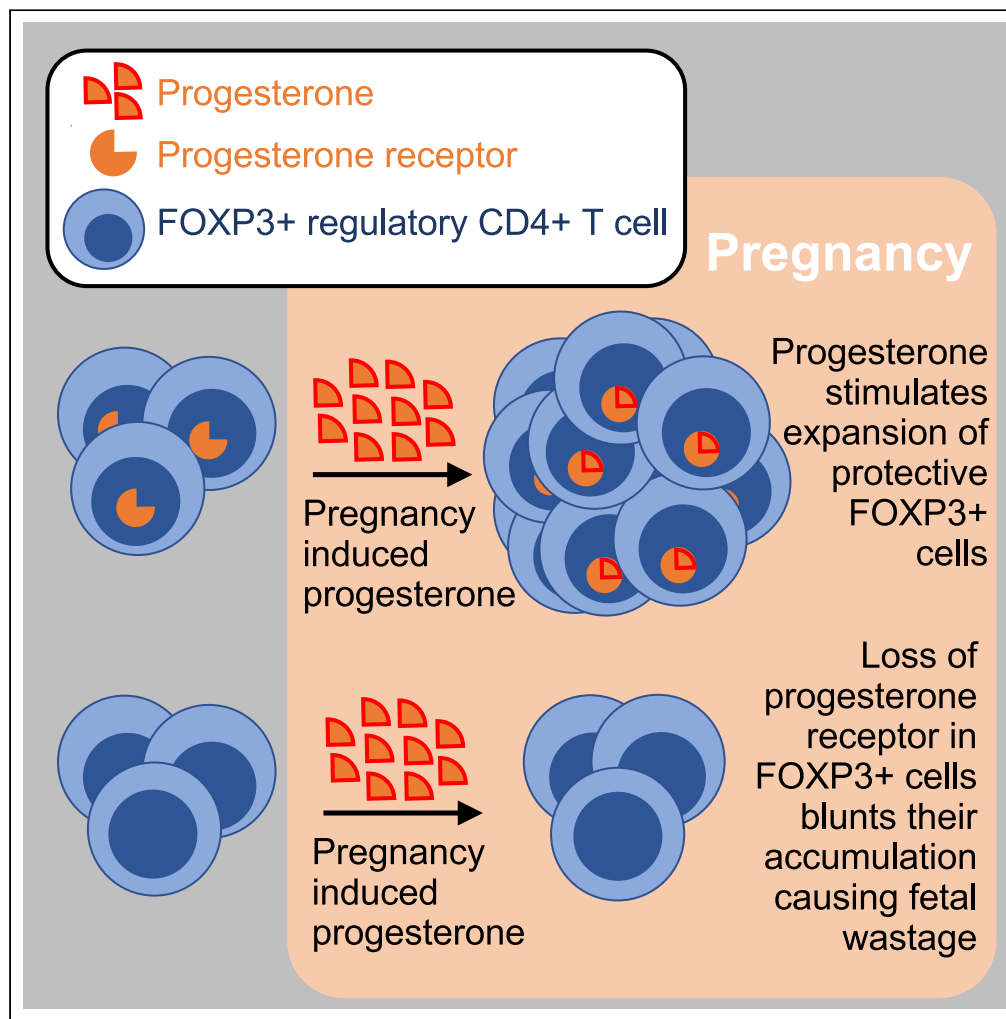## Article

Maternal-fetal conflict averted by progesterone-induced FOXP3+ regulatory T cells

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Highlights

Progesterone receptor in maternal FOXP3+ Tregs protects against fetal wastage

Progesterone receptor stimulation drives maternal Treg proliferation and accumulation

Loss of progesterone receptor unleashes decidual CD8+ T cell infiltration

Exogenous 17-OHPC progesterone reinforces fetal tolerance

Article

Maternal-fetal conflict averted by progesterone-induced FOXP3+ regulatory T cells

Ashley L. Severance,[1,6] Jeremy M. Kinder,[1,6] Lijun Xin,[1] Ashley R. Burg,[1] Tzu-Yu Shao,[1,2] Giang Pham,[1] Tamara Tilburgs,[3] Wendy A. Goodman,[4] Sam Mesiano,[5] and Sing Sing Way[1,7,*]

SUMMARY

Pregnancy stimulates an intricately coordinated assortment of physiological changes to accommodate growth of the developing fetus, while simultaneously averting rejection of genetically foreign fetal cells and tissues. Despite increasing evidence that expansion of immune-suppressive maternal regulatory T cells enforces fetal tolerance and protects against pregnancy complications, the pregnancy-associated signals driving this essential adaptation remain poorly understood. Here we show that the female reproductive hormone, progesterone, coordinates immune tolerance by stimulating expansion of FOXP3+ regulatory T cells. Conditional loss of the canonical nuclear progesterone receptor in maternal FOXP3+ regulatory T cells blunts their proliferation and accumulation, which is associated with fetal wastage and decidual infiltration of activated CD8+ T cells. Reciprocally, the synthetic progestin 17α-hydroxyprogesterone caproate (17-OHPC) administered to pregnant mice reinforces fetal tolerance and protects against fetal wastage. These immune modulatory effects of progesterone that promote fetal tolerance establish a molecular link between immunological and other physiological adaptions during pregnancy.

INTRODUCTION

Pregnancy-associated expansion of maternal FOXP3+ regulatory CD4+ T cells (Tregs) is widely conserved across eutherian mammalian species consistent with a shared requirement for expanded tolerance to foreign paternal-fetal alloantigens (Erlebacher, 2013; Ruocco et al., 2014; Salvany-Celades et al., 2019; Samstein et al., 2012; Tilburgs et al., 2008). Decreased expansion of maternal Tregs is associated with a variety of pregnancy complications, including spontaneous abortion and preeclampsia (Gomez-Lopez et al., 2020; Inada et al., 2013; Jiang et al., 2014; Sasaki et al., 2004; Somerset et al., 2004). Complete or partial depletion of maternal FOXP3+ cells to prepregnancy levels in mice impairs fetal tolerance, leading to fetal wastage (Aluvihare et al., 2004; Rowe et al., 2011). Maternal Treg expansion is initiated as early as conception through paternal antigen stimulation and cytokines such as TGF-β in the seminal fluid (Moldenhauer et al., 2009). Maternal Tregs with fetal specificity further accumulate during pregnancy in mice (Rowe et al., 2012) and humans (Tilburgs et al., 2008, 2009). This necessity for maternal Treg expansion raises important questions regarding the pregnancy-associated factors that promote their expansion in coordination with other pregnancy-induced physiological changes.

Progesterone is a highly conserved female reproductive hormone essential for initiating and maintaining pregnancy across viviparous species (Ramathal et al., 2010; Wu et al., 2018). Rising progesterone levels during the luteal phase of the menstrual cycle prepare the uterine lining for implantation (Graham and Clarke, 1997). Significantly more elevated progesterone levels maintain relaxation of the uterine smooth muscle and closure of the uterine cervix during pregnancy. These actions are mediated by the canonical nuclear progesterone receptor (PR), as female PR-deficient mice are infertile (Lydon et al., 1995), and in all species treatment with the PR antagonist mifepristone (RU486) terminates pregnancy (el-Refaey et al., 1995; Mao et al., 2010; Renthal et al., 2010).

Recent evidence suggests immune-modulatory roles for progesterone that contribute to expanded tolerance during pregnancy. Conditional PR deficiency in CD11c+ antigen-presenting cells causes intrauterine growth restriction that parallels modestly reduced accumulation of uterine Tregs (Thiele et al., 2019).

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Progesterone can also directly stimulate CD4+ T cells to undergo Treg differentiation *in vitro*, which requires PR expression by T cells (Lee et al., 2012; Mao et al., 2010). However, whether progesterone directly stimulates maternal Tregs to promote their accumulation required for sustaining fetal tolerance during pregnancy remains uncertain. This knowledge gap was addressed by evaluating pregnancy outcomes in mice with conditional loss of PR in maternal Treg cells.

RESULTS AND DISCUSSION

To investigate the importance of PR in maternal Tregs, FOXP3^{Cre} and PR$^{flox/flox}$ mice were intercrossed (Fernandez-Valdivia et al., 2010; Rubtsov et al., 2008) to generate FOXP3$^{Cre/Cre}$PR$^{flox/flox}$ (FOXP3-PRKO) mice with selective PR deficiency only in FOXP3+ cells. We found allogeneic pregnancies sired by male BALB/c mice consistently showed increased rates of fetal wastage with reciprocal loss of live pups in female FOXP3-PRKO compared with FOXP3-wild-type (WT) mice (Figure 1A). Similar fertility and numbers of concepti per litter indicate defects in maintaining pregnancy are responsible for negative pregnancy outcomes in FOXP3-PRKO mice (Figure 1A). Normal allogeneic pregnancy in FOXP3$^{Cre/Cre}$ control mice indicate fetal wastage in FOXP3-PRKO mice is not explained by potentially reduced FOXP3 expression in FOXP3^{Cre} knock-in mice (Franckaert et al., 2015; Rubtsov et al., 2008) (Figure 1A).

To more definitively investigate the cause of fetal wastage in FOXP3-PRKO mice, complementation studies evaluated how adoptively transferred PR-sufficient WT cells impact pregnancy outcomes in FOXP3-PRKO recipient mice. Donor splenocytes from FOXP3^{DTR} mice with co-expression of human diphtheria toxin receptor (DTR) with FOXP3 were used for complementation, allowing selective depletion of PR-sufficient FOXP3 cells in recipient FOXP3-PRKO mice after low-dose diphtheria toxin (DT) administration (Kim et al., 2007). These experiments showed fetal wastage, and loss of live pups in FOXP3-PRKO mice is reversed by adoptive transfer of whole splenocytes containing PR-sufficient FOXP3+ cells, whereas pregnancy complications persisted when PR-sufficient donor FOXP3+ cells were selectively eliminated (Figure 1B). Thus, fetal wastage in FOXP3-PRKO mice reflects the selective loss of PR in maternal FOXP3+ cells and is also not explained by potential low-level promiscuous Cre-activity (Bittner-Eddy et al., 2019; Franckaert et al., 2015).

To examine the role of paternal antigens on maternal Treg expansion, BALB/c male mice with constitutive expression of the 2W1S$_{55-68}$ peptide as a recombinant protein in all cells (Balb/c-2W1S/OVA) (Moon et al., 2011) were used to sire allogeneic pregnancy. This approach transforms the MHC class II I-A^b restricted 2W1S$_{55-68}$ peptide into a surrogate fetal antigen, allowing precise tracking of maternal CD4+ T cells responsive to fetal I-A^b:2W1S$_{55-68}$ stimulation (Figure S1) (Rowe et al., 2012). These experiments showed fetal wastage in FOXP3-PRKO mice was associated with sharply reduced percentage and total number of FOXP3+ Tregs with I-A^b:2W1S fetal specificity, whereas accumulation of I-A^b:2W1S FOXP3− CD4+ T cells was similar compared with FOXP3-WT control mice (Figure 1C).

Pregnancy also stimulates functional changes in maternal FOXP3+ cells. A prominent example is expression of cytotoxic T cell Lymphocyte antigen 4 (CTLA-4), which mediates many aspects of immune suppression by FOXP3+ Tregs and is increased in maternal Tregs during healthy pregnancy (Heikkinen et al., 2004; Tilburgs et al., 2008) but reduced with pregnancy complications including preterm birth and spontaneous abortion (Jin et al., 2009; Sasaki et al., 2004). We found increased CTLA-4 expression by maternal FOXP3+ Tregs with I-A^b:2W1S fetal specificity compared with tetramer negative bulk FOXP3+ cells during healthy pregnancies in FOXP3-WT mice but not in FOXP3-PRKO mice (Figure 1D), indicating PR is essential for activation as well as numerical expansion of FOXP3+ cells during pregnancy.

The immune modulatory effects of progesterone were further investigated using RU486, which blocks PR signaling and as a consequence induces parturition in mice within 48 h after administration (Figure 1E). RU486-induced pregnancy termination was accompanied by reductions in both the percentage and number of FOXP3+ Tregs with fetal-I-A^b:2W1S$_{55-68}$ specificity but did not affect accumulation of I-A^b:2W1S FOXP3− CD4+ T cells (Figure 1F). This necessity for PR by maternal FOXP3+ cells, and in particular maternal Tregs with fetal specificity, parallels significantly increased levels of *Pgr* mRNA among splenocytes in pregnant compared with virgin control mice, albeit at sharply reduced levels compared with uterine cells (Figure S2), and is in agreement with prior studies showing only low-level *Pgr* mRNA levels in bulk maternal CD4+ T cells during pregnancy (Engler et al., 2017; Lissauer et al., 2015). Thus, despite RU486 functionally antagonizing progesterone/PR across a variety of maternal cells and tissues, and likely through a variety of receptors including the canonical nuclear PR plus glucocorticoid receptor (Beck et al., 1993;
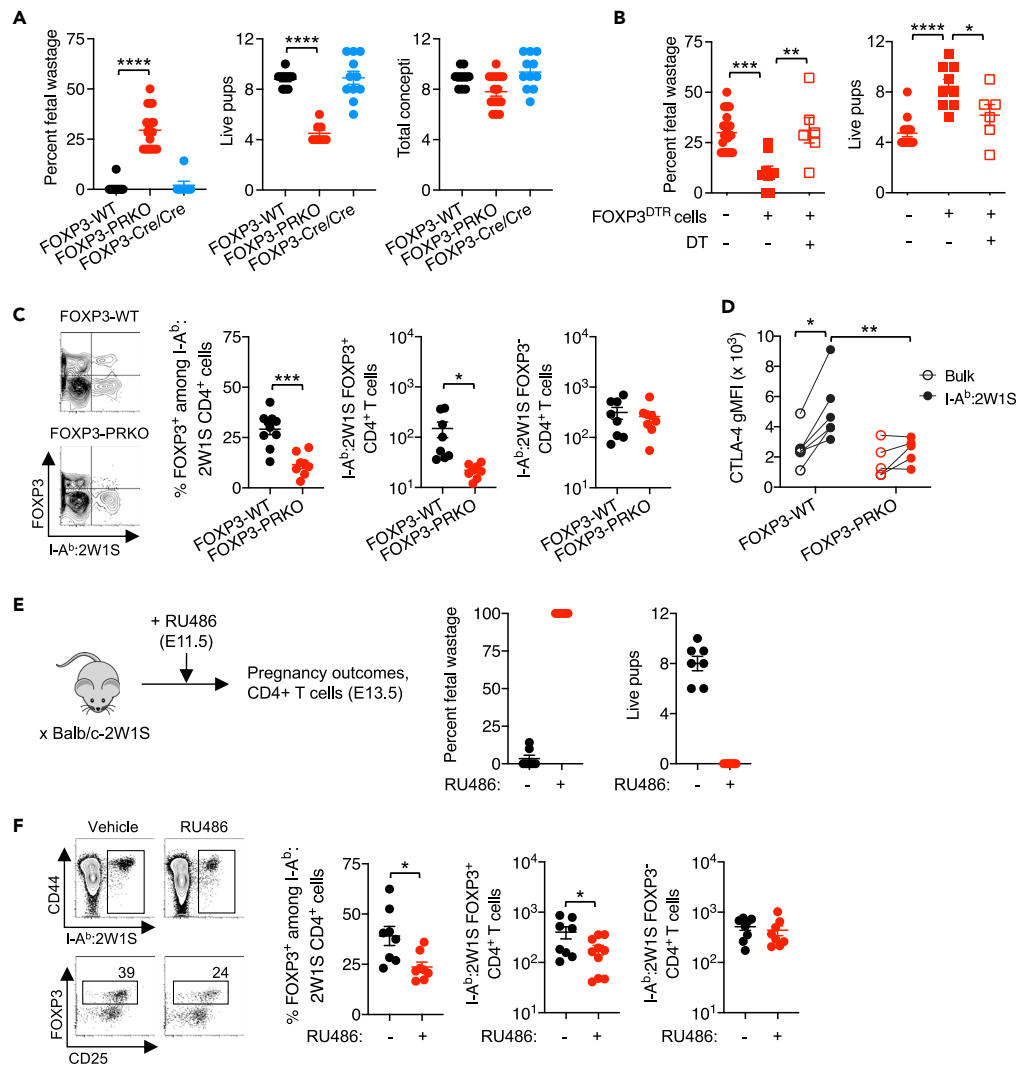
Figure 1. Progesterone receptor signaling in maternal FOXP3+ cells protects against fetal wastage

(A) Percent fetal wastage, number of live pups *in utero*, and concepti in FOXP3-WT (black), FOXP3-PRKO [FOXP3$^{Cre/Cre}$PR$^{flox/flox}$] (red), and FOXP3-Cre/Cre (blue) female mice at mid-gestation (E11.5) during allogeneic pregnancies sired by Balb/c-2W1S/OVA (H-2^d) male mice.

(B) Percent fetal wastage and number of live pups for FOXP3-PRKO female mice at mid-gestation (E11.5) during allogeneic pregnancies sired by Balb/c-2W1S/OVA (H-2^d) male mice, including those adoptively transferred PR-sufficient Treg from FOXP3^{DTR} donor mice prior to mating (squares) and whether or not DT was administered to selectively eliminate donor FOXP3+ Tregs (open).

(C) Frequency of FOXP3+ and number of FOXP3− CD4+ T cells with I-A^b:2W1S$_{55-68}$ specificity in the spleen and pooled peripheral lymph nodes for mice described in panel A.

(D) CTLA-4 expression (gMFI) after cell-surface + intracellular staining among I-A^b:2W1S$_{55-68}$-specific (filled) or bulk (open) CD4+ T cells in the spleen and pooled peripheral lymph nodes for mice described in panel A.

(E) Percent fetal wastage and number of live pups 2 days following administration of RU486 (red) or vehicle (black) in allogeneic pregnant female mice.

(F) Representative FACS plots and summary data showing number of I-A^b:2W1S$_{55-68}$ CD4+ T cells and frequency of FOXP3+ Treg cells in the spleen and pooled peripheral lymph nodes for mice described in panel E. Data are from at least three independent experiments each with similar results, with each point representing data from an individual mouse. Bar, mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.
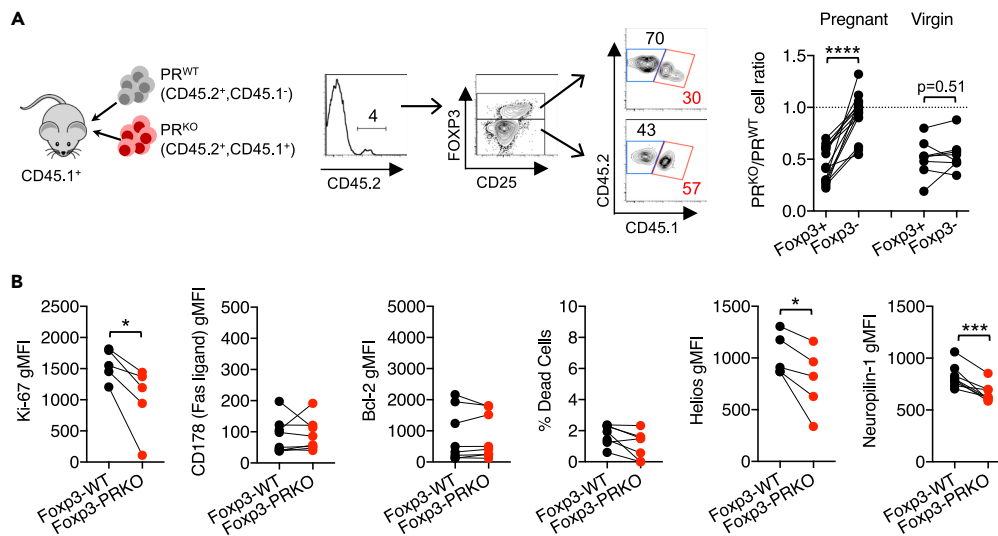
Figure 2. Progesterone receptor stimulation drives proliferation and accumulation of maternal FOXP3+ cells

(A) FACS plots and summary graphs showing ratio of adoptively transferred PR^{KO} [CD45.2+, CD45.1+] (red) to PR^{WT} [CD45.2+, CD45.1−] (gray) FOXP3+ and FOXP3− maternal CD4+ splenocytes during allogeneic pregnancy (E11.5) sired by BALB/c males compared with virgin control mice.

(B) Expression of Ki-67, CD178 (Fas ligand), Bcl-2, Helios, Neuropilin, and percent dead cells identified by staining with a membrane integrity dye by donor PR-sufficient (FOXP3-WT) compared with PR-deficient (FOXP3-PRKO) FOXP3+ cells for mice described in panel A. Data are from at least three independent experiments, each with similar results, with each point representing data from an individual mouse. Bar, mean ± SEM. *p < 0.05, ***p < 0.005, ****p < 0.001.

Zhang et al., 2006), these results together with reduced expansion of fetal-specific Tregs in FOXP3-PRKO mice highlight the importance of progesterone/PR signaling in maintaining expanded accumulation of maternal FOXP3+ Tregs during pregnancy.

To establish the causal relationship between reduced maternal FOXP3+ Treg expansion and fetal wastage in FOXP3-PRKO mice, pregnancy-induced accumulation of donor PR-deficient and PR-sufficient CD4+ T cells was compared after co-transfer into WT recipient mice (Figure 2A). Donor CD4+ T cells were identified based on discordant expression of the CD45.1/CD45.2 congenic markers from endogenous WT cells in recipient mice. We reasoned that if loss of PR-mediated expansion of maternal Tregs drives fetal wastage in FOXP3-PRKO mice, healthy pregnancies in WT recipient mice would exhibit diminished accumulation of donor PRKO compared with PRWT FOXP3+ cells. In agreement, the ratio of PR-deficient to PR-sufficient donor cells was significantly reduced among FOXP3+ compared with FOXP3− cells in pregnant recipient but not virgin control mice (Figure 2A). Reduced expansion of donor PR-deficient FOXP3+ cells reflects diminished proliferation given reduced Ki-67 expression, as opposed to differences in cell death shown by similar levels of the apoptosis markers CD178 (Fas ligand), B-cell lymphoma 2 (Bcl-2), and membrane integrity cell viability staining (Figure 2B) (Allison et al., 2004; Ramaswamy et al., 2009). This platform allowing CD4+ T cell PR stimulation to be evaluated during healthy pregnancy also revealed reduced Helios and Neuropilin-1 (Nrp-1) expression by PR-deficient compared with PR-sufficient FOXP3+ cells, suggesting progesterone selectively promotes accumulation of natural, as opposed to induced, Tregs during pregnancy (Figure 2B) (Curotto de Lafaille and Lafaille, 2009; Rowe et al., 2012). Thus, PR signaling in FOXP3+ cells promotes the proliferation and accumulation of this protective maternal CD4+ T cell subset during pregnancy.

One mechanism whereby Tregs may protect against fetal wastage is through suppressed activation and decidual accumulation of cytolytic CD8+ T cells (Chaturvedi et al., 2015; Samstein et al., 2012). To test this possibility, fetal-specific CD8+ T cells were evaluated in FOXP3-PRKO mice. Co-expression of OVA with 2W1S in Balb/c-2W1S-OVA transgenic mice used to sire allogeneic pregnancy allows endogenous CD8+ T cells with surrogate fetal-OVA specificity to be identified by H-2K^b:OVA$_{257-264}$ tetramer staining (Moon et al., 2011). These experiments showed fetal wastage in FOXP3-PRKO mice paralleled sharply
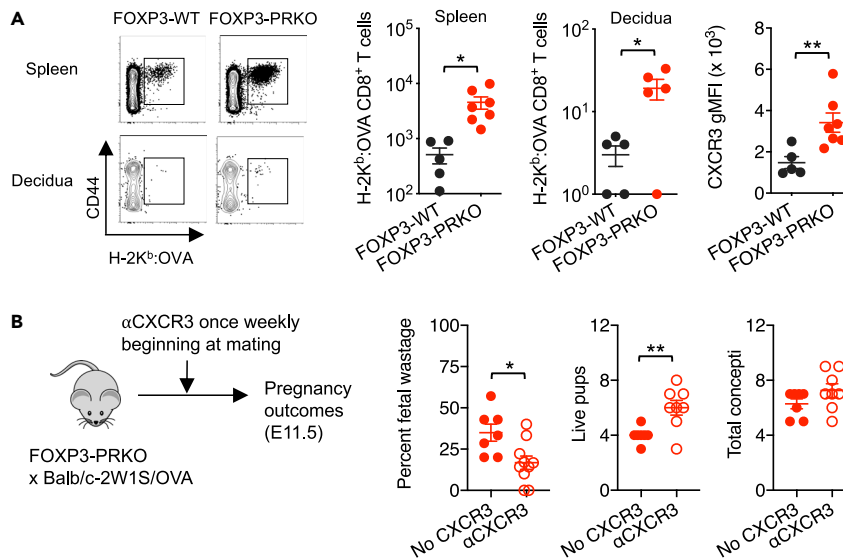
Figure 3. Selective loss of progesterone receptor by maternal FOXP3+ cells unleashes activation and decidual infiltration of CD8+ T cells with fetal specificity

(A) Number of H-2K^b:OVA$_{257-264}$ CD8+ T cells in the spleen and peripheral lymph nodes or decidua and CXCR3 gMFI by H-2K^b:OVA$_{257-264}$ CD8+ T cells in the spleen and lymph nodes of FOXP3-WT (black) or FOXP3-PRKO (red) female during allogeneic pregnancies (E11.5) sired by Balb/c-2W1S/OVA (H-2^d) male mice.

(B) Percent fetal wastage, number of live pups *in utero*, and concepti at mid-gestation following once weekly administration of anti-CXCR3 (open) or isotype (filled) antibodies in FOXP3-PRKO females initiated at time of mating with Balb/c-2W1S/OVA (H-2^d) male mice. Data are from at least three independent experiments, each with similar results, with each point representing data from an individual mouse. Bar, mean ± SEM. *p < 0.05, **p < 0.01.

increased systemic and decidual accumulation of CD8+ T cells with fetal-OVA specificity in comparison with healthy pregnancy in FOXP3-WT mice (Figures 3A and S3). Expanded fetal-OVA-specific CD8+ T cells in FOXP3-PRKO mice also showed increased expression of the chemokine receptor, CXCR3 (Figure 3A), required for decidual infiltration (Chaturvedi et al., 2015). In turn, CXCR3 blockade efficiently overturned fetal wastage in FOXP3-PRKO mice (Figure 3B), similar to fetal wastage triggered by prenatal infection or partial transient depletion of maternal FOXP3+ cells (Chaturvedi et al., 2015). Thus, natural progesterone produced during pregnancy stimulates expansion of maternal Treg through PR that protects against fetal wastage and decidual accumulation of CXCR3+ fetal-specific CD8+ T cells.

A variety of synthetic progestins with unique pharmacodynamic and biological properties have been developed with the goal of therapeutically preventing preterm birth and other pregnancy complications (Coomarasamy et al., 2019; Palacio et al., 2016; Saccone et al., 2017; Stephenson et al., 2017). A widely used synthetic compound is the long-lasting progestin, 17α-hydroxyprogesterone caproate (17-OHPC) (Romero and Stanczyk, 2013). Supplementation with 17-OHPC, but not natural progesterone, reduces the incidence of miscarriage in women with high-risk pregnancies (Saccone et al., 2017). However, how 17-OHPC works remains uncertain. Given the importance of PR in maternal Tregs for sustaining fetal tolerance, the potential for reinforced fetal tolerance by exogenous 17-OHPC was evaluated. Significantly increased accumulation of maternal FOXP3+ Tregs and CD4+ T cells with I-A^b:2W1S fetal specificity was found 48 h after a single dose of 17-OHPC at mid-gestation (E11.5) (Figure 4A). Interestingly, although the frequency of I-A^b:2W1S fetal-specific Tregs did not further increase from expanded pregnancy levels, CTLA-4 expression was significantly increased among FOXP3+ Tregs with fetal-2W1S specificity in 17-OHPC-treated mice (Figure 4A). Thus, exogenous 17-OHPC exerts distinct immune-modulatory properties even in the presence of high endogenous natural progesterone levels during pregnancy.

Protective impacts of 17-OHPC were further investigated using an established model of fetal wastage triggered by partial depletion of maternal FOXP3+ Tregs. FOXP3 is encoded on the X chromosome, and random inactivation of this chromosome in female mice heterozygous for co-expression of diphtheria toxin receptor with FOXP3 (FOXP3$^{DTR/WT}$ mice) allows partial transient depletion of maternal FOXP3+ cells that
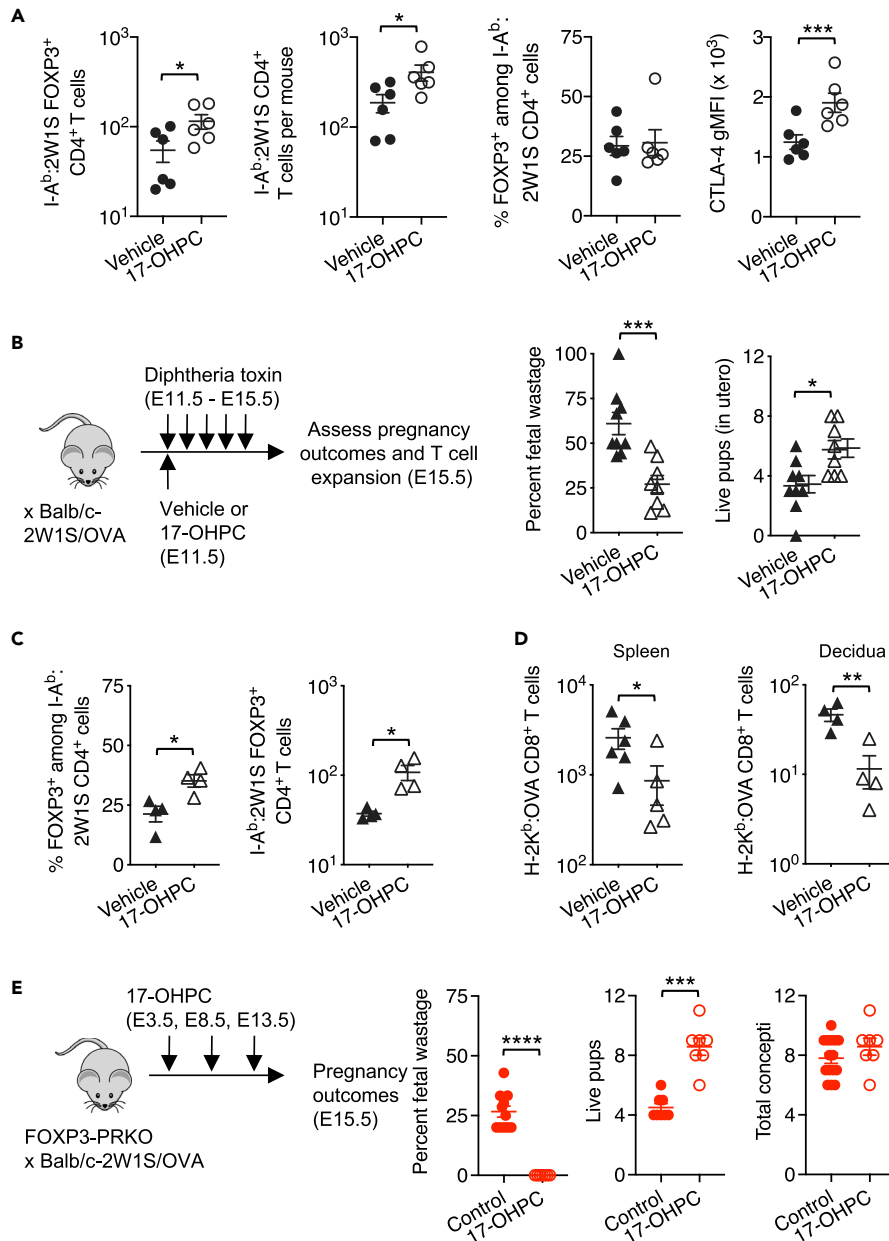
Figure 4. 17-OHPC progesterone protects against fetal wastage

(A) Number of FOXP3+ and CD4+ T cells with I-A^b:2W1S$_{55-64}$ specificity, percent FOXP3+ among CD4+ T cells with I-A^b:2W1S$_{55-64}$ specificity, and CTLA-4 gMFI among 2W1S-specific FOXP3+ cells in the spleen and pooled peripheral lymph nodes 48 h following administration of 17-OHPC (open) or vehicle (filled) in C57BL/6 female mice mid-gestation (E11.5) during allogeneic pregnancy sired by Balb/c-2W1S/OVA (H-2^d) male mice.

(B) Percent fetal wastage, number of live pups *in utero*, and total concepti for 17-OHPC (open black) or vehicle-treated (closed black) Foxp3$^{DTR/WT}$ female mice five days after initiating daily diphtheria toxin (DT) treatment during allogeneic pregnancy sired by Balb/c-2W1S/OVA (H-2^d) male mice.

(C) Percent and number of FOXP3+ Tregs with I-A^b:2W1S$_{55-68}$ specificity one day after initiating DT treatment for groups described in panel b.

(D) Number of H-2K^b:OVA$_{257-264}$-specific CD8+ T cells in the spleen and pooled peripheral lymph nodes or decidua five days after initiation of DT for groups described in panel B.

(E) Percent fetal wastage, number of live pups *in utero*, and total concepti for 17-OHPC-treated FOXP3-PRKO (open) or control FOXP3-PRKO (closed) mice. Data are from at least three independent experiments, each with similar results, with each point representing data from an individual mouse. Bar, mean ± SEM. *p < 0.05, ***p < 0.005, ****p < 0.001.

nadir within the first 24 h after initiating low-dose DT treatment during pregnancy (Rowe et al., 2011, 2012). Remarkably, 17-OHPC reduced fetal wastage and loss of live pups that normally occur with DT administration in FOXP3^DTR/WT pregnant mice (Figure 4B). Protection against fetal wastage paralleled significantly increased percentage and numbers of FOXP3+ Treg cells with I-A^b:2W1S fetal specificity (Figure 4C), with reciprocally reduced systemic and decidual expansion of activated maternal CD8+ T cells with fetal-OVA specificity in 17-OHPC-treated mice (Figure 4D). Thus, exogenous 17-OHPC reinforces fetal tolerance and protects against fetal wastage when maternal Tregs are depleted to prepregnancy levels, to mimic their blunted expansion in human pregnancy complications such as preeclampsia and spontaneous abortion (Jiang et al., 2014; Sasaki et al., 2004, 2007; Somerset et al., 2004).

Along with the canonical nuclear PR, progesterone can stimulate cells through other receptors including membrane bound PRs and glucocorticoid receptor (GR) (Vrachnis et al., 2012), each with considerably higher expression by maternal T cells during pregnancy (Engler et al., 2017; Lissauer et al., 2015). Because 17-OHPC and natural progesterone are molecular distinct with discordant relative affinity to PR and these noncanonical receptors (Attardi et al., 2007; Boucher et al., 2014; Courtin et al., 2012; Romero and Stanczyk, 2013), we evaluated whether exogenous 17-OHPC can bypass the necessity for PR in maternal FOXP3+ Tregs during pregnancy by treating FOXP3-PRKO mice with 17-OHPC. Consistent with this hypothesis, 17-OHPC administered 3 days after mating reversed fetal wastage and loss of live pups in FOXP3-PRKO mice (Figure 4E). Thus, 17-OHPC exerts distinct immune-modulatory properties, even in the presence of endogenous natural progesterone, to reinforce tolerance and protect against fetal wastage.

Taken together, these results indicate that although PR in maternal FOXP3+ is essential for protection against fetal wastage in response to physiologically increased natural progesterone levels during pregnancy, this necessity can be overcome by exogenous progestins. The expanded protective effects of 17-OHPC may reflect stimulation of other cell types through PR and/or noncanonical PR receptors. For example, 17-OHPC, but not micronized progesterone, has been reported to reduce CXCL9 in the placenta and IL-1β in the brain of fetal mice after LPS-induced intrauterine inflammation (Novak et al., 2018), which may explain increased protection by 17-OHPC compared with natural progesterone against miscarriage (Saccone et al., 2017). The importance of nuclear PR expression by CD11c+ antigen-presenting cells for optimal fetal growth (Thiele et al., 2019) suggests these cells are likely candidates to respond to increased progestin levels after 17-OHPC treatment. With regard to noncanonical receptors, GR is a likely candidate given its necessity in maternal T cells for protection against autoimmunity (self-tolerance) during pregnancy (Engler et al., 2017). Thus, although protection against fetal wastage in 17-OHPC-treated mice parallels significantly increased expansion of FOXP3+ Treg cells with I-A^b:2W1S fetal specificity (Figures 4A and 4C), stimulation of other cell types and through noncanonical receptors cannot be excluded and is in fact likely.

Based on this complex division of labor between progesterone responsive cells, and the receptors they utilize for optimal pregnancy outcomes, more comprehensive analyses of how progesterone works temporally throughout pregnancy, and across multiple maternal leukocyte subsets, represent important areas for future investigation. In turn, differences in how natural progesterone and synthetic progestins work may explain the variable success of 17-OHPC for the prevention of preterm birth (Blackwell et al., 2020; Meis et al., 2003). From a broader translational perspective, the immune-modulatory effects of progesterone are likely not restricted only to pregnancy, and further establishing the mechanism for how natural progesterone and synthetic progestins work will likely unveil improved therapeutic approaches for autoimmunity, transplantation, and other physiological contexts where expanded immunological tolerance is desired.

Limitations of the study

Progesterone is known to induce differentiation of CD4+ T cells into FOXP3+ Tregs after stimulation *in vitro*, and PR is essential for CD4+ T cell responsiveness to progesterone stimulation in this context (Lee et al., 2012; Mao et al., 2010). However, maternal T and other leukocyte subsets show only low-level *Pgr* mRNA levels during pregnancy (Engler et al., 2017; Lissauer et al., 2015). One explanation for this discordance is that PR expression in maternal T cells has been evaluated and purified without consideration of fetal-antigen specificity. We found sharply reduced expansion of maternal Tregs when PR is selectively eliminated in maternal FOXP3+ cells using tools that track maternal CD4+ T cells with fetal specificity. Despite the current lack of antibodies required for evaluating PR expression among individual cells by flow cytometry, and the relative scarcity of these cells that precludes their purification by FACS

sorting, selective PR expression by maternal Tregs with fetal specificity is consistent with significantly increased but low-level PR expression among unfractionated maternal splenocytes (Figure S2). More definitive identification of the maternal leukocyte subset(s) that express PR during pregnancy will require the development tools for detecting PR protein, even among potentially rare cells (e.g. maternal T cells with fetal specificity). Another consideration is that although the necessity for PR expression by maternal FOXP3+ cells for averting fetal wastage in response to endogenous natural progesterone physiologically produced during pregnancy is clearly demonstrated (Figures 1, 2 and 3), we also find that exogenous progestins likely work by stimulating unique immune cell types and/or through noncanonical PR receptors. Although this complexity was not evaluated beyond PR expression in maternal FOXP3+ cells, the scientific basis for analysis of bioactive progesterone levels after administration of 17-OHPC compared with natural progesterone during pregnancy and systematic analysis of PR compared with noncanonical receptors among individual maternal leukocyte subsets systemically and at the maternal-fetal interface is nonetheless established.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104400.

AUTHOR CONTRIBUTIONS

A.L.S, J.M.K., L.X., T.T., W.A.G., S.M., and S.S.W. designed the project and experiments; A.L.S, J.M.K., L.X., A.R.B, T-Y.S., and G.P. performed the experiments; A.L.S, J.M.K., T.T., W.A.G., S.M., and S.S.W. organized the data and wrote the paper with editorial input from all the authors.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE/Cy5 anti-mouse/human CD11b Antibody, Clone M1/70	BioLegend	Cat# 101210; RRID:AB_312793
PE/Cy5 anti-mouse/human CD45R/B220 Antibody, Clone RA3-B62	BioLegend	Cat# 103210; RRID:AB_312995
PE/Cy7 anti-mouse CD62L Antibody, Clone MEL-14	BioLegend	Cat# 104418; RRID:AB_313103
APC anti-mouse CD152 (CTLA-4) Antibody, Clone UC10-4B9	BioLegend	Cat# 106310; RRID:AB_2087653
Alexa Fluor 700 anti-mouse/human CD44 Antibody, Clone IM7	BioLegend	Cat# 103026; RRID:AB_493713
PE/Cy7 anti-mouse CD45.1 Antibody, Clone A20	BioLegend	Cat# 110730; RRID:AB_1134168
Brilliant Violet 421 anti-mouse CD45.2 Antibody, Clone 104	BioLegend	Cat# 109832; RRID:AB_2565511
eFluor 450 anti-mouse CD4 Antibody, Clone GK1.5	Invitrogen	Cat# 48-0041-82; RRID:AB_10718983
APC-eFluor 780 anti-mouse CD8alpha Antibody, Clone 53-6.7	Invitrogen	Cat# 47-0081-82; RRID:AB_1272185
PE/Cy5 anti-mouse CD11c Antibody, Clone N418	Invitrogen	Cat# 15-0114-82; RRID:AB_468717
PE/Cy5 anti-mouse F4/80 Antibody, Clone BM8	Invitrogen	Cat# 15-4801-82; RRID:AB_468798
FITC anti-mouse CD4 Antibody, Clone GK1.5	Invitrogen	Cat# 11-0041-82; RRID:AB_464892
PE/Cy7 anti-mouse CD8alpha Antibody, Clone 53-6.7	Invitrogen	Cat# 25-0081-82; RRID:AB_469584
PE/Cy7 anti-mouse CD3epsilon Antibody, Clone 145-2C11	Invitrogen	Cat# 25-0031-82; RRID:AB_469572
FITC anti-mouse FOXP3 Clone FJK-16S	Invitrogen	Cat# 11-5773-82; RRID:AB_465243
eFluor 450 anti-mouse Ki-67 Clone SolA15	Invitrogen	Cat# 48-5698-82; RRID:AB_11149124
APC anti-mouse/human Helios Clone 22F6	BioLegend	Cat# 137222; RRID:AB_10662900
eFluor anti-mouse Neuropilin-1 Clone 3DS304M	Invitrogen	Cat# 48-3041-82; RRID:AB_2574051
PE anti-mouse CD178 (Fas Ligand) Clone MFL3	BioLegend	Cat# 106606; RRID:AB_313279
AF647 anti-mouse Bcl-2 Clone 10C4	BioLegend	Cat# 633510; RRID:AB_2274702
APC anti-CD183 (CXCR3)	Invitrogen	Cat# 17-1831-82; RRID:AB_1210791
InVivoPlus anti-mouse CXCR3 Clone CXCR3-173	BioXCell	Cat# BE0249; RRID:AB_2687730
Chemicals, peptides, and recombinant proteins		
H-2K^b:OVA$_{257-64}$ MHC Class I Monomer	NIH Tetramer Core	N/A
Streptavidin, R-Phycoerythrin Conjugate (SAPE)	Invitrogen	S866
I-A^b:2W1S$_{55-64}$ MHC Class II Tetramer	NIH Tetramer Core	N/A
Mifepristone, RU486	Sigma Aldrich	M8046
17alpha-hydroxyprogesterone caproate	Sigma Aldrich	H5752
LIVE/DEAD Near-IR staining kit	ThermoFisher	L34976
Critical commercial assays		
Fixation/Permeabilization Solution Kit	BD Biosciences	554714
Anti-PE Microbeads	Miltenyi	130-048-801
Experimental models: Organisms/strains		
Balb/c-Act-2W1S/OVA Backcrossed 10 generations to BALB/c background	Marc Jenkins (University of Minnesota)	Published in Moon et al. (2011)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
C57BL/6NCr	Charles River Laboratories (NCI)	#556
Balb/cAnNCr	Charles River Laboratories (NCI)	#555
B6-Ly5.1/Cr	Charles River Laboratories (NCI)	#564
B6.129(Cg)-Foxp3[tm4(YFP/icre)/Ayr]/J	Jackson Laboratory	#016959
PR[flox/flox]	Laboratory of John Lydon and Francisco De-Mayo	Published in Lydon et al. (1995)
Software and algorithms		
FlowJo	BD Biosciences	N/A
GraphPad Prism	GraphPad Software	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources or reagents should be directed to and will be fulfilled by the lead contact, Dr. Sing Sing Way (singsing.way@cchmc.org).

Materials availability

Mouse strains and other reagent describe in this paper are available from the lead contact upon request with a completed Material Transfer Agreement.

Data and code availability

- All data reported will be shared by the lead contact upon request.

- This paper does not report original code. Additional information required to analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

C57BL/6 (B6, H-2[b]; CD45.2), BALB/c (H-2[d]), and B6-Ly5.1/Cr (H-2[b]; CD45.1) mice were purchased from the National Cancer Institute colony at Charles River Laboratories. Balb/c-2W1S/OVA transgenic mice that constitutively express recombinant $2W1S_{55-68}$ and OVA protein behind the β-actin promoter, and Foxp3[DTR/DTR] and FOXP3[DTR/WT] mice where all or half of FOXP3+ cells are susceptible to diphtheria toxin induced ablation have been described (Kim et al., 2007; Rowe et al., 2011, 2012). PR[flox/flox] mice and FOXP3[Cre] mice with *IRES-YFP-Cre* cDNA knocked into the 3′ UTR of the *Foxp3* gene have been described (Fernandez-Valdivia et al., 2010; Rubtsov et al., 2008). FOXP3[CRE] mice were backcrossed >10 times onto the C57BL/6 background prior to intercrossing with PR[flox/flox] mice to generate FOXP3[Cre/Cre] PR[flox/flox] mice (FOXP3-PRKO). For mating, Balb/c-2W1S/OVA male mice backcrossed on the BALB/c background were combined with virgin females for 24 h and visualization of the copulation plug was determined as embryonic day (E) 0.5. Fetal wastage was assessed as *in utero* resorption or fetal death together with placental friability. All mice were housed under specific pathogen-free conditions and used in accordance with the Animal Care and Use Committee of Cincinnati Children's Hospital.

METHOD DETAILS

qPCR analysis

Spleen and uteri were harvested during mid-gestation (E11.5) during allogeneic pregnancy or virgin control mice on the C57BL/6 background. Tissues were cut into several small pieces, immediately submerged in RNAlater solution (Invitrogen), lysed using the RNAaqueous-4PCR kit (Invitrogen), and passed through a homogenizer tube before precipitation. At least 1 μg of isolated RNA was added to each cDNA reaction. cDNA synthesis was performed using both Oligo-d(T)$_{16}$ nucleotide and a PR-specific reverse transcription primers (5′TTGAAATAATGGGTGAAATATA-3′) using the TaqMan Reverse Transcription platform (Applied Biosystems). qPCR reactions were set up using the TaqMan Fast Advanced Master Mix (Applied Biosystems). Four different exon-spanning PR TaqMan probes were used: Mm00435625 (Exon 4–5),

Mm00435626 (Exon 5–6), Mm01176082 (Exon 6–7), and Mm00435628 (Exon 7–8). Samples were run on a 7500 Fast Real-Time PCR system, and normalized to ActB (Mm04394036) within each sample (DCT).

In vivo treatments

For progesterone receptor blockade, pregnant mice were subcutaneously administered 250ug mifepristone [RU486] (Sigma Aldrich, USA) in 100uL sesame oil at E12.5. Treg complementation experiments were performed by adoptively transferring whole splenocytes from FOXP3$^{DTR/DTR}$ donor mice into FOXP3-PRKO recipient mice on the day when pulse mating with allogeneic male mice was initiated. For depletion of PR-sufficient FOXP3$^{DTR/DTR}$ donor Treg cells, FOXP3-PRKO recipient mice were administered DT (0.5 μg) immediately after adoptive cell transfer, and twice weekly (0.5 μg/dose) until pregnancy outcomes evaluated at mid-gestation. For adoptive cell transfer, CD4+ splenocytes from donor PR-sufficient WT CD45.2+ and FOXP3-PRKO CD45.2 + CD45.1 + mice were purified by negative selection (Miltenyi Biotec), mixed at a 1:1 ratio, and intravenously transferred into CD45.1+, CD45.2- recipient mice immediately prior to mating. To neutralize CD8+ T cell decidual infiltration, FOXP3-PRKO females were injected intraperitoneally with 500ug anti-CXCR3 (CXCR3-173, BioXcell) or Hamster IgG once weekly beginning at the time of mating through mid-gestation (E11.5). For exogenous progestin, mice were injected subcutaneously with 2 mg of 17α-hydroxyprogesterone caproate [17-OHPC] (Sigma Aldrich, USA) in 200 μL sesame oil or vehicle control. For partial transient ablation of maternal Treg cells, FOXP3$^{DTR/WT}$ females were administered diphtheria toxin (Sigma Aldrich, USA) [0.5ug first dose, followed by 0.1ug/dose) beginning mid-gestation (E11.5) for five consecutive days with fetal wastage being assessed at E15.5. Where indicated, mice were injected with 17-OHPC (2 mg/200uL) or vehicle 12 h prior to initiation of DT treatment.

Tetramer enrichment and flow cytometry

Single cell suspensions were prepared from the spleen and peripheral (axillary, brachial, cervical, inguinal, mesenteric, pancreatic, para-aortic/uterine) lymph nodes by grinding with glass slides. Decidua tissue was isolated and processed as previously described (Chaturvedi et al., 2015). Specifically, placentas were removed from fetuses and separated at the labyrinth and junctional zone interface. Tissue was placed in RBC lysis buffer and single-cell suspension was generated by grinding between frosted glass slides. Complete DMEM was used to quench the lysis reaction and samples were then filtered through 70 micron cell strainers and pelleted by centrifugation (530 g for 5 min). For tracking, CD4+ I-A^b:2W1S$_{55-68}$ or CD8+ H-2K^b:OVA$_{257-264}$ cells, single cell suspensions were incubated with PE-conjugated or APC-conjugated tetramers and enriched with anti-fluorochrome microbeads (Miltenyi Biotec) as previously described. Bound fractions were eluted and stained with fluorochrome-labeled cell surface antibodies for CD4 (GK1.5), CD8a (53-7.3), CD25 (PC61), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD11b (M1/70), CD11c (N418), B220 (RA3-B62), F4/80 (BM8), CXCR3 (CXCR3-173), CTLA-4 (UC10-4B9). For Treg cell analysis, splenocytes were stained for Ki-67 (SolA15), Helios (22F6), Neuropilin-1 (3DS304M), CD178 (MFL3), Bcl-2 (10C4), and a membrane integrity dye that identified dead cells (Thermo Fisher). For intranuclear staining, cells were fixed and permeabilized (eBioscience) and stained with antibodies against FOXP3 (FJK-16S). Cells were analyzed on a FACSCanto cytometer (BD Biosciences) and analyzed using FlowJo (TreeStar) software.

QUANTIFICATION AND STATISTICAL ANALYSIS

For all experiments, female mice were randomly assigned to experimental groups. Distribution of data points were first evaluated for having a Gaussian normal distribution (Prism, GraphPad). Differences between normally distributed datasets were analyzed using either the Student's paired or unpaired t-test, while non-normally distributed datasets were evaluated using non-parametric Mann-Whitney analysis. A one-way ANOVA was used to analyze mean difference between three or more groups with a single variable and two-way ANOVA was used to analyze multiple groups with more one independent variable (Prism, GraphPad). For each analysis, a p value of <0.05 was taken as statistical significance.