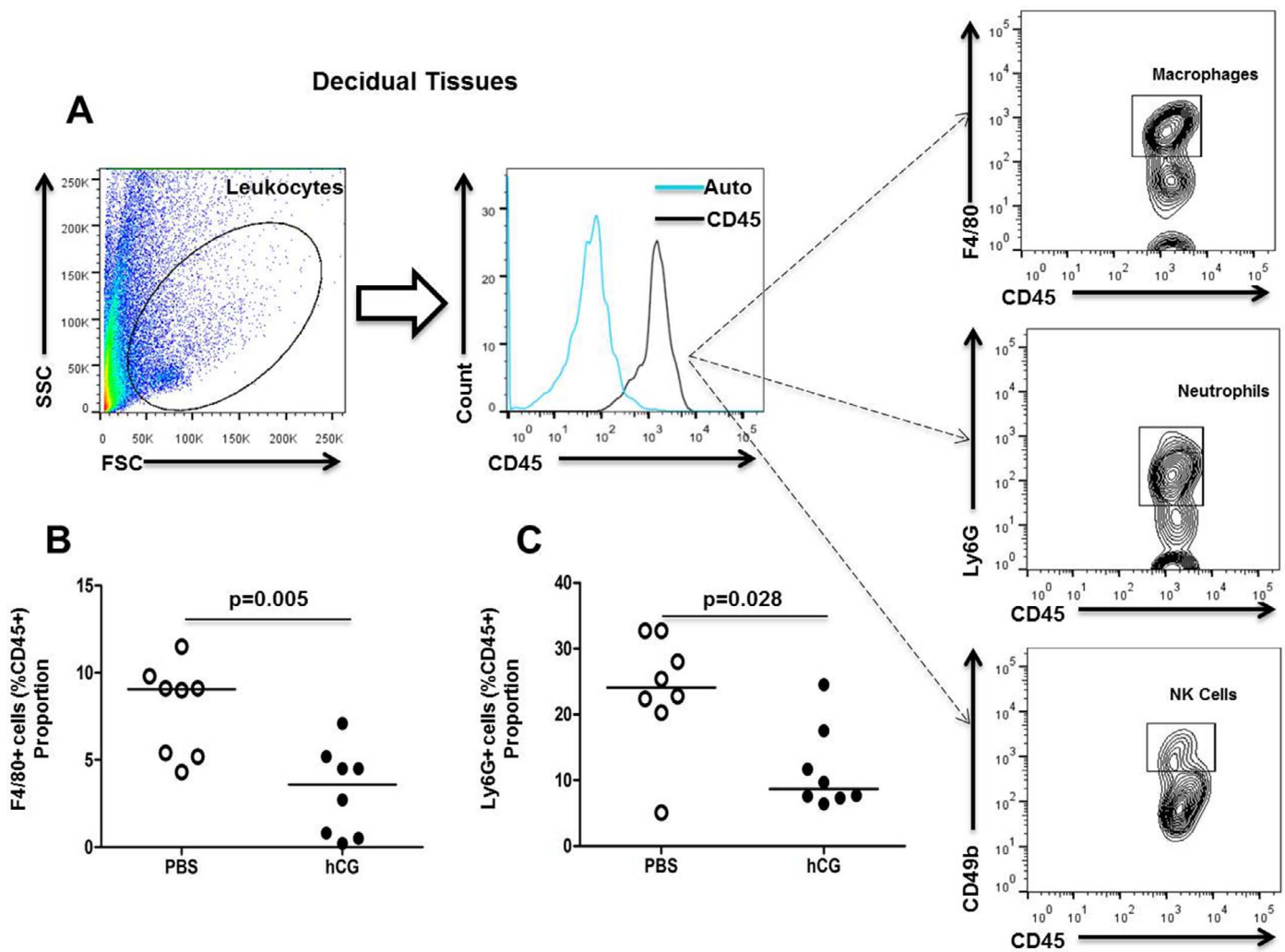


FIG. 3. Innate immune cells at the maternal-fetal interface. **A**) Gating strategy used to identify macrophages ( $CD45^+F4/80^+$  cells), neutrophils ( $CD45^+Ly6G^+$  cells), and NK cells ( $CD45^+CD49b^+$  cells) in decidual tissues. **B**) Proportions of macrophages in decidual tissues from mice injected with PBS or hCG. **C**) Proportions of neutrophils in decidual tissues from mice injected with PBS or hCG.  $n=8$  each. Data are represented as dot plots (median).

#### Human CG Administration Does Not Cause Fetal Death, but Induces Fetal Compromise

All dams injected with hCG underwent dystocia (Table 1), and delivered hypoxic/dead pups (Fig. 7A). This is not surprising, since animals that undergo dystocia deliver dead pups due to complications during the process of labor [67]. In our study, however, another possibility was that hCG had adverse effects on fetuses (in utero), rather than in pups (after delivery). Since abnormal umbilical artery velocimetry and fetal heart rate are associated with fetal compromise [68, 69], Doppler ultrasound was performed in dams injected with hCG or PBS (controls). Fetuses from mice injected with hCG were alive, but umbilical artery pulsatility indexes and fetal heart rates were abnormal compared to those from mice injected with PBS (Fig. 7, B and C). These data demonstrate that, although hCG does not cause fetal death, it causes dystocia and fetal compromise.

#### DISCUSSION

In the present study, we demonstrated that hCG administration has anti-inflammatory effects at the maternal-fetal interface (decidual tissues) by: 1) increasing the proportion of

Tregs; 2) reducing the proportion of macrophages and neutrophils; and 3) inducing an M1  $\rightarrow$  M2 macrophage polarization. Human CG also increased the proportion of decidual Th17 cells. These local changes were not associated with increased systemic concentrations of progesterone or estradiol; however, they were linked to an increased plasma concentration of  $IL1\beta$ . Although pretreatment with hCG prevented endotoxin-induced preterm birth (effect size 44%;  $P=0.04$ ), it caused dystocia and fetal compromise.

#### A Role for hCG in Maternal-Fetal Tolerance During Late Gestation

Tregs are a subset of T cells expressing the activation marker CD25 and the transcriptional factor Foxp3 [70–73]. These cells play a central role in immune responses through their suppressive activity of both self- and non-self-antigens [74], and their function is largely due to the expression of Foxp3 [71, 75]. The most recent hypothesis states that there is an expansion of Tregs during coitus and implantation [76–78], which is maintained throughout gestation in order to sustain a full-term pregnancy [36, 52, 79]. We recently demonstrated that the local administration of progesterone, a hormone produced by the placenta [80], as is hCG [6–9], induces an



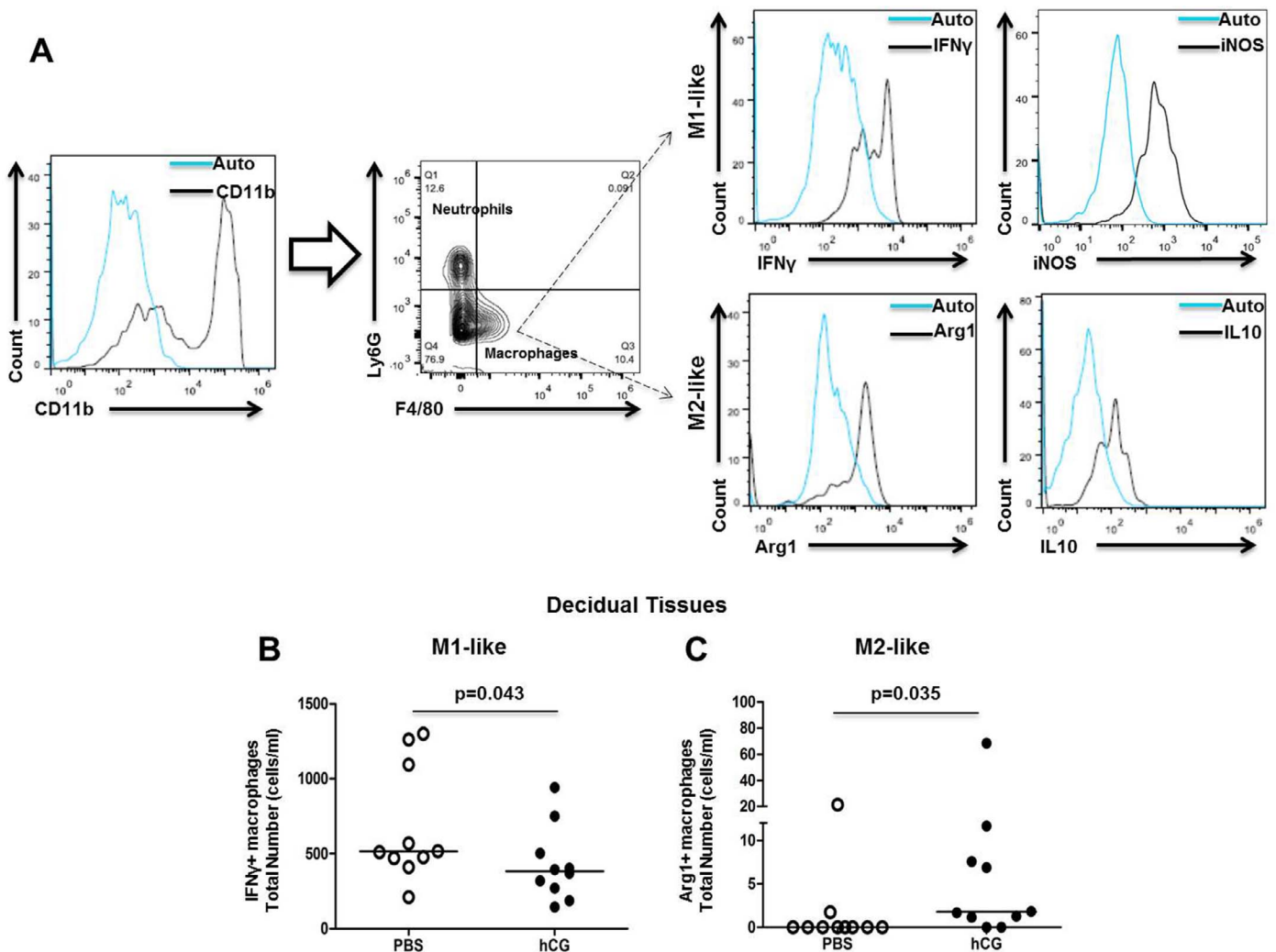


FIG. 4. M1-like and M2-like macrophages at the maternal-fetal interface. **A**) Gating strategy used to identify M1-like (CD11b<sup>+</sup>F4/80<sup>+</sup>IFN $\gamma$ <sup>+</sup> or iNOS<sup>+</sup> cells) and M2-like (CD11b<sup>+</sup>F4/80<sup>+</sup>Arg1<sup>+</sup> or IL10<sup>+</sup> cells) macrophages in decidual tissues. **B**) Number of M1-like (CD11b<sup>+</sup>F4/80<sup>+</sup>IFN $\gamma$ <sup>+</sup>) macrophages in decidual tissues from mice injected with PBS or hCG. **C**) Number of M2-like (CD11b<sup>+</sup>F4/80<sup>+</sup>Arg1<sup>+</sup>) macrophages in decidual tissues from mice injected with PBS or hCG.  $n = 10$  each. Data are represented as dot plots (median).

expansion of Tregs at the maternal-fetal interface [81]. In the present study, an expansion of decidual Tregs in late gestation was observed following hCG administration. This is consistent with previous studies demonstrating that hCG recruits Tregs into the maternal-fetal interface during early pregnancy [26, 28]. In accordance with one of the aforementioned studies [28], we did not observe an expansion of Tregs in the lymphatic organs of MHC-compatible mated mice. Together, these results demonstrate that hCG causes an increase of Tregs in early and late gestation.

In addition to Tregs, immunoregulatory or M2-polarized macrophages also participate in maternal-fetal tolerance [45–47]. M2-polarized macrophages induce homeostasis, dampen inflammation, and promote tissue remodeling and tumor progression [82–84]. M2-polarized macrophages also exhibit an increase in phagocytic activity and express scavenging mannose and galactose receptors, as well as a high production of ornithine and polyamines through the arginase pathway [85, 86]. Recently, we demonstrated that decidual M2-like macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>IL10<sup>+</sup>Arg1<sup>+</sup> cells) are abundant at the maternal-fetal interface prior to iNKT cell activation-induced preterm labor [60]. Our findings suggest that these cells can also have proinflammatory roles during parturition. In

the present study, we demonstrated that hCG administration increases the number of decidual M2-like macrophages in late gestation. Together, these data suggest that hCG induces M2-like macrophages, which may participate in both maternal-fetal tolerance during late gestation and the process of term parturition.

#### *A Role for hCG in Reducing the Proinflammatory Microenvironment at the Maternal-Fetal Interface During Late Gestation*

Proinflammatory macrophages are present in reproductive tissues and at the maternal-fetal interface (decidual tissue) immediately before and during spontaneous term and preterm labor [64, 87–93]. Monocyte/macrophage function and infiltration can be regulated by hCG [94–96]; however, whether this hormone induces or reduces inflammation is controversial. For example, *in vitro* studies demonstrated that hCG increases the secretion of macrophage inhibitory factor in human endometrial cells [97] and granulosa cells [95]. This hormone also enhances the production of NO, ROS, IL6, IL12p40, and phagocytic function in bone marrow-derived macrophages [96], and promotes IL8 secretion by peripheral blood

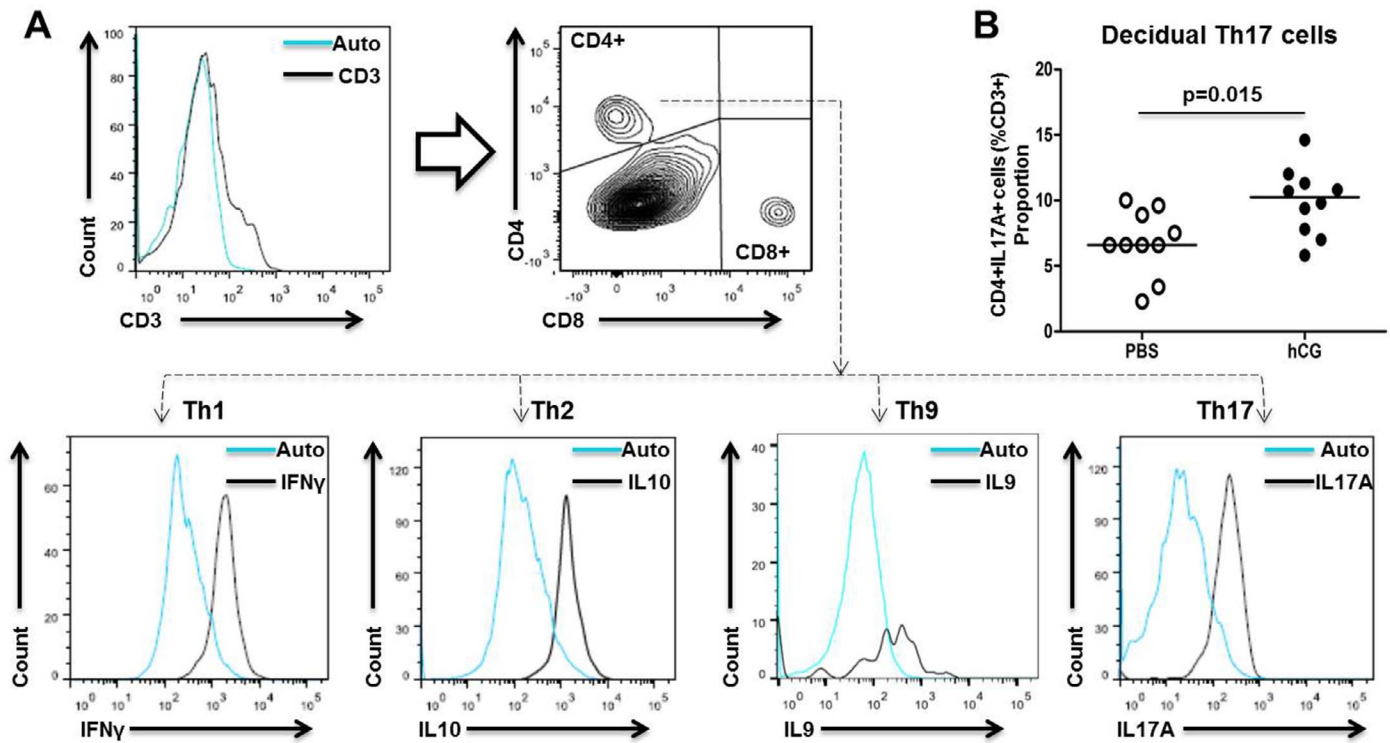


FIG. 5. Th cell subsets at the maternal-fetal interface. **A**) Gating strategy used to identify Th1 (CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells), Th2 (CD4<sup>+</sup>IL10<sup>+</sup> cells), Th9 (CD4<sup>+</sup>IL9<sup>+</sup> cells), and Th17 (CD4<sup>+</sup>IL17A<sup>+</sup> cells) cells in decidual tissues. **B**) Proportions of Th17 cells in decidual tissues from mice injected with PBS or hCG.  $n = 10$  each. Data are represented as dot plots (median).

mononuclear cells from women in early pregnancy [98]. Conversely, an *in vivo* study demonstrated that pretreatment with hCG alleviates thioglycollate-induced peritonitis by reducing TNF $\alpha$ , IL6, pentraxin-related protein 3, CCL3, and CCL5 concentrations in peritoneal lavage fluid [99], which mainly contains macrophages. Here, we demonstrate that pretreatment with hCG from mid- to late gestation reduces the abundance of decidual M1-like macrophages, which provides further evidence of the anti-inflammatory role of this hormone at the maternal-fetal interface.

Neutrophils play an important role during pregnancy, as they release proinflammatory mediators that are associated with the onset of labor [87, 89, 100]. Specifically, in decidual tissues, neutrophils contribute to the proinflammatory microenvironment that accompanies labor during term and preterm gestations by releasing inflammatory cytokines and mediators,

as well as matrix metalloproteinases (MMPs) [101–104]. Similar to macrophages, the effect of hCG on neutrophils is contradictory. *In vitro* studies have demonstrated that hCG is a chemoattractant for human blood neutrophils [94]; however, it was also shown that this hormone induces apoptosis [105] and inhibits phagocytosis and oxidative activity on these innate cells [106]. In this study, we demonstrated that pretreatment with hCG from mid- to late gestation reduces the frequency of decidual neutrophils, which supports the hypothesis that this hormone dampens inflammation at the maternal-fetal interface.

#### *Human CG Administration Increases the Proportion of Th17 Cells in Decidual Tissues*

In addition to regulating effector innate immune cells, such as neutrophils and macrophages, hCG can modulate effector T

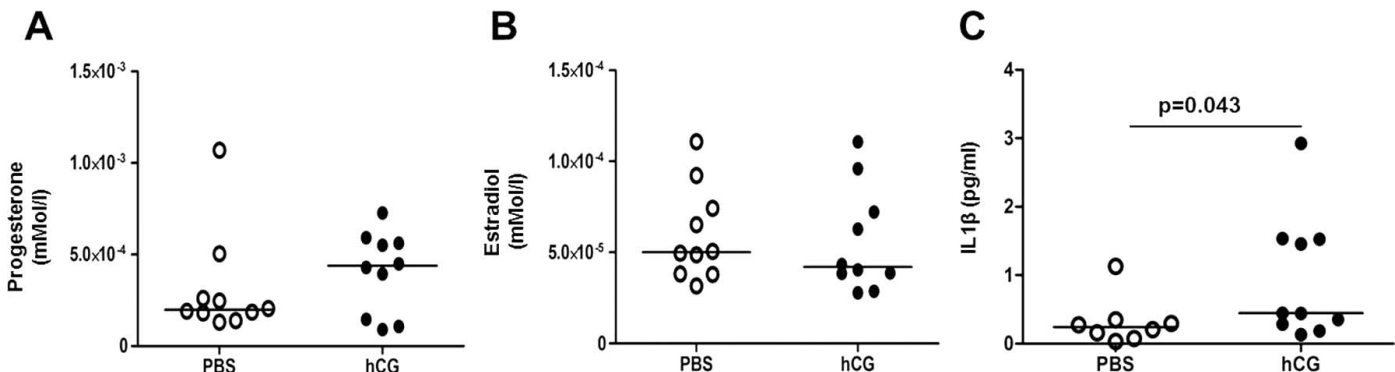


FIG. 6. Plasma concentrations of progesterone, estradiol, and IL1 $\beta$ . **A**) Plasma concentration of progesterone in mice injected with PBS or hCG. **B**) Plasma concentration of estradiol in mice injected with PBS or hCG. **C**) Plasma concentration of IL1 $\beta$  in mice injected with PBS or hCG.  $n = 8$ –10 each. Data are represented as dot plots (median).

TABLE 1. Pretreatment with hCG reduces the rate of endotoxin-induced preterm birth but causes dystocia.

Parameter*	PBS only	Endotoxin only	Endotoxin + hCG	hCG only <sup>†</sup>	P value <sup>‡</sup>
No. of mice per group	10	10	11	7	NS
Gestational age (days; median ± SEM)	19.1 ± 0.09	17.5 ± 0.1	18.3 ± 0.8	22.5 ± 0.3	<sup>a</sup> <i>P</i> < 0.001 <sup>d</sup> <i>P</i> = 0.05
Duration of labor (min; median ± SEM)	91 ± 10.4	90 ± 20.1	80 ± 15.2	n/a	NS
Rate of stillbirth (% ± 95% CI)	9 ± 6.3	100	85.2 ± 8.9	n/a	<sup>a</sup> <i>P</i> < 0.001 <sup>b</sup> <i>P</i> < 0.001
Rate of preterm birth (% ± 95% CI)	0	80 ± 24.7	36.3 ± 28.4	0	<sup>a</sup> <i>P</i> < 0.001 <sup>b</sup> <i>P</i> = 0.03 <sup>d</sup> <i>P</i> = 0.04
Rate of dystocia (% ± 95% CI)	0	10 ± 18.5	36.3 ± 28.4	100	<sup>e</sup> <i>P</i> = 0.001 <sup>b</sup> <i>P</i> = 0.03 <sup>c</sup> <i>P</i> < 0.001 <sup>e</sup> <i>P</i> < 0.001 <sup>f</sup> <i>P</i> = 0.007

\* CI, confidence interval.

<sup>†</sup> n/a, not applicable.

<sup>‡</sup> NS, not significant.

<sup>a</sup> PBS vs. endotoxin.

<sup>b</sup> PBS vs. endotoxin + hCG.

<sup>c</sup> PBS vs. hCG.

<sup>d</sup> Endotoxin vs. endotoxin + hCG.

<sup>e</sup> Endotoxin vs. hCG.

<sup>f</sup> hCG vs. endotoxin + hCG.

cells [107]. Decidual T cells seem to have an effector-memory phenotype during spontaneous term labor, since these cells express CD45RO, IL1 $\beta$ , TNF $\alpha$ , and MMP9 [108, 109]. Among T cell subsets, Th17 cells were found in the decidual tissue of healthy pregnant women [110, 111], and were associated with spontaneous preterm labor [112]. Here, we show that pretreatment with hCG from mid- to late gestation increases the proportion of decidual Th17 cells at term pregnancy. The Th17 cell subset is distinct from Th1 and Th2 cells [113], due to their expression of IL17A, IL17F, and IL22 upon stimulation with IL23 [113–115], TGF $\beta$  plus IL6 [116, 117], or TGF $\beta$  plus IL21 [118, 119]. The role of Th17 cells is unclear, since they participate in tissue inflammation by secreting cytokines, chemokines, and inducing the production of MMPs in surrounding cells [113, 120], and also play a protective role in host defense against infections [121–123]. Therefore, further research is needed in order to investigate whether hCG-induced Th17 cells promote inflammation or have a protective role at the maternal-fetal interface in late gestation.

#### *The Role of hCG in Preventing Endotoxin-Induced Preterm Birth, Inducing Systemic Inflammation, and Influencing Fetal Outcomes*

Pilot studies have shown that hCG suppresses uterine contractions in women undergoing preterm labor, delaying delivery without causing fetal side effects [124, 125]. In addition, pretreatment with hCG delays the time of delivery in mice injected with prostaglandin F<sub>2 $\alpha$</sub>  [126]. Consequently, administration of hCG has been suggested as a strategy for preterm labor prevention [49]. In the present study, we investigated the effectiveness of hCG in preventing endotoxin-induced preterm birth and its safety in pregnancy. Pretreatment with hCG prevents endotoxin-induced preterm birth by 44%. However, mice injected with hCG had high rates of dystocia and increased systemic concentrations of IL1 $\beta$ , which, in turn, can up-regulate the secretion of hCG by the placenta [127, 128]. These data suggest that this cytokine is implicated in the event of hCG-induced dystocia.

The fact that hCG administration increases the systemic concentrations of IL1 $\beta$  is in concordance with a previous study demonstrating that this hormone increases serum concentrations of IL1 $\alpha$  and IL1 $\beta$  in patients undergoing in vitro fertilization-embryo transfer [129]. The systemic proinflammatory effect of hCG is not surprising, since this hormone can activate the immune system [96]. However, hCG has anti-inflammatory effects at the maternal-fetal interface. Taken together, these data suggest that this hormone has differential functionality on decidual and peripheral leukocytes. Given this differential functionality, it is tempting to suggest that the local administration (i.e., vaginally) of hCG could induce anti-inflammation at the maternal-fetal interface without causing systemic proinflammatory effects.

The present study also demonstrates that administration of hCG did not cause fetal death, yet caused fetal compromise, which supports previous animal studies demonstrating that this hormone negatively impacts fetal health [130, 131]. The adverse effect of hCG on fetal heart rate may be explained by the fact that this hormone is produced by the placenta and causes physiological changes in the fetus similar to those seen in the mother [132]. In the mother, hCG inhibits uterine contractility by decreasing intracellular free Ca<sup>+2</sup> levels [30] which, in turn, could lead to myocardial ischemia and tissue injury [133]. These adverse effects are attributed to increased myocardial oxygen consumption, which occurs due to an increased heart rate, stroke volume, and cardiac output [133]. These findings warrant further study of the adverse effects of hCG on the process of labor and the offspring, prior to its implementation as a method for preventing preterm labor/birth.

In summary, the present study demonstrates that hCG administration induces an anti-inflammatory microenvironment at the maternal-fetal interface by increasing Tregs and M2-like macrophages, and by decreasing M1-like macrophages and neutrophils. Human CG administration also increased the proportion of decidual Th17 cells and plasma concentrations of IL1 $\beta$ ; however, their roles require further investigation. These changes were not associated with systemic concentrations of progesterone and estradiol. Pretreatment with hCG reduced the rate of endotoxin-induced preterm birth without causing fetal death; however, this



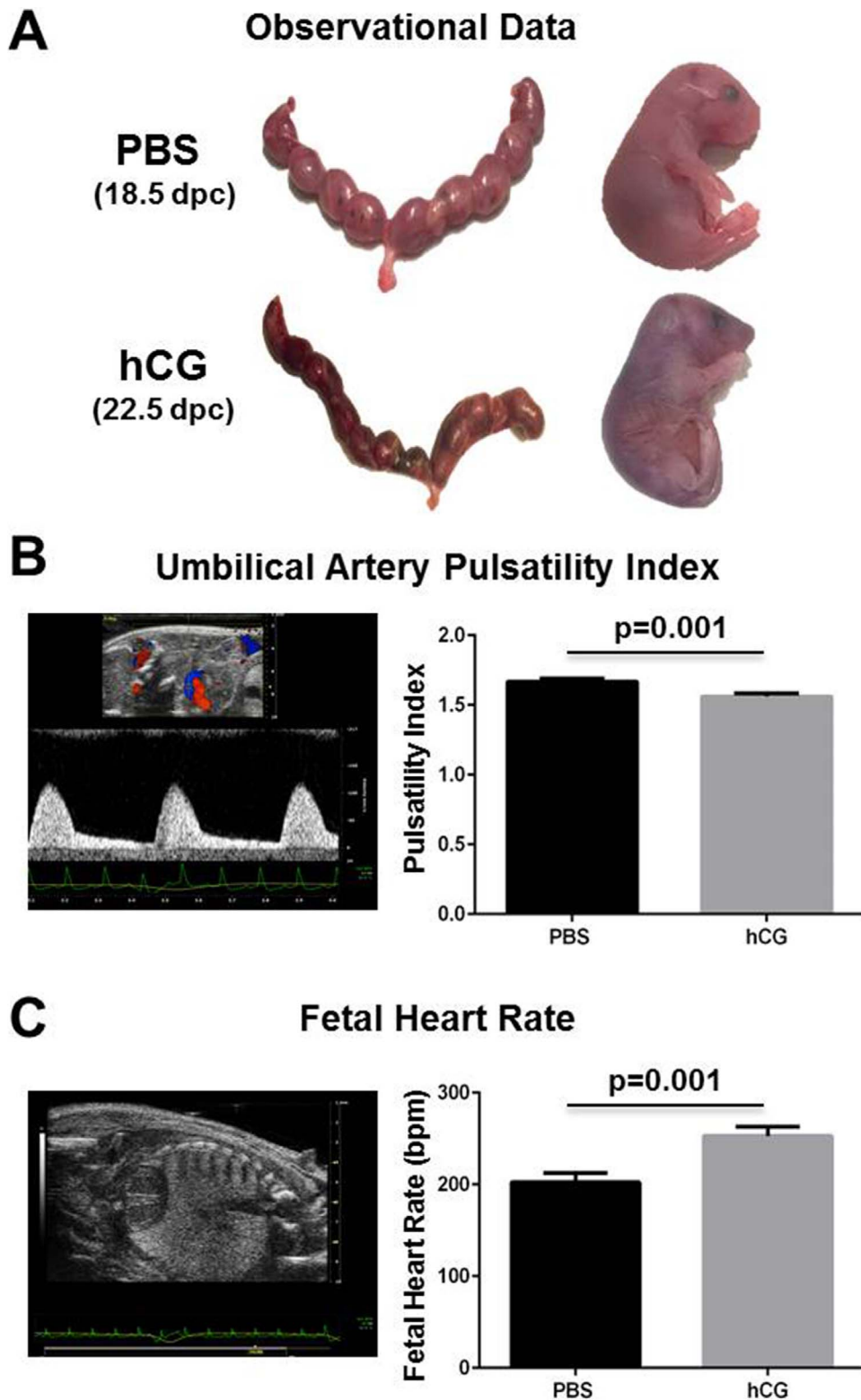


FIG. 7. The effects of hCG on fetuses. **A)** Uterine horns and fetuses from mice injected with PBS or hCG at 18.5 or 22.5 dpc, respectively. **B and C)** Doppler ultrasound was performed on fetuses at 18.5 dpc from mice injected with PBS or hCG. Umbilical artery pulsatility indexes and fetal heart rates were recorded. Data are from four to five independent litters.

hormone caused dystocia and fetal compromise. These results provide insight into the mechanisms whereby hCG induces an anti-inflammatory microenvironment at the maternal-fetal interface during late gestation and prevents preterm labor/birth. However, the fetal compromise induced by hCG may

represent an alteration in the physiology of the fetus that could lead to neonatal mobility and/or mortality. The adverse effects of hCG in human pregnancy are unknown. Nevertheless, the deleterious effects of this hormone in this animal model warrant caution.

## ACKNOWLEDGMENT

We gratefully acknowledge Marcia Arenas-Hernandez, Akshata Naik, Derek Miller, Dr. Zhong Dong, Lorri McLuckie, and Rona Wang for their contributions to the execution of this study, and to Maureen McGerty and George Schwenkel for their critical readings of the manuscript.

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