HHS Public Access

Author manuscript

Am J Obstet Gynecol. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Am J Obstet Gynecol. 2015 December ; 213(6): 846.e1–846.e19. doi:10.1016/j.ajog.2015.08.010.

Vaginal progesterone, but not 17α-hydroxyprogesterone caproate, has antiinflammatory effects at the murine maternal-fetal interface

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Abstract

OBJECTIVE—Progestogen (vaginal progesterone or 17-alpha-hydroxyprogesterone caproate [17OHP-C]) administration to patients at risk for preterm delivery is widely used for the prevention of preterm birth (PTB). The mechanisms by which these agents prevent PTB are poorly understood. Progestogens have immunomodulatory functions; therefore, we investigated the local effects of vaginal progesterone and 17OHP-C on adaptive and innate immune cells implicated in the process of parturition.

STUDY DESIGN—Pregnant C57BL/6J mice received vaginal progesterone (1 mg per 200 μL, n = 10) or Replens (control, 200 μL, n = 10) from 13 to 17 days postcoitum (dpc) or were subcutaneously injected with 17OHP-C (2 mg per 100 μL, n = 10) or castor oil (control, 100 μL, n = 10) on 13, 15, and 17 dpc. Decidual and myometrial leukocytes were isolated prior to term delivery (18.5 dpc) for immunophenotyping by flow cytometry. Cervical tissues were collected to determine matrix metalloproteinase (MMP)-9 activity by in situ zymography and visualization of collagen content by Masson's trichrome staining. Plasma concentrations of progesterone,

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Dr Romero has contributed to this work as part of his official duties as an employee of the United States Government.

The authors report no conflict of interest.

Procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (protocol A09-08-12).

Presented in part at the 62nd Annual Scientific Meeting of the Society for Reproductive Investigation, San Francisco, CA, March 25–28, 2015; the 34th annual meeting of the American Society for Reproductive Immunology, Long Beach, NY, June 2–5, 2014; and the 61st Annual Scientific Meeting of the Society for Gynecological Investigation, Florence, Italy, March 26–29, 2014.

estradiol, and cytokines (interferon [IFN]-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and tumor necrosis factor-α) were quantified by enzyme-linked immunosorbent assays. Pregnant mice pretreated with vaginal progesterone or Replens were injected with 10 μg of an endotoxin on 16.5 dpc (n = 10 each) and monitored via infrared camera until delivery to determine the effect of vaginal progesterone on the rate of PTB.

RESULTS—The following results were found: (1) vaginal progesterone, but not 17OHP-C, increased the proportion of decidual CD4+ T-regulatory cells; (2) vaginal progesterone, but not 17OHP-C, decreased the proportion of decidual CD8+CD25+Foxp3+ T cells and macrophages; (3) vaginal progesterone did not cause an M1→M2 macrophage polarization but reduced the proportion of myometrial IFNγ+ neutrophils and cervical active MMP-9-positive neutrophils and monocytes; (4) 17OHP-C did not reduce the proportion of myometrial IFNy-positive neutrophils; however, it increased the abundance of cervical active MMP-9-positive neutrophils and monocytes; (5) vaginal progesterone immune effects were associated with reduced systemic concentrations of IL-1β but not with alterations in progesterone or estradiol concentrations; and (6) vaginal progesterone pretreatment protected against endotoxin-induced PTB (effect size 50%, *P* = .008).

CONCLUSION—Vaginal progesterone, but not 17OHP-C, has local antiinflammatory effects at the maternal-fetal interface and the cervix and protects against endotoxin-induced PTB.

Keywords

decidua; endotoxin; interleukin 1β; macrophages; matrix metalloproteinase-9; myometrium; neutrophils; preterm birth; preterm labor; regulatory T cells

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality worldwide.[1] The rate of PTB in the United States is 11.39%, which is considered high for a developed nation.[2] Preterm neonates are at an increased risk for short- and long-term morbidity, and prematurity represents a substantial burden for society and the health care system.[3–6] Therefore, the prevention of PTB is a health care priority.

Cervical assessment with ultrasound coupled with the administration of vaginal progesterone represents the main strategy to prevent PTB in nulliparous women and in those without a prior history of prematurity.[7–16] 17-Alpha-hydroxyprogesterone caproate (17OHP-C) has been recommended to prevent PTB in women with a prior history of prematurity.[17–19]

Although the term progesterone has been used to refer to natural progesterone and 17OHP-C,[20] there is evidence that these compounds have different biological activities in the myometrium,[21–24] uterine cervix,[25–30] profile of clinical efficacy,[7–19,26,31–33] and safety[17,34–39]; therefore, these terms should not be used interchangeably.

The mechanisms of action whereby progestogens prevent PTB are unknown. There has been considerable interest in the role of progesterone in the maintenance of myometrial quiescence.[40–44] However, the realization that a short cervix is a risk factor for preterm delivery[45–49] and that a blockage of progesterone action induces cervical ripening in animals and women[50–54] has focused investigation on the role of progesterone on this organ.[55–61]

The current hypothesis is that progesterone acts as an antiinflammatory agent primarily on the uterine cervix.[62–66] This hypothesis is largely based on the known antiinflammatory effects of progesterone,[67–71] and a microarray study demonstrating the differential expression of inflammatory related messenger ribonucleic acid (mRNA) in the cervix of pregnant mice treated with medroxyprogesterone acetate.[55] However, there is no functional evidence that vaginal progesterone has anti-inflammatory effects in vivo.

Inflammation has been implicated in physiological[72–99] and pathological parturition.[100–129] Pathological inflammation can result from the activation of innate immunity in response to microbial products[87,130–138] or activation of the adaptive immune response.[139–144] A breakdown of maternal-fetal tolerance is now recognized as a mechanism of disease for spontaneous premature labor/delivery.[100,145–151] Therefore, the effects of progesterone in the prevention of preterm delivery may be mediated by the innate and/or adaptive immune system.

Indeed, the administration of RU486 (to block progesterone action[152]) during late pregnancy in guinea pigs can increase the release of proinflammatory cytokines by the amniochorion, cervix, and decidual-myometrial tissues,[153] and this hormone can also increase the proportion of CD4+CD25+ regulatory T cells (Tregs), which are key in the control of the adaptive immune response, in the uterine tissues during mid-pregnancy in mice.[154]

The objectives of this study were to determine the effects of vaginal progesterone and 17OHP-C on the following: (1) the proportion of CD4+ Tregs and CD8+CD25+Foxp3+ T cells at the maternal-fetal interface (myometrium and decidua); (2) the proportion and phenotype of macrophages (M1-like or M2-like) at the maternal-fetal interface; (3) the proportion of neutrophils and their cytokine production at the maternal-fetal interface; and (4) matrix metalloproteinase (MMP)-9 activity in the cervix.

Finally, we sought to determine whether pretreatment with vaginal progesterone could prevent endotoxininduced PTB.

MATERIALS AND METHODS

Animals

C57BL/6J mice were bred in the animal care facility at the C. S. Mott Center for Human Growth and Development at Wayne State University (Detroit, MI) and housed under a circadian cycle (12 hours of light and 12 hours of dark). Females 8–12 weeks old were mated with male mice of proven fertility. Female mice were examined daily between 8:00 and 9:00 AM for the presence of a vaginal plug, which denoted 0.5 days postcoitum (dpc). Upon observation of vaginal plugs, the female mice were then separated from the males and were housed in different cages. The weight gain of ≤2 g confirmed the pregnancy at 12.5 dpc. Procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (protocol number A09-08-12).

Progestogen administration

Pregnant females received vaginal progesterone (Crinone 8% vaginal gel; Fleet Laboratories Ltd, Watford, Herts, United Kingdom) at a concentration of 1 mg per 200 μL (n = 10) or 200 μL of Replens (Lil' Drug Store Products, Inc, Cedar Rapids, IA) as a control (n = 10) from 13 to 17 dpc (Figure 1A).

A second group of mice was injected subcutaneously with 2 mg per 100 μL of 17OHP-C (n = 10; Compounding Solutions, Shelby Township, MI) or 100 μL of castor oil (European Pharmacia Grade; ACROS Organics, Thermo Fisher Scientific, Waltham, MA) as a control (n = 10) on 13, 15, and 17 dpc.

We used this source of the 17OHP-C because it is clinically used at the Detroit Medical Center, and previous studies demonstrated that compounded 17OHP-C had adequate potency compared with the Food and Drug Administration—approved agent.[155] The administration of vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc) was performed starting on 13 dpc to mimic the treatment regimen followed by pregnant women with a short cervix.

Vaginal progesterone administration is generally started around 20–23 weeks of gestation in women with a short cervix,[11] which is equivalent to approximately 13 dpc in mice during midgestation. Administration of 17OHP-C or castor oil control started on 13 dpc and continued on alternating days because women receive this synthetic progesterone on a weekly basis.[17] The doses of vaginal progesterone and 17OHP-C were similar to those previously reported in studies using the same animal species.[29,33,55] All mice were euthanized prior to term delivery (18.5 dpc) and decidual, myometrial, and cervical tissues were harvested.

Leukocyte isolation

Immediately after collection, myometrial and decidual tissues were mechanically disaggregated in a cell dissociating reagent (Accutase; Life Technologies, Grand Island, NY) using scissors for approximately 1–2 minutes, as previously described.[156] Samples were then incubated at 37°C for 35 minutes with gentle shaking (MaxQ 4450 benchtop orbital shaker; Thermo Fisher Scientific). The cell suspensions were filtered using a 100 μm cell strainer (Fisher Scientific, Hanover Park, IL) and washed with fatty acyl-CoA synthase (FACS) buffer (bovine-serum albumin 0.1% [Sigma Aldrich, St Louis, MO]), sodium azide 0.05% (Fischer Scientific Bioreagents, Fair Lawn, NJ), and 1× phosphate-buffered saline (PBS; Fischer Scientific Bioreagents)]. The resulting pellet was resuspended in FACS buffer and used for immunophenotyping.

Immunophenotyping

Cell suspensions were incubated with a monoclonal mouse CD16/CD32 antibody (FcγIII/II receptor; BD Biosciences, San Jose, CA) for 10 minutes at 4°C. The cells were then washed with FACS buffer and incubated for 30 minutes at 4°C with the corresponding extracellular and/or intracellular fluorochrome-conjugated antibodies (Supplemental Table). Tregs were determined in decidual and myometrial tissues using the extracellular markers CD3, CD4,

CD8, and CD25 and the transcriptional factor Foxp3. Innate leukocyte populations including macrophages, dendritic cells (DCs), natural killer (NK) cells, and neutrophils were also identified in the decidual and myometrial tissues using the extracellular markers CD45, F4/80, CD11c, CD49b, and Ly6G.

Foxp3 staining was performed using the Foxp3/transcription factor staining buffer set (eBioscience, San Diego, CA). For cytokine staining, the Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences) was used, following the manufacturer's recommendations. Unstained cells were treated with the same protocol and used as autofluorescence controls. Cell suspensions were acquired and analyzed using the LSRFortessa flow cytometer and BD FACSDiva software, version 8.0 (BD Biosciences), respectively. Figures were prepared using FlowJo Software version 10 (FlowJo, LLC, Ashland, OR).

In situ MMP-9 zymography

To determine the MMP-9 activity in cervical tissues, in situ zymography was performed as described by Hadler-Olsen et al.[157] Cervical tissue sections were fixed in ethanol and embedded in paraffin; from these blocks, 5 μm thick sections were cut and mounted on FisherBrand Superfrost microscope slides (Fisher Scientific) and heated to 59°C. Slides were further deparaffinized in xylene and rehydrated in graded alcohol baths. The gelatinase reaction was performed using the EnzChek gelatinase/collagenase assay kit (Life Technologies), and to verify the enzyme specificity, tissue sections were preincubated for 1 hour with 200 μL of 10 mM phenanthroline, a metal chelator and general inhibitor of metalloproteinases.

The remaining slides were preincubated with a reaction buffer, and a substrate was prepared by dissolving 1 mg DQ gelatin (Life Technologies) in 1.0 mL of deionized water and diluted 1:50 with reaction buffer. Substrate solution (200 μL) with or without 10 mM phenanthroline was then added to the tissue sections. All slides were incubated in a dark humidity chamber at 37°C for 2 hours, and the negative control slides were incubated at −20°C for 2 hours.

Following incubation, the sections were rinsed twice with deionized water and fixed in 4% neutral buffered formalin for 10 minutes in the dark and then were rinsed with 1× PBS twice prior to mounting with ProLong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (Life Technologies). The slides were scanned using the Pannoramic MIDI digital slide scanner (PerkinElmer, Inc, Waltham, MA), and annotations were made by laboratory personnel who then utilized 3DHISTECH software (3DHISTECH Kft, Budapest, Hungary) to assess the number of positive cells.

Masson's trichrome staining

Cervical tissue sections were fixed in 4% paraformaldehyde upon harvesting and stored at 4°C in ethanol before being embedded into paraffin blocks. The embedded tissues were then cut into 5 μm thick sections, placed onto salinized slides, deparaffinized with xylene, and hydrated with ethanol and water. The staining was performed on the Dako AutostainerPlus

(Dako, Carpinteria, CA) using Masson's trichrome stain kit (American MasterTech, Lodi, CA), following the manufacturer's protocol. Briefly, the sections were mordanted in Bouin solution overnight at room temperature, rinsed in water, stained with Weigert's hematoxylin for 3 minutes, rinsed again in water, and stained with Biebrich Scarlet-Acid Fuchsin solution for 15 minutes.

After a second rinse, the slides were incubated with phosphomolybdic/phosphotungstic acid for 15 minutes, stained with Aniline Blue stain for 10 minutes, rinsed, and incubated with 1% acetic acid for 5 minutes. The sections were then dehydrated in a series of alcohol baths, and then a coverslip was placed. The images were taken using the Pannoramic MIDI digital slide scanner (PerkinElmer, Inc).

Decidual protein extracts

Decidual tissues were collected from the mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc) at 18.5 dpc and placed in small Petri dishes with sterile 1 × PBS (n = 10 each). Tissues were incubated in a 12-well culture plate (Falcon multiwell plates for cell culture; Becton Dickinson Labware, Franklin Lanes, NJ), using a single well per tissue with 1 mL of Gibco Dulbecco's modified eagle medium (Life Technologies) supplemented with 1% Gibco antibiotic-antimycotic solution (Life Technologies) for 24 hours at 37 C in 5% CO_2. Following incubation, tissues were homogenized using a Tissue Tearor (BioSpec Products, Inc, Bartlesville, OK) and centrifuged at 15,000 × *g* for 30 minutes at 4°C to obtain a cell-free supernatant that contained the protein extract.

Enzyme-linked immunosorbent assays (ELISAs)

Blood samples, obtained by cardiac puncture from the mice that received vaginal progesterone, Replens (Lil' Drug Store Products, Inc), 17OHP-C, or castor oil were placed in tubes containing heparin (Sigma-Aldrich). Plasma samples were then obtained by centrifugation. Plasma progesterone and estradiol concentrations were measured 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), respectively, according to the manufacturer's instructions.

The concentrations of interferon (IFN)-γ, interleukin (IL)-1*β*, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and tumor necrosis factor (TNF)-*α* in plasmawere measuredwith sensitive and specific immunoassays according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). IL-10 was also determined in the decidual protein extracts.

The sensitivities of the assays were as follows: 0.022 pg/mL (IFNγ), 0.104 pg/mL (IL-1*β*), 0.179 pg/mL (IL-2), 0.098 pg/mL (IL-4), 0.066 pg/mL (IL-5), 0.825 pg/mL (IL-6), 0.425 pg/mL (IL-10), 8.578 pg/mL (IL-12p70), 0.218 pg/mL (KC/GRO), and 0.164 pg/mL (TNF*α*), respectively. The interassay and intraassay coefficients of variation were below 7% and 15%, respectively.

Endotoxin-induced preterm birth in animals treated with vaginal progesterone or placebo

Pregnant mice were pretreated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc) from 13 to 17 dpc as previously described (n = 10 each). On 16.5 dpc, the mice were challenged with an intraperitoneal injection of 10 μg of an endotoxin (lipopolysaccharides from *Escherichia coli*, O55:B5; Sigma-Aldrich) in 200 μL of 1 × PBS.

Video recording provided precise measurements of the gestational age, duration of active labor, and rate of stillbirth. Gestational age at birth was calculated from the identification of the vaginal plug (0.5 dpc) through the delivery of the first pup. Active labor was defined as the time elapsed from the delivery of the first pup through the delivery of the last pup. The rate of stillbirth was defined as the number of pups that were born dead of the total number of pups born. PTB was defined as fetal delivery before 18 dpc.

Statistical analysis

Statistical analyses were performed using SPSS, version 21.0 (IBM Corp, Armonk, NY). A Shapiro-Wilk test was performed to determine whether data were normally distributed. Because the data did not have a normal distribution, Mann-Whitney *U* tests were performed. A χ^2 test was used to compare proportions. Graphical data were presented as mean ± SEM. A value of $P < .05$ was considered statistically significant.

RESULTS

Administration of vaginal progesterone, but not 17OHP-C, increases the proportion of CD4D+ Tregs in decidual tissues

We first determined the proportions of CD4+ Tregs (CD4+CD25+Foxp3+ T cells) and CD8+CD25+Foxp3+ T cells in myometrial and decidual tissues following vaginal progesterone or 17OHP-C administration to pregnant mice. Figure 1B shows the gating strategy used to analyze CD4+ Tregs and CD8+CD25+Foxp3+ T cells in myometrial and decidual tissues.

Vaginal progesterone administration increased the proportion of decidual CD4+ Tregs when compared with the group receiving Replens (control; Lil' Drug Store Products, Inc) (Figure 1C); however, it decreased the proportion of decidual CD8+CD25+Foxp3+ T cells (Figure 1D)

Administration of 17OHP-C did not have such effects (Figures 1, E and F, $P > .05$). Moreover, the vaginal progesterone administration did not alter the proportion of myometrial CD4+ Tregs or CD8+CD25+Foxp3+ T cells (Figure 2). Therefore, the administration of vaginal progesterone, but not 17OHP-C, increased the proportion of CD4+ Tregs in the decidual tissues.

To explore whether IL-10 (an antiinflammatory cytokine and a differentiation factor of Tregs[158]) could mediate an increase in CD4+ Tregs, we determined the concentration of this cytokine in decidual tissues. No differences were observed in the concentration of IL-10 between the decidual protein extracts upon vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc) administration (Supplemental Figure). These results do not

support a role for IL-10 in the increase of decidual CD4+ Tregs upon administration of vaginal progesterone.

Administration of vaginal progesterone, but not 17OHP-C, decreases the proportion of macrophages in decidual tissues

To further characterize the decidual microenvironment following vaginal progesterone or 17OHP-C administration, the proportion of innate immune cells was determined. The gating strategy used to analyze NK cells (CD45+CD49b+ cells), DCs (CD45+CD11c+ cells), neutrophils (CD45+Ly6G+ cells), and macrophages (CD45+F4/80+ cells) in decidual tissues is shown in Figure 3A.

Vaginal progesterone administration reduced the proportion of macrophages in decidual tissues when compared with Replens (control; Lil' Drug Store Products, Inc) (Figure 3B). In contrast, 17OHP-C administration did not alter the proportion of decidual macrophages (Figure 3C). No differences were found in the proportions of decidual neutrophils, NK cells, or DCs between these 2 groups of mice (data not shown).

To characterize the phenotype of macrophages that were reduced in decidual tissues upon vaginal progesterone administration, we determined the expression of M1-like and M2-like markers including inducible NO synthase (iNOS), IFNγ, Arg1 cells, and IL-4.[159] The gating strategy used to determine M1-like (CD11b+Ly6G-F4/80+iNOS+ or IFNγ cells) and M2-like (CD11b+Ly6G-F4/80+Arg1+ or IL4+ cells) macrophages in decidual tissues is shown in Figure 4A.

We hypothesized that vaginal progesterone administration would reduce the proportion of M1-like macrophages and/or would cause an M1→M2 macrophage polarization. Administration of vaginal progesterone did not change the proportion of M1-like (Figure 4, B and C) or M2-like (Figure 4, D and E) macrophages. Vaginal progesterone administration reduced the proportion of decidual macrophages, yet these results do not support the hypothesis that vaginal progesterone reduces M1-like macrophages or causes an M1→M2 macrophage polarization.

Administration of vaginal progesterone, but not 17OHP-C, reduces the proportion of IFNγ+ neutrophils in myometrium

Uterine/myometrial macrophages and neutrophils have been implicated in the onset of term and preterm labor.[84,87] We therefore sought to determine whether vaginal progesterone or 17OHP-C administration alters the proportion of these innate immune cells in myometrial tissues.

The gating strategy used to determine macrophages, neutrophils, and their expression of IFNγ or IL-4 was similar to the strategy used in Figure 4A. Administration of vaginal progesterone tended to reduce the proportion of myometrial macrophages; however, this reduction did not reach statistical significance (Figure 5A). Although the administration of vaginal progesterone did not reduce the proportion of total neutrophils (Figure 5B), it decreased the proportion of IFNγ -positive neutrophils (CD11b+Ly6G+F4/80– cells) in myometrium (Figure 5C). 17OHP-C administration did not reduce the proportion of IFNγ -

positive neutrophils in myometrium (data not shown). These results demonstrate that vaginal progesterone administration reduced the proportion of proinflammatory neutrophils in myometrium.

Administration of vaginal progesterone, but not 17OHP-C, reduces the abundance of active MMP-9-positive cells in the cervix

We further investigated whether vaginal progesterone and 17OHP-C had effects on MMP-9 activity and collagen content in the cervical tissues. Administration of vaginal progesterone or 17OHP-C increased MMP-9 activity (green staining) (Figure 6, A and B) and reduced collagen content (blue staining; Figure 6, C and D) in the cervical tissues.

While analyzing the images, we observed that the cervices in the Replens group (control; Lil' Drug Store Products, Inc) had an abundant number of active MMP-9-positive cells (white arrows). Semiquantification of these cells revealed that vaginal progesterone administration reduced the abundance of active MMP-9-positive cells when compared with Replens (control) (Figure 6E).

In contrast, 17OHP-C administration increased the abundance of active MMP-9-positive cells when compared with castor oil (control) (Figure 6F). Magnification of active MMP-9-positive cells in Replens (control) revealed these cells to be neutrophils and monocytes (Figure 6G). Therefore, vaginal progesterone and 17OHP-C increased MMP-9 activity and reduced collagen content in the cervix. However, only vaginal progesterone reduced the infiltration of active MMP-9-positive neutrophils and monocytes.

Administration of vaginal progesterone or 17OHP-C is not associated with changes in the systemic concentrations of progesterone or estradiol

To investigate whether the immune effects of vaginal progesterone or 17OHP-C were associated with a change in the systemic levels of sex steroids, we quantified the concentrations of progesterone and estradiol in the plasma. Administration of vaginal progesterone or 17OHP-C did not change the systemic concentrations of progesterone or estradiol (Figure 7, A and B). These results demonstrate that the local immunomodulatory effects of vaginal progesterone in decidual, myometrial, and cervical tissues were not associated with systemic changes in sex steroids.

Administration of vaginal progesterone, but not 17OHP-C, reduces the systemic concentration of IL-1β

Preterm labor is associated with a systemic inflammatory response[125,126] and the systemic or intraamniotic administration of IL-1β leads to PTB in mice.[160,161] Therefore, we evaluated whether the administration of vaginal progesterone or 17OHP-C had an effect on the systemic concentration of IL-1β. Vaginal progesterone reduced by 20% the plasma concentrations of IL-1β (Figure 8A); however, the administration of 17OHP-C did not alter the concentration of this cytokine (Figure 8B).

Pretreatment with vaginal progesterone conferred partial protection (50%) against endotoxin-induced preterm birth

Finally, we evaluated the efficacy of vaginal progesterone in preventing endotoxin-induced preterm birth. Mice pretreated with vaginal progesterone had lower rates of endotoxin-induced preterm birth than mice pretreated with Replens (control; Lil' Drug Store Products, Inc) (40% vs 90%, $P = .008$; Table). These results demonstrate that vaginal progesterone administration may be an effective treatment for reducing inflammation-associated preterm labor.

COMMENT

Principal findings of the study

The principal findings of the study included the following: (1) the administration of vaginal progesterone, but not 17OHP-C, increased the proportion of decidual CD4+ Tregs and decreased the proportions of CD8+CD25+Foxp3+ T cells and macrophages in decidual tissues; (2) administration of vaginal progesterone did not cause an M1→M2 macrophage polarization; however, it reduced the proportion of IFNγ -positive neutrophils in the myometrium and active MMP-9-positive neutrophils and monocytes in the cervix; (3) in contrast, the administration of 17OHP-C increased the abundance of active MMP-9-positive neutrophils and monocytes in the cervix; (4) the immune effects of vaginal progesterone were associated with reduced systemic concentrations of IL-1β but not with alterations in progesterone or estradiol concentrations; and (5) pretreatment with vaginal progesterone was associated with a 50% reduction in endotoxin-induced PTB.

Vaginal progesterone increases the proportion of decidual CD4D+ Tregs

Lymphocytes with immunoregulatory properties were described more than 4 decades ago[162–166]; however, the lack of specific markers for these cells precluded their characterization using immunophenotypic techniques. CD4+ Tregs are an important subset of T cells, which express CD25 and Foxp3.[167–170]

The 2 main Treg subsets are thymic Tregs and peripheral Tregs.[168,171] These cells play a central role in immune responses through their suppressive activity of both self- and noneself-antigens,[172–174] and this suppressive function largely is due to their expression of the transcription factor Foxp3.[168,171]

During pregnancy, there is an expansion of antigen-specific CD4+ Tregs that exhibit suppressive functions. This is thought to promote maternal-fetal tolerance and pregnancy maintenance.[175–178] A breakdown of this tolerance during late pregnancy is considered a mechanism of disease for spontaneous preterm labor,[100,151] which might be due to the diminished suppressive function of CD4+ Tregs in preterm labor.[179–182] Indeed, we recently presented evidence that the administration of endotoxin, which causes PTB in mice, leads to a reduction of CD4+ Tregs at the maternal-fetal interface.[144]

Because progesterone plays a central role in pregnancy maintenance[40–44] and increases CD4+ Tregs with a suppressive function during midgestation,[154] we hypothesized that administration of vaginal progesterone and 17OHP-C from midgestation to late gestation

would lead to an expansion of CD4+ Tregsat the maternalfetal interface. In the study herein, administration of vaginal progesterone, but not 17OHP-C, increased the proportion of decidual CD4+ Tregs.

Altogether these findings suggest that vaginal progesterone administration during late gestation fosters local maternal-fetal tolerance by increasing the proportion of decidual CD4+ Tregs.

Vaginal progesterone reduces the proportion of decidual CD8+CD25+Foxp3+ T cells

In addition to increasing the proportion of CD4+ Tregs, vaginal progesterone administration to pregnant mice reduced the proportion of CD8+CD25+Foxp3+ T cells in decidual tissues. This finding is consistent with previous reports demonstrating that progesterone regulates CD8+ T cell cytokine release and cytotoxicity during pregnancy.[183,184] CD8+CD25+ T cells expressing Foxp3 seem to share phenotypic, functional, and mechanistic actions with the classical CD4+ Tregs; therefore, they were named CD8+ Tregs.[185] CD8+Foxp3+ T cells increased in vivo in response to inflammation induced by IL-6.[186] These cells inhibit T-cell responses in vitro and Th17 cell-mediated immune arthritis in vivo.[186]

During midgestation, CD8+Foxp3+ T cells expressing CD103 are found in the spleen in which they suppress immune responses via ICOS-B7h.[187] Recently we reported that splenic CD8+CD25+Foxp3+ T cells, which produce IL-10, increased in endotoxin-induced PTB.[144] In addition, we found that CD8+CD25+Foxp3+ T cells are present in both decidual tissues and maternal circulation during term pregnancy and that their proportions are increased by exogenous administration of IL-6, which restores parturition on time in *Il6*−/− mice.[188] This supports a role for these cells in the proinflammatory milieu that is associated with the process of labor.

As a whole, these data suggest that CD8+CD25+Foxp3+ T cells have a proinflammatory phenotype rather than a suppressive phenotype and that vaginal progesterone administration reduces the proportion of these cells in the decidua, thereby having an antiinflammatory role.

Vaginal progesterone decreases the proportion of decidual macrophages

Macrophages/monocytes play central roles in the maintenance of pregnancy and term and preterm parturition including uterine contractility, cervical ripening, and the rupture of membranes as well as in uterine involution during the postpartum period.[78,84,189–199] Macrophage/monocyte neutralization using an anti-F4/80 antibody prevents endotoxin-induced PTB,[195] which demonstrates that macrophages/monocytes participate in the process of microbial-induced preterm labor. Macrophages/monocytes express progesterone receptors[200,201]; therefore, it is possible that the infiltration and/or function of these cells are regulated by progesterone.

In the study herein, we found that administration of vaginal progesterone decreased the proportion of macrophages in the decidual tissues. These data are consistent with previous reports demonstrating that the administration of progesterone reduces the infiltration and migration of macrophages/mono-cytes into the reproductive tissues.[56,64] Altogether these

data suggest that vaginal progesterone regulates the infiltration of macrophages/monocytes into the decidual tissues, which fosters an antiinflammatory microenvironment at the maternal-fetal interface.

Vaginal progesterone reduces the proportion of IFNγ -positive neutrophils in myometrium

Neutrophils play an important role during term and preterm parturition because they release proinflammatory mediators that are associated with the onset of labor.[72,78,84,202–204] In the myometrium, the mRNA expression of *CXCL8*, a neutrophil chemokine, is higher in women who underwent labor than in those who did not undergo labor at term, suggesting a role for neutrophils in myometrial contractions.[78,82]

Recently we were able to support this hypothesis by demonstrating that the percentage and total number of myometrial neutrophils increase in endotoxin-induced PTB.[144] Indeed, myometrial neutrophils express inflammatory cytokines such as IL-6, IL-8, TNFα, IFNγ, and IL-4,[77,144,205] which is a characteristic phenotype of activated neutrophils.[206,207] Therefore, we hypothesize that vaginal progesterone administration to pregnant mice would reduce the infiltration of activated neutrophils into the maternal-fetal interface.

In accordance with our hypothesis, we found that vaginal progesterone administration reduces the proportion of IFNγ -positive neutrophils in myometrium. Previous in vitro studies demonstrated that incubation with progesterone reduces the release of chemokine ligand-8, which attracts neutrophils, in human myometrial biopsies or rabbit uterine cervical fibroblasts.[62,208] Collectively these data suggest that vaginal progesterone administration to pregnant mice reduces the infiltration of activated neutrophils into the myometrial tissues, which may be mediated by chemokine ligand-8.

Vaginal progesterone, but not 17OHP-C, reduces active MMP-9-positive neutrophils and monocytes in the cervix

We next evaluated whether vaginal progesterone or 17OHP-C administration had effects on MMP-9 activity and collagen content. MMPs are a super-family of zinc enzymes that participate in the degradation of the extracellular matrix.[209–211] MMP-9 (also known as gelatinase B) was discovered in polymorphonuclear leukocytes and monocytes.[212] During pregnancy, MMP-9 is expressed by resident cells and infiltrating leukocytes at the maternal-fetal interface and has been associated with the process of labor.[74,95,97,213–217]

The expression of active MMP-9 is increased at term pregnancy in humans and rabbits,[63,218] and it was localized in human infiltrating leukocytes and murine columnar epithelial cells and fibroblasts.[196,218] In vitro experimentation has demonstrated that the incubation of columnar epithelial cells or fibroblasts with progesterone inhibits MMP-9 activity.[63,196] However, in vitro incubation of human myometrial muscle cells with progesterone is not able to reduce IL-1β or TNFα-induced MMP-9 activity.[219]

Alternatively, in vitro incubation with progesterone reduces collagen synthesis in a 3-dimensional culture system with human cervical fibroblasts.[220] Herein the in vivo administration of vaginal progesterone increased MMP-9 activity and reduced collagen content in the cervical stroma. Vaginal progesterone also reduced the infiltration of active

MMP-9-positive neutrophils and monocytes. In contrast, 17OHP-C administration increased MMP-9 activity in the cervical stroma, reduced collagen content, and increased infiltration of active MMP-9-positive neutrophils and monocytes.

Collectively these data demonstrate that administration of vaginal progesterone or 17OHP-C increases MMP-9 activity in the cervical stroma and decreases collagen content, yet administration of natural progesterone reduces the infiltration of neutrophils and monocytes expressing active MMP-9. Infiltration may be the key element in determining changes in the biomechanical properties of the cervix, which favor parturition.

Pretreatment with vaginal progesterone reduces the rate of endotoxin-induced preterm birth

Vaginal progesterone administration to women with a sonographic short cervix reduces the rate of PTB.[7,9,11,221] In addition, pretreatment by injection of natural or medroxyprogesterone acetate prevents endotoxin-induced PTB in mice, which is associated with the down-regulation of the mRNA expression of the inflammatory cytokines *Il1β* and *Tnf*.[23,33]

It is interesting that the systemic administration of IL-1b induces PTB in mice, and pretreatment with the IL-1 receptor antagonist abrogrates this effect.[160] In the current study, pretreatment with vaginal progesterone reduced the frequency of endotoxin-induced PTB by 50% and reduced the systemic concentrations of IL-1*β*. Altogether these data suggest that pretreatment with vaginal progesterone fosters a local and systemic antiinflammatory response, preventing endotoxin-induced preterm birth.

Although previous studies had reported that systemic administration of progesterone reduces the rate of endotoxin-induced preterm birth by 28%,[23] the current study is the first to demonstrate that vaginal progesterone has this effect. The fact that vaginal progesterone does not prevent endotoxin-induced preterm delivery in all cases is not unexpected, given that even in women with a short cervix, the administration of vaginal progesterone reduced the rate of preterm delivery by only 45%.[11] The mechanisms responsible for the protection against preterm birth in some animals and women, and not in others, remain to be determined.

Administration of 17OHP-C to women with multiple gestations has been reported to increase the rates of midtrimester fetal loss[38] and PTB before 32 weeks.[39] Similarly, pretreatment with 17OHP-C before endotoxin exposure has adverse effects on pregnant mice, including behavioral changes (lethargy or piloerection) and maternal death.[33] For these reasons, we did not study the effect of 17OHP-C on endotoxin-induced preterm birth. However, in contrast to previous reports with 17OHP-C, vaginal progesterone followed by endotoxin did not result in demonstrable maternal morbidity or death.

A previous study demonstrated that progesterone binds with more avidity to progesterone receptors than 17OHP-C; however, both progestogens are comparable in eliciting the transactivation of reporter genes as assessed by luciferase activity in the T47D-2963.1 and T47Dco carcinoma cell lines.[222] Progesterone and 17OHP-C also induced similar

stimulation of endogenous alkaline phosphatase activity.[222] The equivalent biological effect per unit mass of 17OHP-C and progesterone in preventing preterm delivery induced by an inhibitor of nitric oxide synthase has also been shown in CD-1 mice.[223]

These findings suggest that the progestational activity of 17OHP-C and progesterone as measured by these assays are similar. However, this does not seem to translate into changes in the immune cell composition at the maternal-fetal interface. Specifically, the total exposure to 17OHP-C was greater than the total exposure to vaginal progesterone. Yet we observed antiinflammatory effects only with vaginal progesterone.

Conclusion

Our results demonstrate that the administration of vaginal progesterone fosters an antiinflammatory microenvironment at the maternal-fetal interface by increasing CD4+ Tregs and reducing CD8+CD25+Foxp3+ T cells, macrophages, and IFNγ-positive neutrophils. In addition, the administration of vaginal progesterone decreases the infiltration of active MMP-9-positive neutrophils and monocytes in the cervix, marginally reduces the plasma concentration of IL-1*β*, and reduces the frequency of endotoxin-induced PTB. Administration of 17OHP-C did not have the same effects as vaginal progesterone. These results provide insight into the mechanisms whereby vaginal progesterone prevents preterm birth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Akshata Naik, Elly Sanchez-Rodriguez, Dr Eleazar Soto, Marcia Arenas-Hernandez, Nakisha Rutledge, Tamara Roumayah, Yang Jiang, Amapola Balancio, Stella Dewar, Dr Zhong Dong, Lorri McLuckie, Rona Wang, and Sunjay Modi for their contributions to the execution of this study and to Maureen McGerty for her critical readings of the manuscript.

This study was supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal, and Child Health and by the Perinatology Research Branch, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services.

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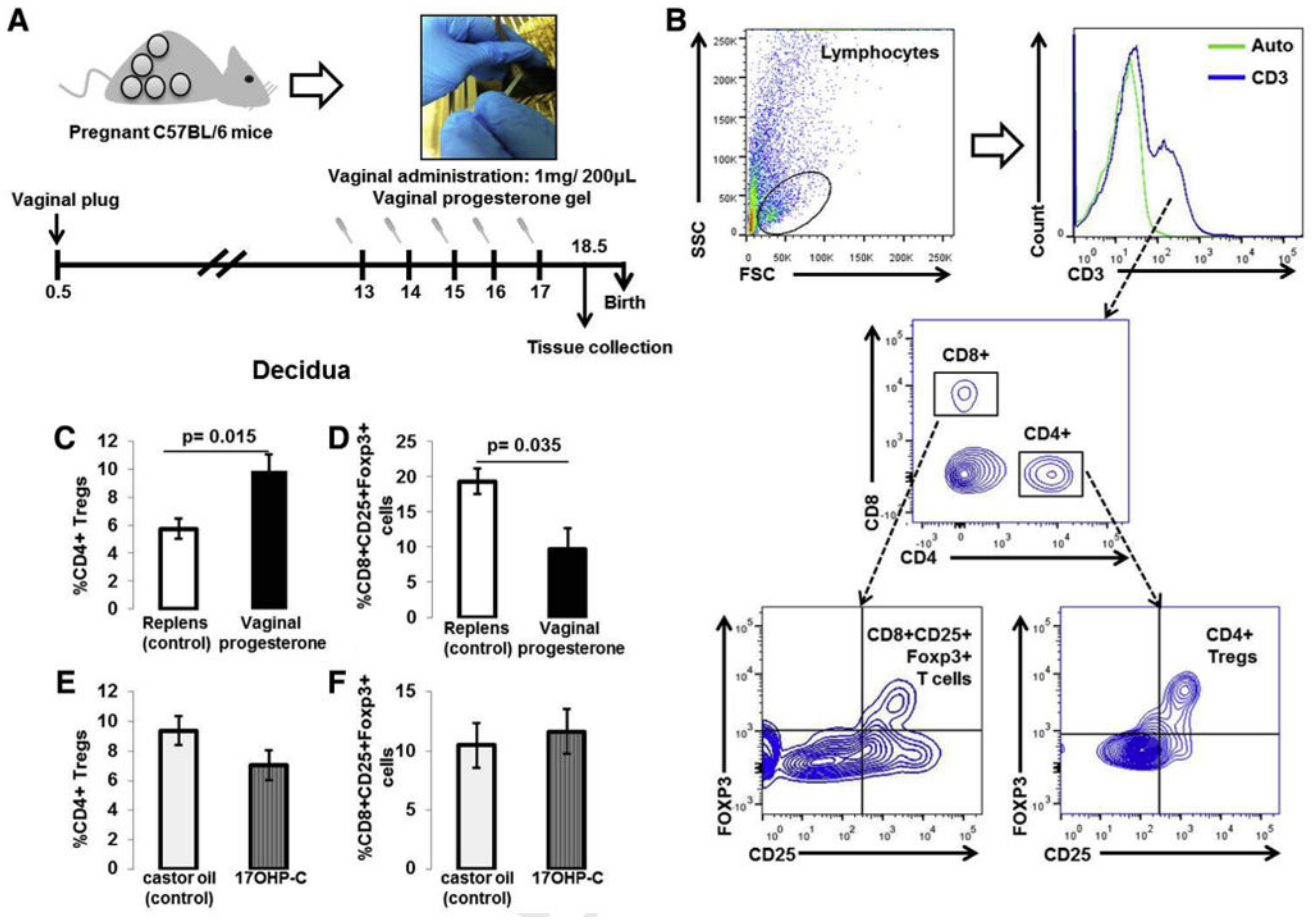

FIGURE 1. Animal model and identification of decidual CD4+ Tregs and CD8+CD25+Foxp3+ cells

A, Vaginal progesterone administration scheme. **B**, Gating strategy used to identify CD4+ Tregs (CD4+CD25+Foxp3+ cells) and CD8+CD25+Foxp3+ cells in decidual tissues. CD3+ T cells were gated within the total lymphocyte gate (FSC vs SSC). The green histogram represents the autofluorescence control. CD4+ Tregs and CD8+CD25+Foxp3+ cells were gated within the CD4+ and CD8+ gates, respectively. **C**, Proportions of decidual CD4+ Tregs in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **D**, Proportions of decidual CD8+CD25+Foxp3+ cells in mice treated with vaginal progesterone or Replens (control). **E**, Proportions of decidual CD4+ Tregs in mice injected with 17OHP-C or castor oil (control). **F**, Proportions of decidual CD8+CD25+Foxp3+ cells in mice injected with 17OHP-C or castor oil (control) (n = 10 each). Data are represented as mean ± SEM.

FSC,; *17OHP-C*, 17-alpha-hydroxyprogesterone caproate; *SSC*, saline sodium citrate; *Treg*, regulatory T cell.

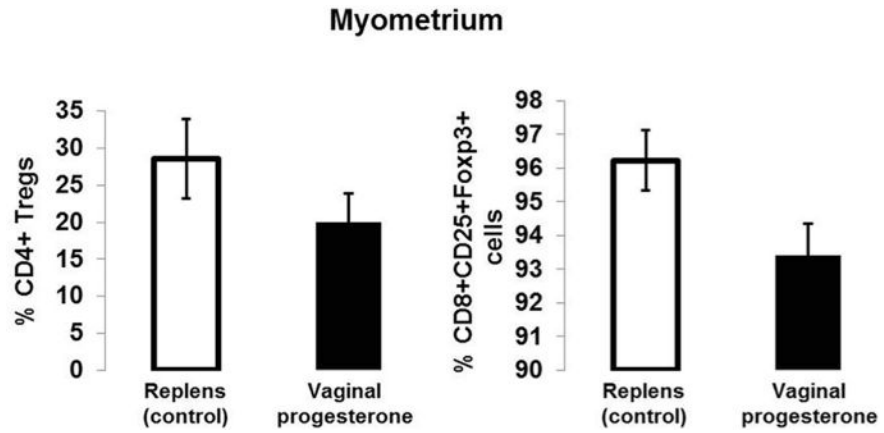

FIGURE 2. Proportions of myometrial CD4+ Tregs and CD8+CD25+Foxp3+ cells

Proportions of myometrial CD4+ Tregs (CD4+CD25+Foxp3+ cells) and CD8+CD25+Foxp3+ cells in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc) (n = 10 each). Data are represented as mean ± SEM. *Treg*, regulatory T cell.

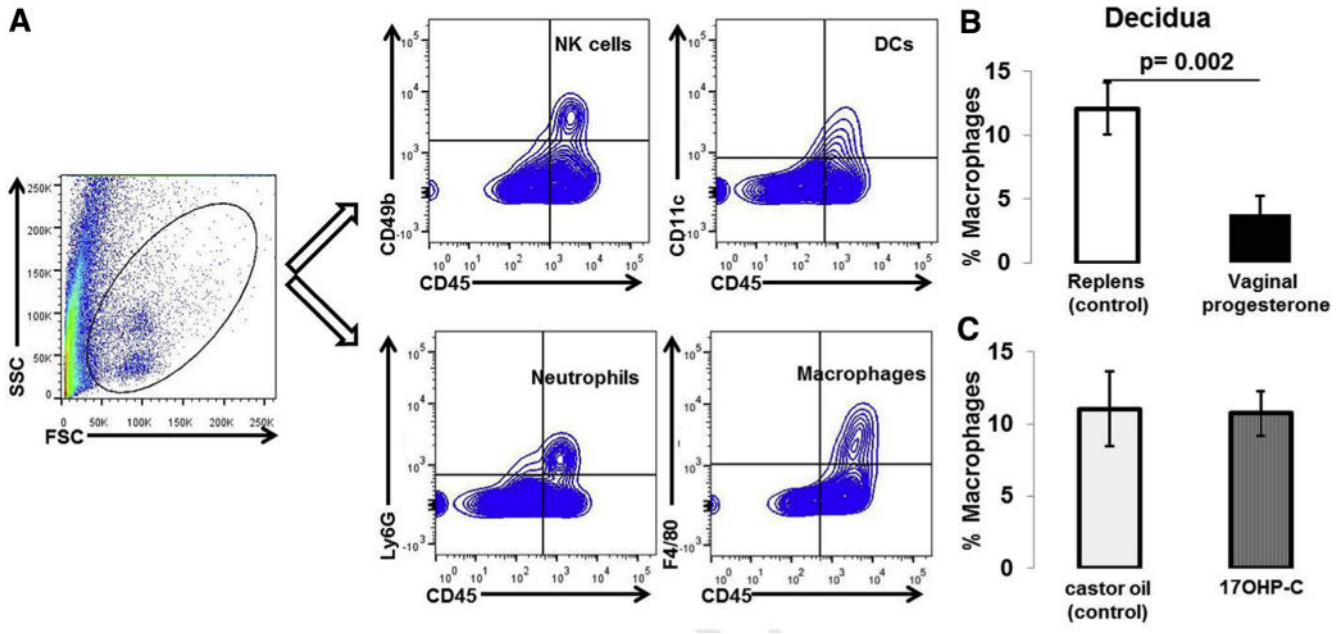

FIGURE 3. Immunophenotyping of innate immune cells in decidual tissues

A, Gating strategy used to identify NK cells (CD45+CD49b+ cells), DCs (CD45+CD11c+ cells), neutrophils (CD45+Ly6G+ cells), and macrophages (CD45+F4/80 cells) in decidual tissues. **B**, Proportions of decidual macrophages in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **C**, Proportions of decidual macrophages in mice injected with 17OHP-C or castor oil (control) (n = 10 each). Data are represented as mean ± SEM.

DC, dendritic cell; *FSC,; NK*, natural killer; *17OHP-C*, 17-alpha-hydroxyprogesterone caproate; *SSC*, saline sodium citrate.

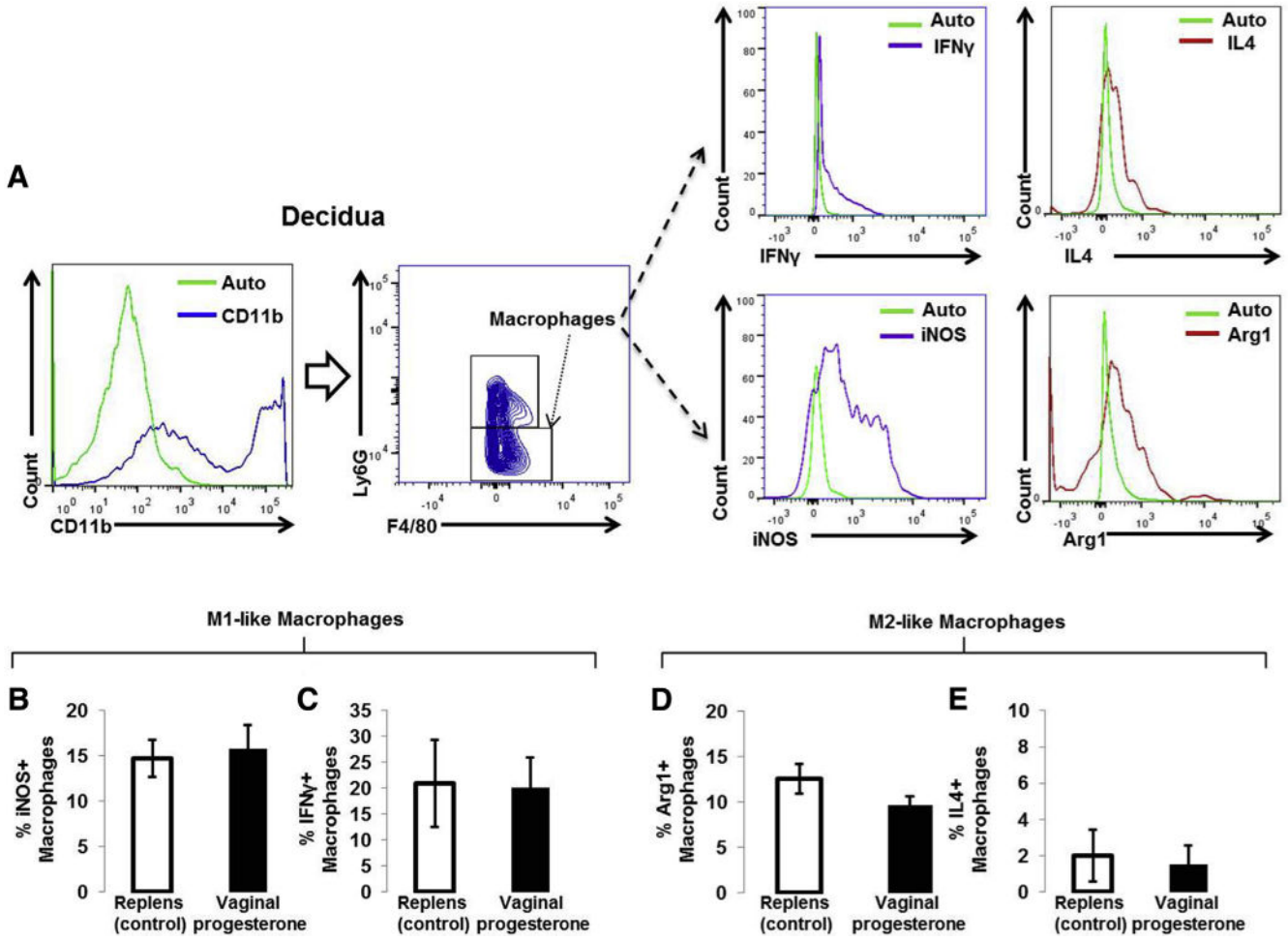

FIGURE 4. M1- and M2-like macrophages in decidual tissues

A, Gating strategy used to identify M1-like (CD11b+ Ly6G-F4/80+ IFNγ -positive or iNOS-positive cells) and M2-like (CD11b Ly6G-F4/80+ IL4-positive or Arg1-positive cells) macrophages. The green histogram represents the autofluorescence control. **B** and **C**, Proportions of M1-like (CD11b+Ly6G-F4/80+ IFNγ-positive or iNOS-positive cells) macrophages in decidual tissues from mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **D** and **E**, Proportions of M2-like (CD11b+Ly6G-F4/80+ IL4-positive or Arg1-positive cells) macrophages in decidual tissues from mice treated with vaginal progesterone or Replens (control) (n = 10 each). Data are represented as mean ±SEM.

IFN, interferon; *IL*, interleukin; *iNOS*, inducible nitric oxide synthase.

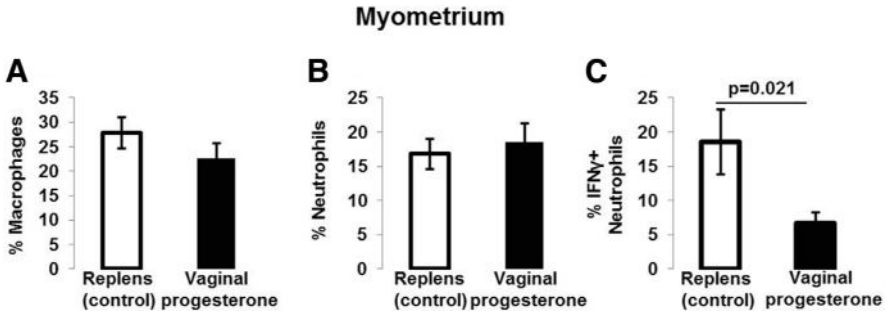

FIGURE 5. Macrophages and neutrophils in myometrium

A, Proportions of myometrial macrophages in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **B**, Proportions of myometrial neutrophils in mice treated with vaginal progesterone or Replens (control). **C**, Proportions of myometrial IFNγ -positive neutrophils (CD11b+Ly6G+F4/80− cells) in mice treated with vaginal progesterone or Replens (control) (n = 10 each). Data are represented as mean ±SEM.

IFN, interferon.

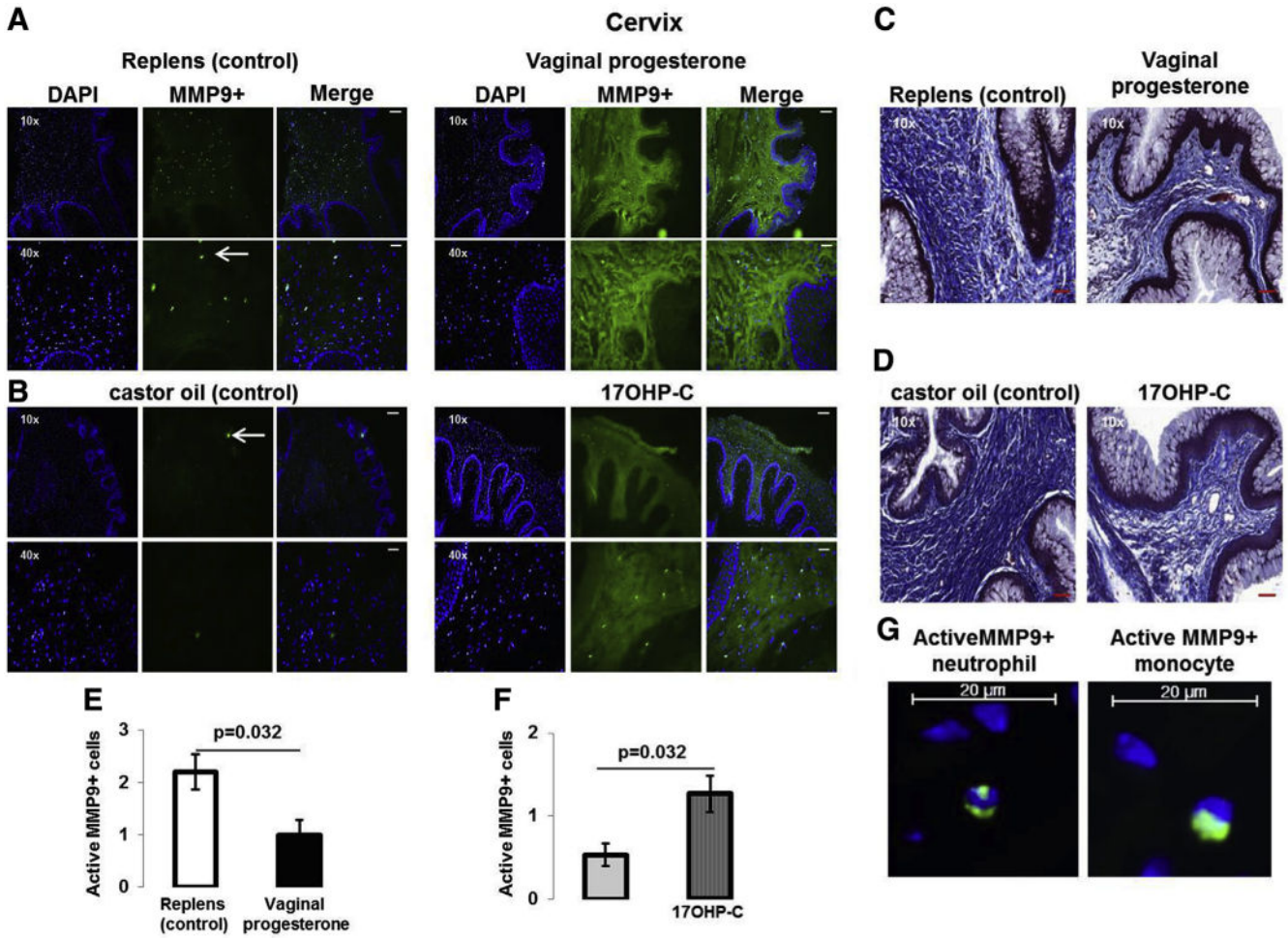

FIGURE 6. MMP-9 activity and collagen content in cervical tissues

A, MMP-9 activity (*green staining*) in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **B**, MMP-9 activity (*green staining*) in mice injected with 17OHP-C or castor oil (control). Nuclei were stained with DAPI. White arrows represent active MMP-9-positive cells. Scale bars in ×10 and ×40: 200 μm and 50 μm, respectively. **C**, Masson's trichrome staining of the cervical tissues from mice treated with vaginal progesterone or Replens (control). Scale bars in ×10 and ×40: 200 μm and 50 μm, respectively. **D**, Masson's trichrome staining of the cervical tissues from mice injected with 17OHP-C or castor oil (control). Collagen fibers are stained in *blue*. Scale bars in ×10 and ×40: 200 μm and 50 μm, respectively. **E**, Semiquantification of active MMP-9-positive cells in the cervices from mice treated with vaginal progesterone or Replens (control). **F**, Semiquantification of active MMP-9-positive cells in cervices from mice injected with 17OHP-C or castor oil (control) (n = 5 each). Data are represented as mean ±SEM. **G**, Magnified image of active MMP-9-positive neutrophils and monocytes in cervical tissues from control mice. Scale bars: 20 μm.

DAPI, 4′,6-diamidino-2-phenylindole; *MMP*, matrix metalloproteinase; *17OHP-C*, 17-alpha-hydroxyprogesterone caproate.

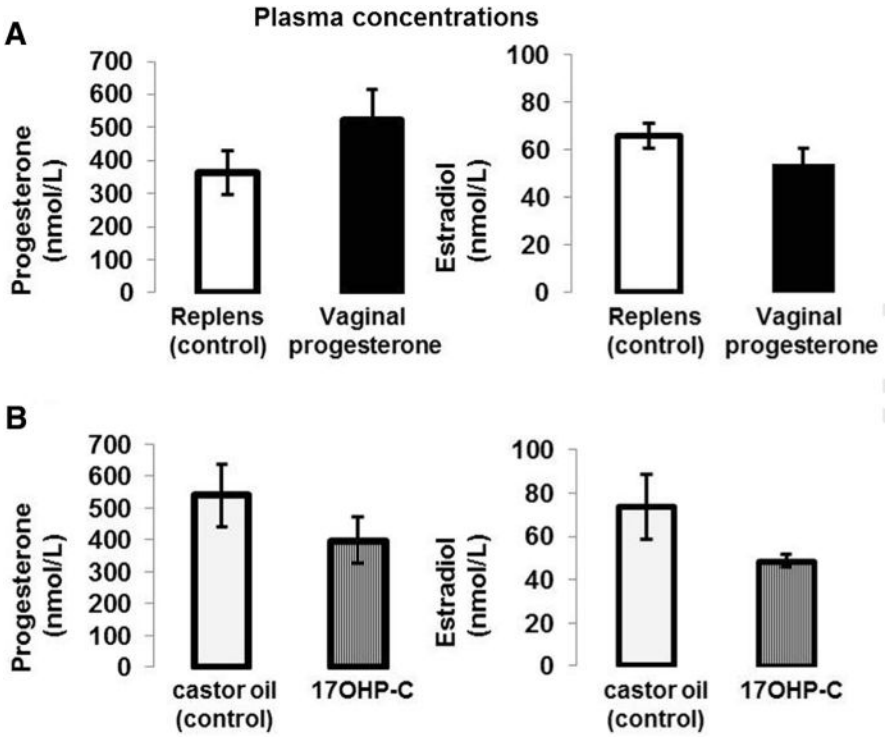

FIGURE 7. Plasma concentrations of progesterone and estradiol

A, Progesterone and estradiol concentrations in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **B**, Progesterone and estradiol concentrations in mice injected with 17OHP-C or castor oil (control). Plasma samples were collected at 18.5 dpc (n = 10 each). Data are represented as mean ± SEM.

dpc, days postcoitum; *17OHP-C*, 17-alpha-hydroxyprogesterone caproate.

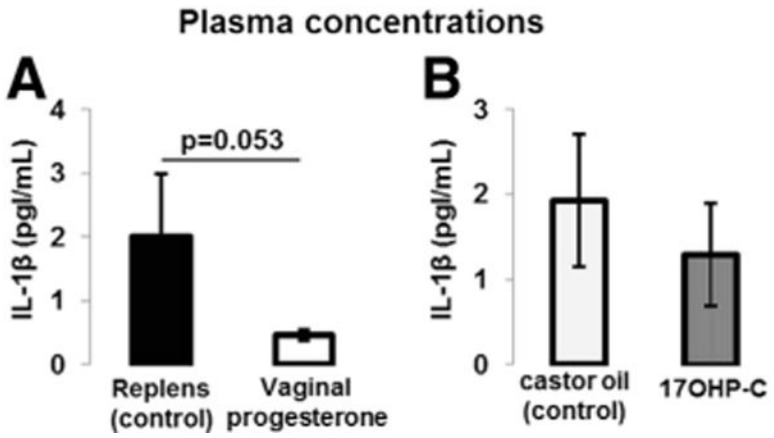

FIGURE 8. Plasma concentration of IL-1β

A, IL-1β concentrations in mice treated with vaginal progesterone or Replens (control; Lil' Drug Store Products, Inc). **B**, IL-1β concentrations in mice injected with 17OHP-C or castor oil (control). Plasma samples were collected at 18.5 dpc (n = 10 each). Data are represented as mean ± SEM.

dpc, days postcoitum; *IL*, interleukin; *17OHP-C*, 17-alpha-hydroxyprogesterone caproate.

TABLE

Vaginal progesterone administration decreases the rate of endotoxin-induced preterm birth

	Replens (control) plus endotoxin	Vaginal progesterone plus endotoxin	*P* value
Number of mice	10	10	—
Preterm birth, %[a,b]	90	40	.008
Gestational length, d[c,d]	17.7 ± 0.6	18.4 ± 0.8	.038
Duration of labor, min[e,d]	37 ± 20.7	40.5 ± 30.1	NS

Replens is manufactured by Lil' Drug Store Products, Inc. *dpc*, days postcoitum; *NS*, not significant. *PTB*, preterm birth.

[a] The rate of PTB was defined as the percentage of dams delivering at <18.0 dpc among all births;

[b] χ^2 test;

[c] Days elapsed from the detection of a vaginal plug (0.5 dpc) to the delivery of the first pup. Data are shown as mean ± SD;

[d] Mann-Whitney *U* test;

[e] Time elapsed from the delivery of the first pup to the last pup. Data are shown as mean ± SD.