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Research Article

Effects of macrophage depletion on characteristics of cervix remodeling and pregnancy in CD11b-*dtr* mice

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Abstract

To test the hypothesis that macrophages are essential for remodeling the cervix in preparation for birth, pregnant homozygous CD11b-*dtr* mice were injected with diphtheria toxin (DT) on days 14 and 16 postbreeding. On day 15 postbreeding, macrophages (F4/80+) were depleted in cervix and kidney, but not in liver, ovary, or other non-reproductive tissues in DT—compared to saline—treated *dtr* mice or wild-type controls given DT or saline. Within 24 h of DT-treatment, the density of cell nuclei and macrophages declined in cervix stroma in *dtr* mice versus controls, but birefringence of collagen, as an indication of extracellular cross-linked structure, remained unchanged. Only in the cervix of DT-treated *dtr* mice was an apoptotic morphology evident in macrophages. DT-treatment did not alter the sparse presence or morphology of neutrophils. By day 18 postbreeding, macrophages repopulated the cervix in DT-treated *dtr* mice so that the numbers were comparable to that in controls. However, at term, evidence of fetal mortality without cervix ripening occurred in most *dtr* mice given DT—a possible consequence of treatment effects on placental function. These findings suggest that $CD11b^+$ $F4/80^+$ macrophages are important to sustain pregnancy and are required for processes that remodel the cervix in preparation for parturition.

Summary Sentence

Conditional depletion of macrophages in CD11b-*dtr* mice during the critical period for cervix remodeling interfered with ripening and the progress of pregnancy.

Key words: parturition, diphtheria toxin, collagen, monocytes, ripening, preterm birth.

Introduction

More than 10% of all pregnancies worldwide end prematurely (<37 weeks gestation), while at term medical interventions occur in upwards of 30% of deliveries in developed countries [1, 2]. Whether advanced in preterm birth or delayed and possibly incomplete in some women at term, cervix remodeling is essentially a gatekeeper for birth in viviparous species [3–5]. Although availability of biopsies limited studies of the cervix in women to the peripartum period and preterm birth [6], the shift from soft to ripening in rodents is associated with morphological and other biomolecular changes many days before birth. Analogous to an inflammatory process [7–11], ripening is characterized by increased biomechanical compliance [12, 13], degradation of cross-linked extracellular collagen [14–16], reduced

cell nuclei density [5, 17, 18], and increased presence of mature macrophages [17, 19]. Similar to other mammals, this transition occurs while progesterone is at or near peak concentrations in circulation and well before uterine contractile activity increases with labor [4, 20]. Although progesterone promotes the progressive softening and structural changes in the extracellular matrix that occur before ripening [5, 21], loss of progesterone efficacy, so-called progesterone withdrawal [4, 22–24], and evidence for local inflammation appear to be critical for ripening and birth both at term, as well as with preterm birth [17, 19, 25, 26].

The importance of inflammatory processes that control remodeling and degradation of the extracellular collagen matrix led us to consider the conditional depletion of myeloid cells as an approach to understand organ-specific functions of tissue-resident macrophages. Macrophages and their production of proinflammatory factors, as well as neutrophils to a lesser and later extent, but not other lymphocytes, are associated with cervix ripening [4]. Although mice are typically insensitive to diphtheria toxin (DT) [25], in transgenic mice with the human DT receptor linked to the lineage-specific CD11b promoter, a temporary conditional depletion of macrophages occurs systemically and, to an extent, in certain organs in response to injection of a small amounts of DT [27–29]. Specifically, DT-treatment of non-pregnant or male *dtr* mice, selectively and acutely, induces apoptosis in some myeloid cells and macrophages in particular that express CD11b receptors in the kidney, peritoneal cavity, and skin while an abundance of macrophages persist in liver and spleen [30–33]. In these studies, this model helped identify a role for macrophages, whether tissue resident or from circulation, in the genesis and resolution of inflammation-induced disease in the lung, kidney, and liver of non-pregnant mice. For the ovary, DT-treatment of non-pregnant *dtr* mice established an essential role for macrophages to maintain ovarian vascularity and corpus luteum function [30, 33]. Use of this model for studying inflammatory processes that are associated with cervix remodeling as pregnancy nears term and parturition is lacking. Given the heterogeneity of functions and phenotype of macrophages in various anatomical locations and physiological states, the main objective of this study was to test the hypothesis that macrophages are essential to ripen the cervix in preparation for birth. Findings indicate that fewer and impaired macrophages in the cervix of DT-treated pregnant CD11b-*dtr* mice may forestall characteristics of ripening that occur before birth in controls. In addition, adverse consequences of DT-treatment on fetal viability have broader implications for the importance of macrophages to sustain pregnancy.

Materials and methods

Experimental design

Transgenic homozygous male and female CD11b-*dtr* (*dtr*) mice and wild-type (WT) controls of the FVB strain were obtained from a breeding colony at the University of Edinburgh. Origin of this murine model for macrophage depletion has been previously described [30–32, 34]. In previous reports, no signs of diminished well-being were found following DT injections in *dtr* mice or WT controls. Other approaches may differentially deplete macrophages from some tissues or circulation, but lack the specificity to eliminate a particular subtype or to affect the physiological functions related to the F4/80 phenotype [29, 35]. All mice were bred and maintained in the vivarium with free access to food and water in 12 h of light per day (lights on at 7am). Experiments were in compliance with UK

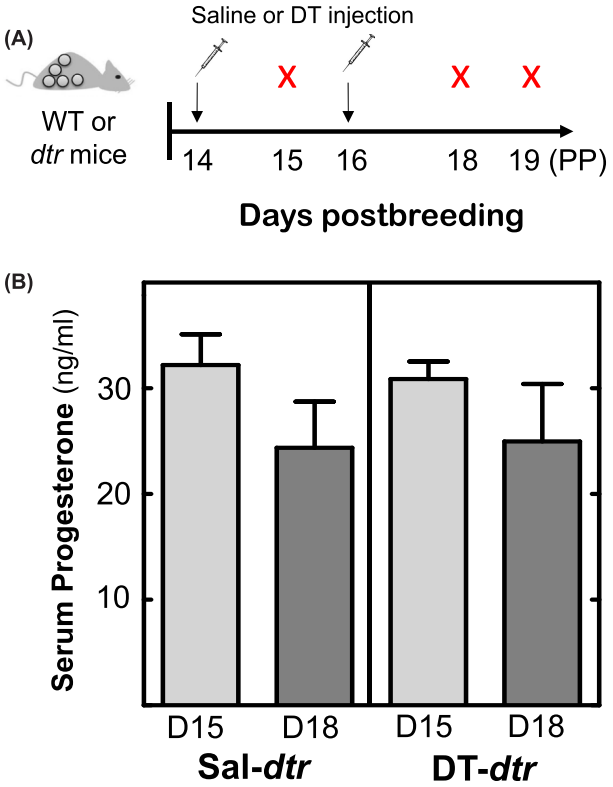

Figure 1. **A** Timeline of injection of saline (Sal) or diphtheria toxin (DT) administered intraperitoneal on days 14 and 16 postbreeding into homozygous *dtr* mice. X indicated day of euthanasia when blood and tissues were collected for study (n = 4–10 mice/group). **B.** Serum progesterone concentrations on days 15 and 18 postbreeding in Saline- or DT-treated *dtr* mice. Treatments described in Methods. $P > 0.05$ two-way ANOVA (n = 4–5 mice/group).

Home Office guidelines under approved Project licenses and Veterinarian supervision.

For this study, the approach was to treat pregnant *dtr* transgenic mice with DT to conditionally deplete macrophages during the critical period for ripening of the cervix. The focus on resident macrophages in the cervix stems is in part from previous studies in multiple strains of pregnant mice [4] and a 2012 flow cytometry study [36]. Residency in various tissues by other myeloid cells that express CD11b receptors does not change in response to DT in previous studies [29, 30]. Moreover, lymphocytes and NK cells are scarce or absent in the cervix stroma of mice [37]. Saline-treated *dtr* mice served as controls for DT-treatment. As an additional control, FVB mice, the background strain for this *dtr* transgenic model, were similarly treated with saline or DT during pregnancy. Accordingly, groups of *dtr* or WT mice were injected on days 14 and 16 postbreeding with saline vehicle or DT (20 ng/g body weight in 0.1 ml vehicle i.p.; D0564; Sigma Aldrich). Given evidence for repopulation of tissue resident macrophages following depletion [29, 30], this treatment regimen, based upon a previous protocol [34], was intended to extend macrophage depletion beyond the acute response, assessed on day 15 postbreeding, through the prepartum period when the cervix transitions from soft to ripening [4]. Mice were euthanized on days 15, 18, and 19 postbreeding (Figure 1A Treatment schema) to assess the acute and prolonged responses to DT injections, i.e., D15 group (after treatment on day 14 postbreeding) and D18 or D19 groups (after treatment on days 14 and 16 postbreeding), respectively. Immediately postmortem, an intra-cardiac blood sample

was collected for serum progesterone assay (DEV9988 ELISA kit, Dimeditec Diagnostics). Assay sensitivity was 0.12 ng/ml with inter- and intra-assay variability of <12%. The cervix, including a portion of attached vagina, uterus, and ovaries, as well as liver, kidney, placenta, spleen, and thymus were harvested, fixed in fresh 4% paraformaldehyde, and transferred within 24 h to 70% ethanol.

Tissue processing and analyses

All tissues were paraffin-embedded, sectioned (6 μm), and stained by immunohistochemistry to identify F4/80-stained macrophages (1:800 dilution, T-2006; Bachem) or neutrophils (7/4-neutrophil, 1:50, MCA771GA, Bio-Rad) and counterstained with methyl green to visualize cell nuclei as previously described [17, 19]. Sections were imaged with an Aperio ScanScope microscope (Leica Biosystems) and 8–16 photomicrographs (300 × 417μm) taken from each of two longitudinal sections of cervix/mouse to survey an area from the ectocervix to striated transition zone before appearance of uterine glands and smooth muscle. Cell nuclei and macrophages in stroma were counted using NIH Image J. Blood vessel lumen, epithelia, and other atypical structures were excluded from counted areas. As in previous studies, macrophages were defined as brown stain within the confines of a delineated cell membrane in close proximity to a methyl-green-stained cell nucleus. In addition to further understanding the effects of DT on macrophages in the cervix of day 15 *dtr* mice compared to saline-treated or WT controls, cell morphology was assessed for distinctive characteristics of apoptosis [38]. An impaired macrophage was defined as monomorphic cell body with reduced pseudopodia, indistinct methyl-green-counterstained nucleus boundary, or evidence of nuclear condensations (blebbing) as previously described [39].

Other sections were stained with picrosirius red to identify collagen in cross-linked structures [4]. Assessment of optical density (OD) of circular polarized light birefringence from picrosirius red stained sections has proven useful as a measure that is inversely proportional to fibrillary collagen in cross-linked structure in tissues including cervix [4, 40, 41]. Collagen and number of macrophages/area were normalized to cell nuclei density for each animal to account for variability in cellular hypertrophy within sections, as well as among sections and individuals due to heterogeneity of cervix anatomy with respect to progression of remodeling with pregnancy and treatment. Levene's test was used to determine whether data were normally distributed (Levene's test $P > 0.05$). Differences were evaluated by Student *t*-test or one-way ANOVA followed by LSD or Tukey's post-hoc test for individual comparisons (SPSS Statistics Software, IBM). $P < 0.05$ was considered significant.

Results

Effects of DT on serum progesterone, pregnancy, and parturition

Serum progesterone concentrations were not significantly different in CD11b-*dtr* mice whether treated with saline or DT (Figure 1B). Compared to saline controls, progesterone in circulation of CD11b-*dtr* mice on day 15 postbreeding was not affected by DT-treatment given 24 h earlier on day 14 of pregnancy. Serum progesterone concentrations were also equivalent in CD11b-*dtr* mice given saline or DT on the morning of day 18 postbreeding, i.e., 96 h and 48 h after the first and second treatment with saline or DT. Thus, DT had no acute or more long-term effect on systemic concentrations of progesterone as compared to saline-treated controls.

In pregnant *dtr* mice on day 15 postbreeding (D15), 24 h after saline injection, the reproductive tract appeared indistinguishable from that in WT controls. The uterus was vascularized with multiple distinct fluid-filled sacs, each containing a fetus that appeared viable (Figure 2A top panel). By comparison in 3 of 11 DT-treated *dtr* mice, the uterus contained fewer segments. For example, 24 h after DT injection, the 2 segments in each uterine horn of this *dtr* mouse each containing two fetal compartments (see arrows in Figure 2B bottom panel) separated by a vascular-dense zone (presumably fetal membranes from postmortem observation). Based upon shape and firmness to touch, the uterus seemed contracted. The reproductive tract and uterine contents in the other eight of DT-treated *dtr* mice were similar to that in saline controls. For all *dtr* mice, irrespective saline or DT-treatment, the cervix appeared unripe as a dense firm fibrous structure and preterm birth did not occur.

With the progress of pregnancy, the reproductive tract in saline-treated *dtr* mice on day 18 postbreeding (D18) was unremarkable for this gestational age (Figure 2B top panel). By contrast, five of seven *dtr* mice injected with DT on days 14 and 16 postbreeding, i.e., 4 and 2 days earlier, had compacted uterus that contained, at each implantation site, a gelatinous encapsulated dark haemorrhagic mass that was likely, as previously described, the resorbing remnants of fetal tissues [42]. These observations suggested fetal mortality had occurred without preterm birth. The gross uterine morphology in the remaining 2 DT-treated *dtr* mice was indistinguishable from saline controls. On the morning of day 19 postbreeding, all 5 saline-treated *dtr* mice had given birth to viable pups (each showed movement and contained milk in stomach) while 7 of 10 DT-treated mice had not delivered by that evening when the study was concluded as per protocol. The uterus in each of these seven DT-treated mice was compact and contained resorbing tissue (presumably fetuses). Of the 3 DT-treated *dtr* mice that delivered on day 19 postbreeding, 2 litters had one stillborn pup each, while in the third litter, 2 of 10 pups were stillborn and 8 had been cannibalized based upon number of implantation sites in this dam's postpartum uterus. Based upon evidence of resorption and dark color of encapsulate at intrauterine implantation sites, less than 30% of DT-treated *dtr* dams sustained pregnancy past day 18 postbreeding (Figure 3C).

Short-term effects of DT on cervix morphology

In WT mice, 24 h after treatment with saline or DT on day 14 postbreeding, there were no differences on the distribution, morphology, or density of cell nuclei or macrophages in the cervix stroma (Figure 3A top panels and insets). Similarly, for *dtr* mice, saline treatment did not appear to alter the distribution, morphology, or density of cell nuclei of macrophages compared to WT controls. However, a difference in density and morphology of macrophages in *dtr* versus WT was apparent (Figure 3A bottom panels and insets). Strain differences are consistent findings in previous studies of WT background controls and genetically altered mutant mice [16, 43]. For *dtr* mice after DT injection, macrophages were smaller, most without elongated pseudopodia, and sparsely distributed compared to the same field of view in D15 saline-treated *dtr* mice. Analysis of macrophage morphology indicated most had presented apoptotic characteristics of compacted cell body, rounded shape, nucleus condensation, and indistinct nucleus boundary compared to saline or WT controls

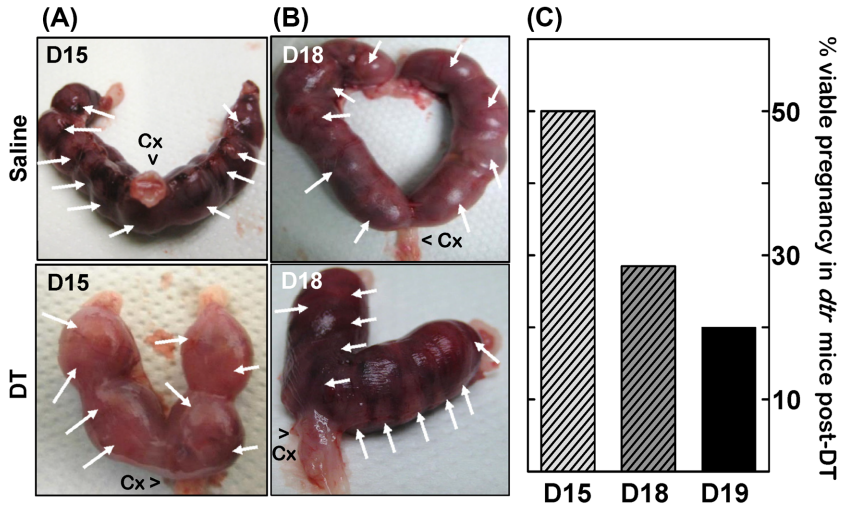

Figure 2. **A** Representative photographs of the reproductive tract from *dtr* mice on day 15 (D15) postbreeding that had been injected 24 h earlier with Saline (7 of 7) or DT (3 of 11). Distinct uterine compartments, each with a single fetal sac, are indicated by white arrows in saline-treated mice compared with compact compartments demarcated by a sinuous vascularized boundary in DT-treated *dtr* mice. **B** Photographs of *dtr* mice on day 18 postbreeding treated with saline (5 of 5; 1 day before expected delivery) or DT (5 of 7) given 4 and 2 days earlier on days 14 and 16 postbreeding. Note the 9 fetal compartments in the saline-injected mouse compared to the estimated 11 compact segments with resorbing fetuses in the DT-treated *dtr* mouse. **C** Histogram of the percentage of viable pregnancy in DT-treated *dtr* mice based upon morphology and firmness of uterus, as well as assessment of fetal viability with respect to presence of dark deoxygenated blood, compactness, resorption, and diminished segment size. On day 19 postbreeding, all saline *dtr* controls gave birth in the morning (<9a), while 7 of 10 DT-treated CD11b-*dtr* mice had not delivered by 4 pm in the afternoon when the study concluded.

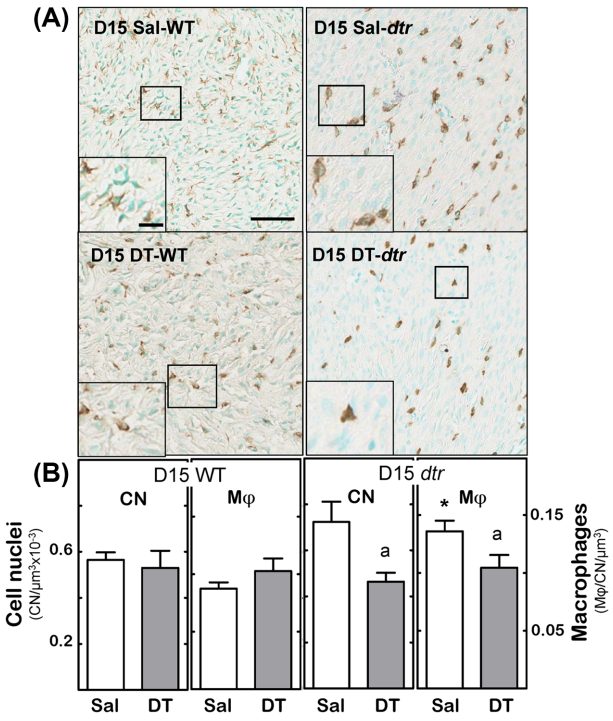

Figure 3. **A** Photomicrographs of cervix sections on day 15 (D15) from wild-type (WT) or *dtr* mice that were stained for F4/80 macrophages (Mφ) and counterstained with methyl green to identify cell nuclei (CN) as described in Methods. Scale bar = 50 μm or 6.5 μm (inset). **B** Histograms of the density of cell nuclei/volume and macrophages/cell nuclei/volume of WT (left) or *dtr* mice (right) injected 24 h earlier with saline (Sal) or DT.$^{*}P < 0.05$ D15 WT vs *dtr* mice Mφ/CN, $^{a}P < 0.05$ vs day 15 saline *dtr* mice (Student *t*-test, n = 4–9 mice/group).

(Supplement Figure 1). As in previous studies, macrophages per field were normalized to cell nuclei density to account for heterogeneity of tissue morphology within cervix sections and among mice. Treatment with DT had no effect on the density of cell nuclei or macrophages in the cervix from WT mice compared to that in saline-treated controls (Figure 3B). By contrast, in DT-treated *dtr* mice, the density of cell nuclei and macrophages were reduced compared to that in saline *dtr* controls.

Among all groups, neutrophils were sparse and diffusely distributed throughout the cervix in WT and *dtr* mice 24 h after saline or DT-treatment. There were no differences in appearance of neutrophils in the cervix with respect to distribution, cellular morphology, evidence of apoptosis, or stain/cell with respect to treatment (data not shown).

Longitudinal sections from the external to internal os, allowed assessment of cell nuclei and macrophage densities in cervix subregions that were categorized as ectocervix (vaginal tissue present), endocervix, and transition zone before appearance of smooth muscle bundles or endometrial glands of uterus. There were no statistical differences in densities of cell nuclei or macrophages (normalized to cell nuclei) between the different subregions with respect to saline or DT-treatment. However, across all three subregions, the density of macrophages was reduced in DT- versus saline-treated *dtr* mice (Supplement Figure 2).

For cross-linked collagen fibers in the extracellular stroma, DT injection had no effect on picrosirius red stain birefringence (Figure 4). Optical density was not different 24 h after DT or saline treatment in WT controls or *dtr* mice. This was not unexpected given the latency between treatment and assessment. Thus, the apparent effects of DT on macrophage morphology and reduction in macrophages/area of cervix stroma were not associated with a change in cross-linked collagen in the extracellular matrix.

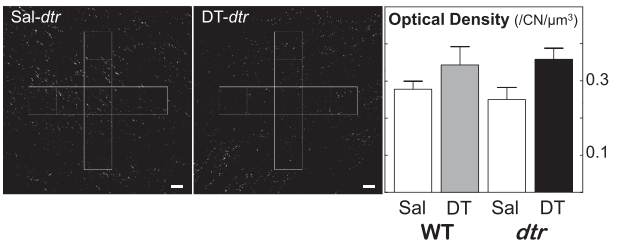

Figure 4. Photograph of a picrosirius red-stained section of cervix from a *dtr* mice on day 15 postbreeding, 24 h after injection of Saline (Sal) or DT. The 9 non-overlapping boxes represent the area analyzed for optical density (9 photomicrographs in each of 3 sections/cervix). Scale bar = 50 μm. The histogram is the optical density assessment of polarized light birefringence (OD/CN), an indication of collagen content and structure degradation. Details provided in Methods and previous studies [19, 41] $P > 0.05$ for all comparisons (two-way ANOVA, n = 4–9 mice/group).

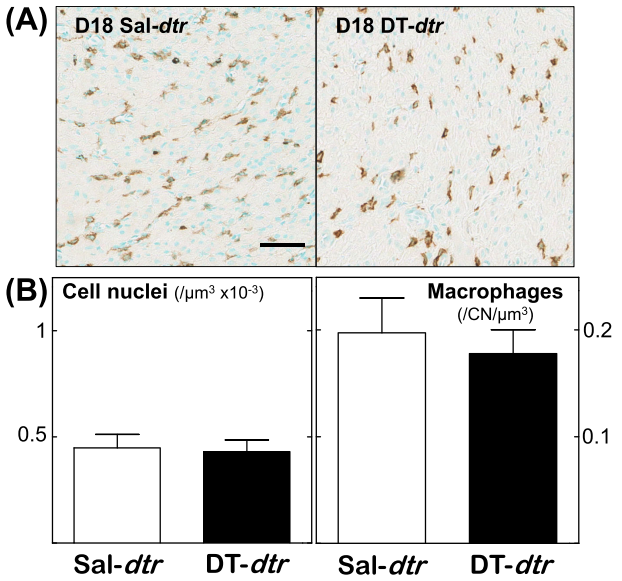

Figure 5. **A** Photomicrographs of cervix sections stained for cell nuclei and macrophages from a day 18 (D18) postbreeding saline (Sal)- or DT-treated *dtr* mouse. Scale bar = 50 μm. **B** Histograms of the density of cell nuclei or macrophages/cell nuclei/area in the cervix stroma of *dtr* mice on day 18 postbreeding that had been injected 4 and 2 days earlier with saline or DT (n = 5–7 mice/group). Note scale change for macrophages/CN compared to Figure 4, an indication of increased abundance as pregnancy neared term. $P > 0.05$ vs Sal group (Student *t*-test).

Long-term effects of DT-treatments on cervix morphology

On day 18 postbreeding, no apparent effects of saline or DT-treatment on days 14 and 16 were evident in WT mice for cellular morphology, distribution, or staining of cells in the cervix stroma (data not shown). Similar variations in these morphological characteristics appeared to be within the typical range in saline-treated controls and DT-treated *dtr* mice (Figure 5A). Specifically, the densities of cell nuclei and macrophages/cell nuclei in the prepartum cervix at term were not different with respect to treatment (Figure 5B). For collagen as well, OD of picrosirius red-stained collagen was not different in cervix sections from groups of day 18 mice irrespective of treatment (data not shown; $P > 0.05$ Student *t*-test). For groups of mice on day 19 postbreeding, evaluation of photomicrographs indicated no difference in cell nuclei, macrophage, or optical densities with respect to treatment even though 7 of 10 DT-treated DT mice had not delivered.

In other tissues, treatment with DT had varied effects on the presence of F4/80-stained macrophages in *dtr* mice. Consistent with previous reports in non-pregnant or male mice, fewer macrophages were found in the kidney within 24 h of DT injection in *dtr* mice compared to saline controls on day 15 postbreeding (Figure 6). For liver, macrophages were evenly dispersed and neither depleted nor morphology appeared to be affected by DT-treatment. In the ovary, macrophages were predominantly located in interstitial tissue between corpora luteum. Although distribution of macrophages varied within each ovary and among *dtr* mice in each group, neither the abundance nor morphology of cell nuclei or macrophages appeared to be affected by DT-treatment compared to that in ovaries from saline controls. In thymus, macrophages were sparsely distributed in sections from *dtr* mice on day 15 postbreeding, predominantly in the capsule and cortex regions of the tissue. The distribution and residency by macrophages appeared similar regardless of saline or DT-treatment in *dtr* mice. By day 19, macrophages seemed more abundant in these regions as well as in more peripheral and medullary areas (data not shown).

In the placenta of *dtr* mice on day 15 postbreeding, macrophages were widely distributed across subregions. The greater prevalence of macrophages in the labyrinth and chorionic plate did not appear to be affected by treatment with DT compared to that of saline *dtr* mice (Figure 7). Other effects of DT-treatment were apparent in the subset of *dtr* mice with evidence of fetal demise, i.e., dark deoxygenated blood, or compacted uterus. In these DT-treated *dtr* mice, the decidua was condensed with greater vascularity, and nearby, an increased presence of deoxygenated dark red blood cells. The sparse presence of resident macrophages limited an accurate census/region, though stain per cell appeared reduced compared to saline controls or DT-treated *dtr* mice in which pregnancy was sustained (Figure 7 right panels). Neutrophils were also sparsely distributed throughout the placenta and morphologically similar in shape, size, and staining of the nucleus irrespective of treatment (data not shown). No morphological characteristics of apoptosis were observed in macrophages or neutrophils in placenta across treatment groups or with respect to fetal morbidity after DT-treatment in *dtr* mice. Thus, DT-treated *dtr* mice with evidence of pregnancy loss may be associated with a change in placental morphology that may reflect impaired function.

Discussion

The hypothesis that macrophages promote remodeling of the cervix was tested by conditional depletion of resident CD11b macrophages in *dtr* mice with the human DT receptor linked to CD11b cells. These findings are the first to establish that treatment with DT depleted F4/80-stained-differentiated macrophages in the cervix of pregnant *dtr* mice. By comparison, DT had no effects on the census of macrophages in saline-treated *dtr* mice or in WT mice that lack the DT receptor. The impact of DT to reduce the density of macrophages in cervix stroma within 24 h of DT-treatment in pregnant *dtr* mice was also found to induce characteristic apoptotic morphology in most remaining F4/80-stained cells. However, no such effects of DT were evident in controls or in neutrophils in any group. This finding contrasts with results in multiple strains of mice in which cell density is temporally associated with reduced cross-linked collagen in the cervix stroma between days 15 and 17 of pregnancy [4]. This period when the cervix transitions from soft to ripening coincides with an increase in density of macrophages that is proposed to

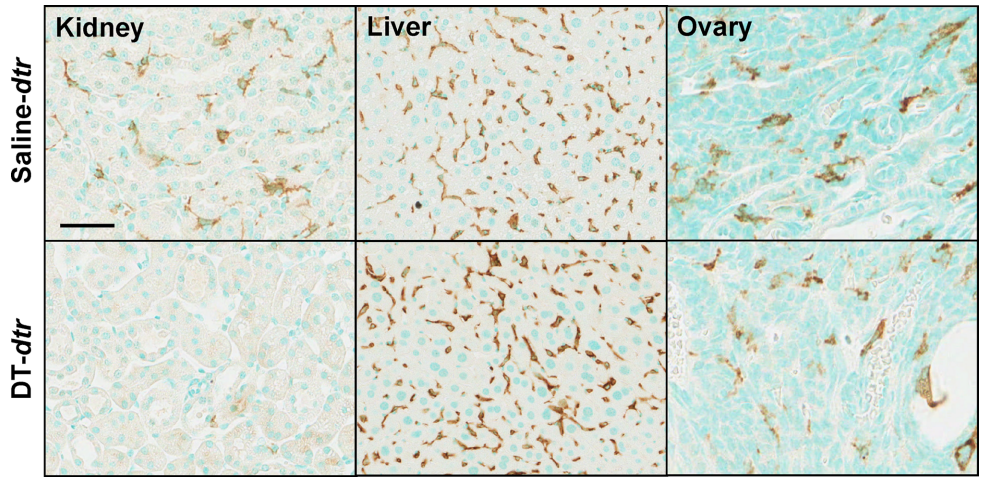

Figure 6. Photomicrographs of other tissues from saline- or DT-treated *dtr* mice on day 15 postbreeding stained for F4/80 macrophages and cell nuclei. Scale bar is 50 μm. Note the diminished density of macrophages in kidney, but not liver or ovary.

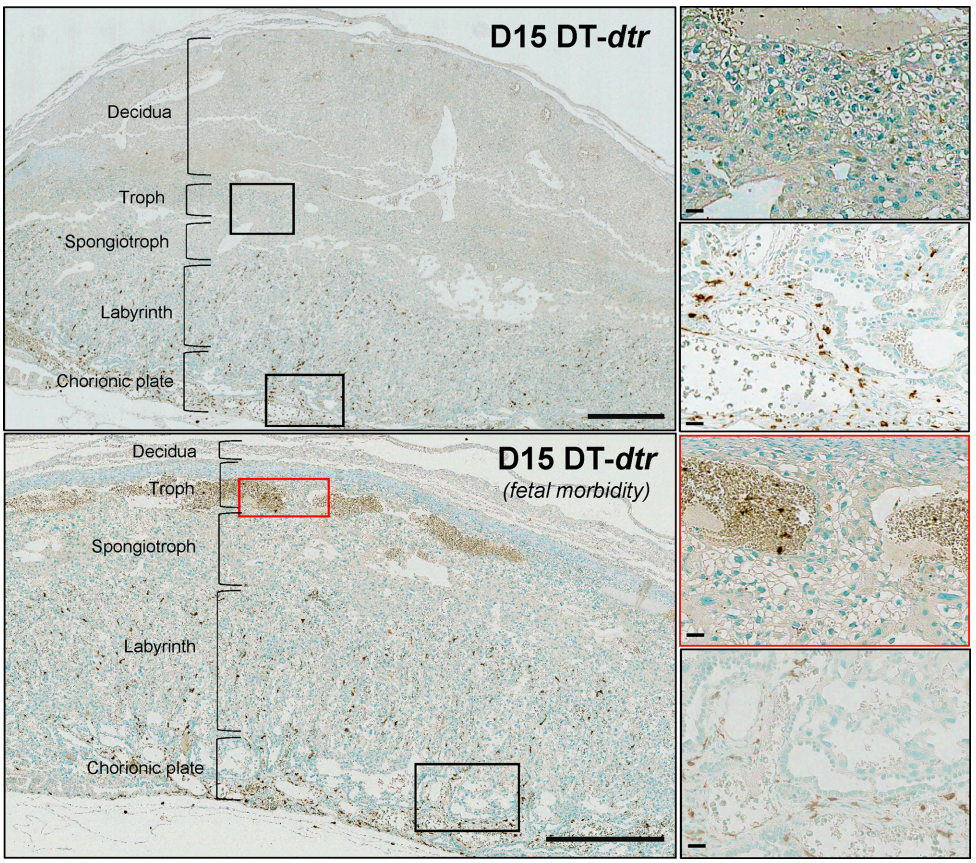

Figure 7. Photomicrographs of placenta sections from CD11b-*dtr* mice on D 15 postbreeding treated 24 h earlier with DT without or with evidence of fetal morbidity (described in Figure 2 legend). Sub-regions are demarcated by brackets, i.e., Troph = Trophoblast layer, Spongiotroph = Spongiotrophoblast layer. Boxes are magnified at right. Scale bar is 500 μm or 25 μm in right 4 panels.

be driven by local factors that promote phenotypic activities and extracellular collagen degradation. Thus, results in the present study suggest a deficit in macrophages throughout various subregions and impaired activities within 24 h of DT-treatment may eliminate an essential drive for collagen degradation and prepartum cervix remodeling.

In a broader context, other consequences of macrophage depletion on pregnancy in this model complicate interpretation of findings. Evidence for repopulation of macrophages in the cervix of *dtr* mice, 2 days after the second DT injection on day 16 postbreeding, may interfere with the ripening process and account for delayed parturition beyond that in controls in 70% of DT treated mice.

Specifically, the phenotype of repopulated macrophage may not be the same as that in residence of the cervix during normal term. In the present study, the cervix presented a firm unripe appearance similar to that in controls on days 15 and 18 postbreeding that gave birth at term. Moreover, premature cervix ripening clearly did not occur even though cell nuclei density declined. This finding raises the possibility that inflammation resulting from macrophage depletion and presumed impaired function of monomorphic stained cells may not be the same as inflammatory processes during the shift from soft to ripening before term in the cervix. Further investigation is needed to determine if during this prepartum transition is associated with alterations in macrophage morphology that characterize phenotypic inflammatory (M1) or wound-healing (M2) activities [44, 45].

Another consideration is the unanticipated effects of macrophage depletion that was associated with fetal morbidity and loss of pregnancy without preterm birth. The consequences of this pregnancy loss on resident immune cells and cervix structure are not known. In 54% (15/28) of DT-treated *dtr* mice, evidence for preterm labor was suggested by the observation of compact fetal sacks and shortened uterine horns. Although reduced cell nuclei density is found with inflammation induced by infection in gut smooth muscle [46], whether products of macrophage apoptosis induce a similar inflammatory reaction by repopulating recruited macrophages is not known. Moreover, the apparent reduced thickness of decidua and increased vascularity in the trophoblast layers of placenta in fetal morbidity and pregnancy loss in *dtr* mice after the initial DT-treatment provides anecdotal evidence that placental function may be compromised. CD11b monocytes have been proposed as communicators of sprouting vessels in decidua, and depletion of this cell may have had unintended consequences [47]. Further analyses of macrophages and placenta from DT-treated *dtr* mice would be worthwhile to understand the relationship of immune cell trophoblast interactions to maintain fetal well-being and sustain pregnancy.

Other contributions of this study include the recognition that the cervix is a separate and distinct component of the reproductive tract during pregnancy. Continued use of the term uterine cervix is difficult to justify because the cervix is highly innervated compared to the uterus [48, 49]. In addition, despite a heterogeneity in structure, the present study suggests more prepartum uniformity in morphological remodeling from the ectocervix (interface with the external vaginal biome), to endocervix (internal conduit to the maternal womb), and isthmus (transitional region into the lower uterus). Subregional differences in collagen organization and smooth muscle content in the cervix were well-recognized [50] and are consistent with findings that cross-linked collagen is decreased before labor and birth in mice [4, 13, 41]. This period when the cervix transitions from soft to ripening coincides with reduced cell density and increase macrophage density, evidence of an inflammatory process that is proposed to reflect a uniformity of prepartum remodeling that precedes dilation, effacement, and a transformation into the lower uterine segment, a term that lacks any reported structural identity across species. These latter changes in peripartum cervix morphology have been associated with an increased presence of neutrophils [17, 51], but little or no change in residency in the present study or previous studies do not suggest a role for this immune cell in the transition to ripening [19]. The possibility that immune cells other than macrophages may contribute to preterm or postterm cervix remodeling in pathophysiological conditions remains to be a focus of study.

In summary, this study focused on the importance of resident macrophages in the overarching concept that inflammation drives the shift from a soft to ripening cervix while progesterone in circulation is at or near peak concentrations of pregnancy. This period in rodents and, in all likelihood, other mammalian species including human occurs at an earlier time than previously appreciated and extends from Csapo's progesterone block hypothesis that progesterone becomes unable to sustain an unripe cervix [23]. The present findings advance the importance of the presence and function of sufficient numbers of F4/80 macrophages for the ripening process given that their depletion/impairment after DT-treatment was not associated with ripening or birth acutely or in most *dtr* mice at term. The implication is that macrophages may be effector cells in the ripening process because of known capabilities to produce molecules including prostaglandins, vasodilators, inflammatory cytokines, and collagen degrading enzymes. These actions may be guided, in part, by the convergence of local factors that regulate differentiated-macrophage phenotypes, and perhaps more importantly, by the stromal cells that integrate various inputs to diminish PR-mediated effects to sustain an unripe cervix [52]. The contribution of other hormones to sustain pregnancy, as well as fetal development, parturition, and newborn well-being are also important considerations for further study. Thus, focus on signals that drive macrophage-mediated inflammation and regulate PR activity of stromal cells hold promise as sentinels or points for interventions that may promote barrier functions of an unripe cervix and prolongation of a pregnancy at risk for preterm birth.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplement Figure 1. Histograms of percentage (%) of macrophages with evidence of apoptosis. Macrophage morphology was evaluated in photomicrographs of cervix from the 4 groups in Figure 3, i.e., D15 saline- or DT-treated WT or CD11b *dtr* mice (2 sections each, n = 3–5/group). Macrophages lacking pseudopodia, with nucleus condensate, and indistinct nucleus boundary were scored as impaired compared to polymorphic-shaped cells with well-delineated nucleus. $^{*}P < 0.05$ one-way ANOVA.

Supplement Figure 2. Histograms of macrophages/cell nuclei/area in the stroma ectocervix (Ecto), endocervix (Endo), or transition zone(TZ) before presence of uterine smooth muscle or glands of wild-type (WT) and *dtr* mice on day 15 postbreeding that had been injected 24 h earlier with saline (Sal) or DT (n = 3–5/group, $^{*}P < 0.05$ DT vs Sal). Cell nuclei densities for subregions of cervix in these day 15 groups are in the same range as that for Figure 3. $P > 0.05$ DT vs Saline group within each strain (Student *t*-test).

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References

1. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, Kinney M, Lawn J, Born Too Soon Preterm Birth Action G. Born too soon: the global epidemiology of 15 million preterm births. *Reprod Health* 2013; **10**:S2.
2. Martin JA, Hamilton BE, Osterman MJ. Births in the United States, 2014. *NCHS Data Brief* 2015:1–8.
3. Word RA, Li XH, Hnat M, Carrick K. Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. *Semin Reprod Med* 2007; **25**:69–79.
4. Yellon SM. Contributions to the dynamics of cervix remodeling prior to term and preterm birth. *Biol Reprod* 2017; **96**:13–23.
5. Yellon SM, Burns AE, See JL, Lechuga TJ, Kirby MA. Progesterone withdrawal promotes remodeling processes in the nonpregnant mouse cervix. *Biol Reprod* 2009; **81**:1–6.
6. Dubicke A, Ekman-Ordeberg G, Mazurek P, Miller L, Yellon SM. Density of stromal cells and macrophages associated with collagen remodeling in the human cervix in preterm and term birth. *Reprod Sci* 2016; **23**:595–603.
7. Dobyns AE, Goyal R, Carpenter LG, Freeman TC, Longo LD, Yellon SM. Macrophage gene expression associated with remodeling of the prepartum rat cervix: microarray and pathway analyses. *PLoS One* 2015; **10**:e0119782.
8. Mackler AM, Iezza G, Akin MR, McMillan P, Yellon SM. Macrophage trafficking in the uterus and cervix precedes parturition in the mouse. *Biol Reprod* 1999; **61**:879–883.
9. Stjernholm-Vladic Y, Stygar D, Mansson C, Masironi B, Akerberg S, Wang H, Ekman-Ordeberg G, Sahlin L. Factors involved in the inflammatory events of cervical ripening in humans. *Reprod Biol Endocrinol* 2004; **2**:74.
10. Sennstrom MB, Ekman G, Westergren-Thorsson G, Malmstrom A, Bystrom B, Endresen U, Mlambo N, Norman M, Stabi B, Brauner A. Human cervical ripening, an inflammatory process mediated by cytokines. *Mol Hum Reprod* 2000; **6**:375–381.
11. Menon R, Mesiano S, Taylor RN. Programmed fetal membrane senescence and exosome-mediated signaling: a mechanism associated with timing of human parturition. *Front Endocrinol* 2017; **8**:196.
12. Myers KM, Feltovich H, Mazza E, Vink J, Bajka M, Wapner RJ, Hall TJ, House M. The mechanical role of the cervix in pregnancy. *J Biomech* 2015; **48**:1511–1523.
13. Yoshida K, Jiang H, Kim M, Vink J, Cremers S, Paik D, Wapner R, Mahendroo M, Myers K. Quantitative evaluation of collagen crosslinks and corresponding tensile mechanical properties in mouse cervical tissue during normal pregnancy. *PLoS One* 2014; **9**(11):e112391.
14. Myers K, Socrate S, Tzeranis D, House M. Changes in the biochemical constituents and morphologic appearance of the human cervical stroma during pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2009; **14**: S82–S89.
15. Yoshida K, Mahendroo M, Vink J, Wapner R, Myers K. Material properties of mouse cervical tissue in normal gestation. *Acta Biomater* 2016; **36**:195–209.
16. Yellon SM, Oshiro BT, Chhaya TY, Lechuga TJ, Dias RM, Burns AE, Force L, Apostolakis EM. Remodeling of the cervix and parturition in mice lacking the progesterone receptor B isoform. *Biol Reprod* 2011; **85**:498–502.
17. Yellon SM, Dobyns AE, Beck HL, Kurtzman JT, Garfield RE, Kirby MA. Loss of progesterone receptor-mediated actions induce preterm cellular and structural remodeling of the cervix and premature birth. *PLoS One* 2013; **8**:e81340.
18. Myers DA. The recruitment and activation of leukocytes into the immune cervix: further support that cervical remodeling involves an immune and inflammatory mechanism. *Biol Reprod* 2012; **87**:107.
19. Kirby MA, Heuerman AC, Custer M, Dobyns AE, Strilaeff R, Stutz KN, Cooperrider J, Elsissy JG, Yellon SM. Progesterone receptor-mediated actions regulate remodeling of the cervix in preparation for preterm parturition. *Reprod Sci* 2016; **23**:1473–1483.
20. Mitchell BF, Taggart MJ. Are animal models relevant to key aspects of human parturition? *Am J Physiol Regul Integr Comp Physiol* 2009; **297**:R525–R545.
21. Larsen B, Hwang J. Progesterone interactions with the cervix: translational implications for term and preterm birth. *Infect Dis Obstet Gynecol* 2011; **2011**:1–13.
22. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. *J Clin Endocrinol Metab* 2002; **87**:2924–2930.
23. Csapo A. Progesterone block. *Am J Anat* 1956; **98**:273–291.
24. Zakar T, Hertelendy F. Progesterone withdrawal: key to parturition. *Am J Obstet Gynecol* 2007; **196**:289–296.
25. Cha JH, Chang MY, Richardson JA, Eidels L. Transgenic mice expressing the diphtheria toxin receptor are sensitive to the toxin. *Mol Microbiol* 2003; **49**:235–240.
26. Golightly E, Jabbour HN, Norman JE. Endocrine immune interactions in human parturition. *Mol Cell Endocrinol* 2011; **335**:52–59.
27. Duffield JS, Tipping PG, Kipari T, Cailhier JF, Clay S, Lang R, Bonventre JV, Hughes J. Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am J Pathol* 2005; **167**:1207–1219.
28. Cailhier JF, Sawatzky DA, Kipari T, Houlberg K, Walbaum D, Watson S, Lang RA, Clay S, Kluth D, Savill J, Hughes J. Resident pleural macrophages are key orchestrators of neutrophil recruitment in pleural inflammation. *Am J Respir Crit Care Med* 2006; **173**:540–547.
29. Ferenbach DA, Sheldrake TA, Dhaliwal K, Kipari TM, Marson LP, Kluth DC, Hughes J. Macrophage/monocyte depletion by clodronate, but not diphtheria toxin, improves renal ischemia/reperfusion injury in mice. *Kidney Int* 2012; **82**:928–933.
30. Turner EC, Hughes J, Wilson H, Clay M, Mylonas KJ, Kipari T, Duncan WC, Fraser HM. Conditional ablation of macrophages disrupts ovarian vasculature. *Reproduction* 2011; **141**:821–831.
31. Mirza R, DiPietro LA, Koh TJ. Selective and specific macrophage ablation is detrimental to wound healing in mice. *Am J Pathol* 2009; **175**:2454–2462.
32. Cailhier JF, Partolina M, Vuthoori S, Wu S, Ko K, Watson S, Savill J, Hughes J, Lang RA. Conditional macrophage ablation demonstrates that resident macrophages initiate acute peritoneal inflammation. *J Immunol* 2005; **174**:2336–2342.
33. Care AS, Diener KR, Jasper MJ, Brown HM, Ingman WV, Robertson SA. Macrophages regulate corpus luteum development during embryo implantation in mice. *J Clin Invest* 2013; **123**:3472–3487.
34. Stoneman V, Braganza D, Figg N, Mercer J, Lang R, Goddard M, Bennett M. Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques. *Circ Res* 2007; **100**:884–893.
35. Van der Hoek KH, Maddocks S, Woodhouse CM, van Rooijen N, Robertson SA, Norman RJ. Intrabursal Injection of Clodronate Liposomes Causes Macrophage Depletion and Inhibits Ovulation in the Mouse Ovary. *Biol Reprod* 2000; **62**:1059–1066.
36. Payne KJ, Clyde LA, Weldon AJ, Milford TA, Yellon SM. Residency and activation of myeloid cells during remodeling of the prepartum murine cervix. *Biol Reprod* 2012; **87**:106.
37. Shapiro DB. An overview of GnRH antagonists in infertility treatments. Introduction. *Fertil Steril*. 2003; **80**:1–7.
38. Rello S, Stockert JC, Moreno V, Gamez A, Pacheco M, Juarranz A, Canete M, Villanueva A. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* 2005; **10**:201–208.
39. Meszaros AJ, Reichner JS, Albina JE. Macrophage phagocytosis of wound neutrophils. *J Leukoc Biol* 1999; **65**:35–42.

40. Feltovich H, Ji H, Janowski JW, Delance NC, Moran CC, Chien EK. Effects of selective and nonselective PGE2 receptor agonists on cervical tensile strength and collagen organization and microstructure in the pregnant rat at term. *Am J Obstet Gynecol* 2005; **192**:753–760.
41. Kirby MA, Heuerman AC, Yellon SM. Utility of Optical Density of Picrosirius Red Birefringence for Analysis of Cross-Linked Collagen in Remodeling of the Peripartum Cervix for Parturition. *Integr Gynecol Obstet J* 2018; **1**:1–3.
42. Croy BA, Yamada AT, Adamson SL, DeMayo FJ. *The Guide to Investigation of Mouse Pregnancy*. Chap 1, Fig 13, San Diego, Ca: Academic Press; 2014:18.
43. Yellon SM, Ebner CA, Sugimoto Y. Parturition and recruitment of macrophages in cervix of mice lacking the prostaglandin f receptor. *Biol Reprod* 2008; **78**:438–444.
44. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci* 2013; **110**:17253–17258.
45. McWhorter FY, Davis CT, Liu WF. Physical and mechanical regulation of macrophage phenotype and function. *Cell Mol Life Sci* 2015; **72**:1303–1316.
46. Blennerhassett MG, Vignjevic P, Vermillion DL, Collins SM. Inflammation causes hyperplasia and hypertrophy in smooth muscle of rat small intestine. *Am J Physiol* 1992; **262**:G1041–G1046.
47. Croy BA, Chen Z, Hofmann AP, Lord EM, Sedlacek AL, Gerber SA. Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. *Biol Reprod* 2012; **87**:125 (1–11).
48. Kirby LS, Kirby MA, Warren JW, Tran LT, Yellon SM. Increased innervation and ripening of the prepartum murine cervix. *J Soc Gynecol Investig* 2005; **12**:578–585.
49. Di Tommaso S, Cavallotti C, Malvasi A, Vergara D, Rizzello A, De Nuccio F, Tinelli A. A qualitative and quantitative study of the innervation of the human non pregnant uterus. *CPPS* 2017; **18**:140–148.
50. Leppert PC. Anatomy and physiology of cervical ripening. *Clin Obstet Gynecol* 1995; **38**:267–279.
51. Timmons BC, Mahendroo MS. Timing of neutrophil activation and expression of proinflammatory markers do not support a role for neutrophils in cervical ripening in the mouse. *Biol Reprod* 2006; **74**:236–245.
52. Heuerman AC, Hollinger TT, Mesiano S, Menon R, Yellon SM. Role of progesterone receptor isoforms in the pregnant mouse cervix during prepartum remodeling. *Reprod Sci*. 2019 Jan 17:1933719118820462. doi: 10.1177/1933719118820462 [Epub ahead of print].