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

Biology of Reproduction

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

--Manuscript Draft--

Manuscript Number:	BIOLRE-2018-0423R1
Full Title:	Effects of macrophage depletion on characteristics of cervix remodeling and pregnancy in CD11b-dtr mice
Short Title:	Macrophage depletion and cervix remodeling
Article Type:	Research Article
Section/Category:	Pregnancy
Keywords:	parturition; diphtheria toxin; collagen; monocytes; , ripening; preterm birth
Corresponding Author:	Steven M. Yellon Loma Linda University School of Medicine Loma Linda, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Loma Linda University School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Steven M. Yellon
First Author Secondary Information:	
Order of Authors:	Steven M. Yellon
	Erin Greaves
	Anne C Heuerman
	Abigail E Dobyns
	Jane E Norman
Order of Authors Secondary Information:	
Manuscript Region of Origin:	UNITED STATES
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, kidney, but not liver, ovary, or other non-reproductive tissues in DT- compared to saline-treated dtr mice or wildtype controls given DT or saline. Within 24h 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 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.
Suggested Reviewers:	Dean Myers Oklahoma State University Center for Health Sciences Dean-Myers@ouhsc.edu
	Nardy Gomez-Lopez

	Wayne State University Health Sciences Center ngomezlo@med.wayne.edu
	David Olson Alberta Health dmolson@ualberta.ca
	Jeff Keelan University of Western Australia Faculty of Science jeff.keelan@uwa.edu.au
	Robert Taylor Wake Forest University School of Medicine rtaylor@wakehealth.net
	George Saade University of Texas Medical Branch Galveston gsaade@UTMB.edu
	Yoel Sadovsky Magee-Womens Research Institute ysadovsky@mwri.magee.edu
Opposed Reviewers:	Ann Word University of Texas Southwestern Medical Center at Dallas ruth.word@utsouthwestern.edu conflict of interest
	Mala Mahendroo University of Texas Southwestern Medical Center at Dallas Mala.Mahendroo@UTSouthwestern.edu conflict of interest
Additional Information:	
Question	**Response**

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

Short title: Macrophage depletion and cervix remodeling

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.

Keywords: parturition, diphtheria toxin, collagen, monocytes, ripening, preterm birth

S.M. Yellon[1,2], E. Greaves[3], A.C. Heuerman[1], A.E. Dobyns[1], J.E Norman[3]

[1] Longo Center for Perinatal Biology, [2] Division of Physiology, Departments of Basic Sciences, and Pediatrics,
Loma Linda University School of Medicine, Loma Linda, CA 92350, and
[3] MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh, Scotland EH16 4TJ United Kingdom

Correspondence: Steven M. Yellon, Ph.D., Longo Center for Perinatal Biology, MRW A572, Loma Linda University School of Medicine, Loma Linda, CA 92350. PHONE: 909-558-4325; FAX: 909-558-4029; e-mail: syellon@LLU.edu

Grant support: NIH HD954931, 2013-14 UK-US Fulbright Commission, All Disciplines Scholar award, and the Department of Pediatrics Research Fund

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, kidney, but not liver, ovary, or other non-reproductive tissues in DT- compared to saline-treated *dtr* mice or wildtype controls given DT or saline. Within 24h 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 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.

Introduction

More than 10% of all pregnancies world-wide 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 occurs 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 nonpregnant 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 nonpregnant mice. For the ovary, DT-treatment of nonpregnant *dtr* mice established an essential role for macrophages to maintain ovarian vascularity and corpus luteum function [30, 33]. Use of this model for the study 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

Experiment 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 Home Office guidelines under approved Project licenses and Veterinarian supervision.

For the 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, 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 0.1ml 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

response 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 x 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 understand 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 structure [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's 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 24h 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., 96h and 48h 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), 24h 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. By example, 24h after DT injection, the 2 segments in each uterine horn of this *dtr* mouse each contained two fetal compartments (see arrows in Figure 2B bottom panel) separated by a vascular-dense zone (presumably fetal membranes from post-mortem observation). Based upon shape and firmness to touch, the uterus seemed contracted. The reproductive tract and uterine contents in the other 8 of DT-treated *dtr* mice were similar to that in saline controls. For all *dtr* mice, irrespective of 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, 5 of 7 *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 7 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 post-partum 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, 24h 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 (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 24h 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 3 subregions, the density of macrophages were 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 24h 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.

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, optical density 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's 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 nonpregnant or male mice, fewer macrophages were found in the kidney within 24h 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 the 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 24h 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 increased in density of macrophages that is proposed to 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 24h 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 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 a 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 post-term 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 differentiate 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.

Acknowledgments

We are thankful for the assistance in conduct of experiments by the technical staff at the University of Edinburgh Centres for Reproductive Health and Inflammation Research (Jean Wade, Spike Clay, Gary Bothwick). Professor Forbes Howie helped generate the serum progesterone data and the SuRF Core Staff (Michael Millar, Ruth Hamblin, Laura Johnstone, Melanie McMillan, and Robert Morris) contributed to process of tissues for histology. The help of Victoria Magloire to evaluate resident macrophages in non-reproductive tissues and of Brigitte Vazquez in manuscript preparation was appreciated. We are grateful for the help of Jeremy Hughes for procurement of the *dtr* mice (University of Edinburgh MRC Centre for Inflammation Research, Queen's Medical Research Institute), Professor Graham Burton for the consult about the placenta (Cambridge University, Director of the Centre for Trophoblast Research), and to Donald R. Chase, M.D. at Loma Linda University (past Director of the California Tum Ferenbach or Tissue Registry for help with microscopy.

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 Suppl 1: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 (1-17).

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 (Lausanne) 2017; 8:196 (1-7).

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: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; 144 Suppl 1:S82-89.

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:R525-545.
21. Larsen B, Hwang J. Progesterone interactions with the cervix: translational implications for term and preterm birth. Infect Dis Obstet Gynecol 2011; 2011:353297 (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 Suppl 1:S1-S7.

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; 1-3.

42. Croy BA, Yamada AT, Adamson SL, DeMayo FJ. The Guide to Investigation of Mouse Pregnancy. Academic Press; 2014: Chapter 1 Fig 13 p18.

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 U S A 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:G1041-1046.

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. Curr Protein Pept Sci 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. Reproductive Sciences (in press 2018).

Figure Legends

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/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).

Figure 2. A. Representative photographs of the reproductive tract from *dtr* mice on day 15 (D15) postbreeding that had been injected 24h 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 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 % 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 4pm 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 24h 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's t-test, n=4-9/group).

Figure 4. Photograph of a picrosirius red-stained section of cervix from a *dtr* mice on day 15 postbreeding, 24h after injection of Saline (Sal) or DT. The 9 non-overlapping boxes represents 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/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/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 (Students t-test).

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 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 24h 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.

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 24h 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 (Students t-test).

Responses to Reviewers comments

The Reviewers recognized the importance of this study and our efforts to advance understanding of the role of macrophages in cervix remodeling. The remarkable finding that this immune cell may be important to sustain pregnancy was pure serendipity. This unanticipated effect of macrophage depletion/impairment on fetal morbidity and loss of pregnancy without birth diverted some attention from the original hypothesis and focus of study on the cervix. This revision aspires to clarify appreciation of the novel contribution and importance of results that advances understanding of the role of this immune cell in prepartum cervix remodeling. Specifically clarified and highlighted in this revision are the innovative analyses of remodeling characteristics and subregions, as well as new insights from addition of a more comprehensive evaluation of macrophage morphology. This study also applied the same high standards of analyses from previous publications to establish the usefulness of the CD11b-*dtr* mice model to conditionally arrest macrophage activities (by acute reduction in numbers and impaired function of remaining resident cells) - an effective block of both cervix ripening and birth (final paragraph in Discussion). Although outcomes were not necessarily as expected, this is the 1st report of findings in this transgenic conditional macrophage ablation model in pregnant mice and provides evidence for a direct link of the importance of CD11b^{+}, F4/80^{+} resident macrophages for cervix ripening and sustaining pregnancy.

Reviewer 1

1. *Did the authors consider that the administration of DT could be removing other cell types besides macrophages? CD11b is also expressed in neutrophils and dendritic cells.… consider changing "macrophage depletion" with "CD11b+ cell depletion" in the title and throughout the manuscript.*

Yes, this was a consideration, but an extensive literature for the *dtr* mouse model (refs #25, 27-34 particularly relevant to this study) specified macrophages in the title and none included the term 'CD11b cells'. This is likely due, in part, to findings that DT treatments of *dtr* mice do not deplete neutrophils (refs 29,30) or appear to affect most other resident CD11b cells. There are many similarities between the effects of DT to deplete F4/80 macrophages in various tissues among these studies and the present report with the exception of the ovary, a likely consequence of a role for macrophages to regulate changes in estrous cycle vasculature but not the maintenance of the corpus luteum during pregnancy. In the present study, as well, the sparse presence of neutrophils in the prepartum cervix was not further diminished by DT treatments. As for dendritic cells, information is quite limited about their presence in the cervix of any species (e.g., PMID10929950 at term or nonpregnant human cervix). Perhaps of relevance, a study using DT treatment of nonpregnant *dtr* mice determined that myeloid cells (mainly macrophages), but not dendritic cells are important for development of renal fibrosis (PMID21127386). Another circumstantial note is that our previous flow cytometry study found little change in other leukocyte populations in the prepartum cervix of mice (PMID22914314). Thus despite the possibility of CD11b expression in other cells, there is little to link these myeloid cells or other leukocytes to cervix remodeling compared to resident F4/80-stained macrophages, which have been implicated as a characteristic associated with the transition from a soft to ripening cervix. These points are clarified in the revision (lines 83-85, 308-10). Thus, the title reflects a current consensus that is consistent with past publications.

2. *Methods. Lines 79-80. Why were the mice injected on day 14 and/or 16?*

Macrophages are reported to substantially repopulate other tissues with 48-96h of a single DT injection into CD11b dtr mice (refs 29,30). Thus, the intent was to extend the duration of CD11b cell depletion to include the period from day 15-18 when the cervix transitions from soft to ripening by injecting DT (or vehicle as the control) on days 14 and 16 postbreeding into *dtr* mice in D18 and D19 groups. The 2 DT injections 48h apart follows a previous protocol that prolonged diminished tissue resident macrophages (ref 34). The sentence is revised to include this explanation (lines 88-93).

3. *Results. Lines 80-81 and Figure 1. Please explain why tissue analysis occurred 48 hours after the injection of DT on day 16, but only 24 hours after the injection of DT on day 14.*

The study was designed with the goal to assess prolonged (item 2 rationale above) compared to acute (24h) effects of DT treatment on the abundance of resident macrophages in the cervix during the critical transition from soft to ripening (lines 88-93). The hypothesis was that prolonged reduced density of macrophages blocked activated phenotypes that drive characteristics associated with structural remodeling. The remarkable finding that fetal mortality occurred without preterm birth in a significant proportion of DT-treated CD11b mice and pregnancy was not sustained, confounded interpretation of findings about the remodeling process. The explanation has been added (lines 267-79).

4. *Results. Lines 119-121. Please consider re-writing this paragraph, it is a little confusing.*

Thank you for the opportunity to clarify and revise this paragraph (lines 134-40).

5. *Results. Lines 125-136. Please consider re-writing this paragraph in order to clarify the importance of these observations. In addition, can the authors add a reference supporting that the characteristics such as "dark haemorrhagic mass", fetal compartment compaction, and diminished uterine segment size are related to fetal viability?*

With appreciation for this recommendation, the paragraph is re-written to better explain the evidence that indicates fetal demise without preterm delivery (lines 142-52). These observations to indicate fetal mortality and resorption (Fig 3B bottom panel) are typical of images found in a search of Google images with the key words 'fetal resorption mice', as well as in PMID16148158 Fig 1 and in a photo in the outstanding 2014 book edited by Croy, Yamada, DeMayo, and Adamson entitled 'The Guide to Investigation of Mouse Pregnancy' (Chapter 1 Fig 13 p18, new ref 42). Although none of these reports reference fetal mortality or resorption later in gestation as in the present study, the suggestion for a reference is welcomed.

6. *Results. Line 138-140. What happened to the 7 DT-treated mice which had not delivered by day 19? Was the viability evaluated?*

The 7 DT-treated *dtr* mice had not delivered by the late afternoon of day 19 postbreeding. The study was concluded at this time after consultation with the institutional veterinarian because saline-treated *dtr* controls had given birth about 12-15h earlier. Text (now lines 162-65) and the Fig 3 legend are revised accordingly.

7. *Results. Figure 3* [now Fig 2]*. Could you please increase the resolution of this image?*

Done. With apologies, this commendation was appreciated (and necessary).

8. *Results. Figure 4* [now Fig 3]. *The morphology of the cervical tissues does not look comparable between WT and dtr mice as is stated in the text (Lines 149-151). Please explain*

Good point. Strain variations in densities of cells and macrophages in the cervix of mice are common and noted in previous studies (PMIDs18003949 and 21613631). The chance to correct this oversight is appreciated (see lines 177-178, Figure 3 and legend). Moreover, this comment led to the discovery that macrophage morphology is predictive of impairment leading to apoptosis (PMIDs24101477, 25504084, 9886244, 15711936 detailed in Responses to Reviewer 3). These insights led to analyses of DT effects on macrophage morphology as new findings that strengthen the main focus of the report on cervix remodeling (lines 11, 114-16, 180-83, 189-91, 244-46, 257, revised Figure 3, and new Supplement Figure 1).

9. Figures. Figures 1 and 2 can be merged in one figure.

Done.

Reviewer 2

1. *The authors just observed the density of macrophages in cervix and other reproductive tissues. However, they should determine other immune cells, such as lymphocytes and NK cells in cervix, uterus, deciduas, placenta and ovary.*

With limited resources, time, and personnel for this study at the University of Edinburgh, we focused on the highest priority endpoints for analyses of cervix remodeling. The presence of neutrophils in cervix and placenta of saline- and DT-treated *dtr* mice was found not to support a major role for this immune cell in the remodeling process (lines 288-91, 242-46, 309-10). There is also little to suggest a role for lymphocytes or NK cells in processes related to cervix ripening during the shift from a soft to ripening cervix in women, due in part to a lack of biopsy material, while in the cervix stroma mice, a paucity of lymphocytes and no NK cells has been reported (PMID2015366). By contrast, the focus on resident macrophages in the cervix comes from previous replicable microscopy studies (ref 4 review), as well as a 2012 flow cytometry study in which the increased presence of leukocytes in the prepartum cervix resulted, for the most part, from an increased presence of macrophages (PMID22914314, Fig 3C). This conclusion was based upon evidence from methods in which systemic blood was removed, a live cell marker actually used, and careful setting of gates that did not exclude monocytes or macrophages (important criteria that were absent in PMID19234164). Thus with all due respect to priorities and objectives related to cervix remodeling, the study of other immune cells among reproductive tissues was outside the scope and resources of this study's focus. In appreciation of this constructive suggestion, the revision includes a strengthened rationale for the focus of study on macrophages (lines 45-47, 81-83) and highlights the need to follow up with study other immune cells and tissues (lines 311-12).

2. *They just described the morphology of cervix and roughly described morphology of placenta and made a conclusion that pregnancy loss is associated with altered morphology of pregnancy… should describe the detailed morphology of deciduas and placenta…uterus and ovary. In addition, the expression levels of contraction-associated proteins in uterus should also be determined.*

Although these suggestions would be interesting, they are well beyond the scope or feasibility of this study. In due consideration, these parameters and tissues would be worthy to explore of further study using this model (now mentioned in the Discussion lines 288-94).

3. *They should also determine the estrogens, PGs and glucocorticoids in circulation besides progesterone because these hormones are critical for maintenance of pregnancy.*

This list of systemic hormones that are important to various aspects of pregnancy is extensive. However, there is clearly consensus that progestogens, specifically progesterone, is the only hormone that is critical to maintain pregnancy, i.e., completely capable of maintaining pregnancy to term. Other endocrine studies were not possible due to limited serum volume, lack of resources for additional mice and personnel to explore finding related to fetal mortality, and an uncertain direct link of these to specific characteristics related to prepartum cervix remodeling. The present study does not exclude the important contribution of these hormones for aspects of pregnancy, fetal development, parturition, and newborn well-being. Accordingly, this important point has been added (lines 327-28).

Reviewer 3

The authors are sincerely grateful for the comments that *this manuscript is well written and presented and the methodologies are clear. Appropriate controls are included throughout the experimental protocols, and the inclusion of a methodological schematic figure was appreciated. I found the figures and the introduction, methods, and results sections to be clear and informative*.

Comments

1. *decreased cell nuclei density, uterine compaction and pregnancy loss, could be in response to an inflammatory reaction induced by apoptotic fragments from the ablation* [of macrophages] *and not due to the conditional absence of macrophages themselves. Could the authors have measured the comparative expression of apoptotic markers and inflammatory cytokines/chemokines in the cervical tissues in saline and DT-treated CD11b-dtr and WT mice?*

This summary is insightful and accurate. This comment led us to a more comprehensive analysis of macrophage morphology and apoptosis, as well as to discover a recent literature about the relationship between macrophage morphology and function. First, there is a well-characterized consensus about the distinctive morphological characteristics of a cell undergoing apoptosis (PMID15711936). As previously applied (PMID9886244), the entire set of photomicrographs for cervix from D15 *dtr* and WT mice treated with saline or DT was reanalyze and according to specified criteria (Methods lines 115-18) produced new results (revised Fig 3 insets, lines 176-83, Supplement Figure 1) that clearly indicate apoptosis was ongoing in mostly all remaining resident macrophages in the cervix of *dtr* mice treated with DT treatment (Discussion lines 255-257). Despite an outstanding study of cCaspase-3 in the murine endometrium (PMID29234102), there was insufficient time and limited sections of cervix from *dtr* mice to properly develop this method to assess macrophage apoptosis or other approaches to evaluate inflammation. Day 18 groups were also not analyzed due to the potential confound of DT effects on fetal mortality and viability of pregnancy. Second, a recent information suggests a link between macrophage shape and polarity of function (PMIDs24101477, 25504084) - a link previously noted in our past replicated studies of macrophage density changes during the shift from a soft to ripening cervix. This concept is mentioned as an additional endpoint that supports the contention that DT treatment blocked phenotypic macrophage functions in the cervix in D15 *dtr* mice (lines 277-79). Collectively these findings suggest that absence or loss of activities by CD11b-related F4/80 macrophages are essential for inflammatory processes that reduce cell nuclei density and ultimately ripens the cervix in preparation for birth.

2. *Although 54% of DT-treated dtr mice demonstrated uterine morphologies suggestive of preterm labour, there was minimal evidence of premature ripening of the cervix, and few differences between the saline and DT-treated groups in reference to cervical ripening.*

We agree that premature conclusion of pregnancy was not associated with premature ripening of the cervix. The acuity of this wording improves on what was written and is in the revision (lines 281-85). Also appreciated is the opportunity to clarify that the 'few' differences, i.e., cell nuclei and macrophage densities, as well as new evidence for apoptotic/impaired macrophages in DT- versus saline-treated D15 *dtr* mice were significant (new Supplement Figure 1), the impact of which may likely contribute to observed lack of cervix remodeling or birth in most DT-treated mice at term. These represent 3 of 4 study endpoints that have a collective impact to suggest the arrest of cervix remodeling and the possibility that this may contribute to the lack of birth in most DT-treated mice at term. Subregion analyses appears to support this conclusion, as well. As for cross-linked collagen, the increase in optical density of birefringence, an indication of degradation (PMID30175325), was not significant 24h after DT treatment in cervix sections from D15 DT- vs saline- mice (Fig 4). Although it is conceivable that this finding reflects the loss of an inflammatory drive to stimulate prepartum degradation of the extracellular matrix, this finding was not unexpected because 24h may be an insufficient latency for DT treatment to produce significant differences in the extracellular collagen as a result of reduced or impaired macrophages (mentioned lines 203-04). The Reviewer's comment led us to appreciate that this period of pregnancy has not been a focus of past studies. To improve resolution of cross-linked collagen, further investigation of the period prior to day15 postbreeding could help advance understanding of

cervix structure when major changes in biomechanical compliance/dispensability of cervix occur (Myers et al 2014). Thank you for this constructive and impactful comment.

From these results my understanding is that resident macrophages may be important for sustaining pregnancy and trophoblast function,

Agreed and well-stated. This finding was unanticipated and the attention could overshadow the important advances provided by the principal focus on cervix remodeling. As mentioned in responses to Reviewer 2, pursuit of an explanation for this finding was beyond the focus and resources for this study.

…but the study did not seem to add much additional knowledge about the role of macrophages in the process of cervical ripening specifically.

We believe this statement about the original submission more reflects the lack of clear explanations, a need for context, and possibly the shadow of serendipity of findings relating to fetal morbidity. Explanations in Comments 1 & 2 above are provided to correct this issue. Additional data and a more comprehensive analyses have more clearly explained the essential role for macrophages in cervix remodeling because preterm ripening or birth did not occur when resident macrophages were reduced/impaired by DT treatment and effects on characteristics of remodeling were associated with early conclusion of pregnancy without birth at term in most DT-treated *dtr* mice. This Reviewers comments helped motivate efforts to improve appreciation of the direct link between the presence of macrophages, their phenotypic function based upon presence and shift in morphology, as well as relevance of remodeling characteristics to the transition from a soft to ripe cervix between days 15 and 18 of pregnancy.

(lines 291-293, now lines 322-24*) "The present findings advance the importance of F4/80 macrophages as local producers of effector molecules, including prostaglandins, vasodilators, inflammatory cytokines and collagen degrading enzymes for the ripening process." I don't understand how the authors can make this statement based on their results.*

Sentence revised to raise the possibility that macrophages may be a source for various effector molecules for remodeling.

For their statistical analysis, the authors use student's t-tests or one-way ANOVA. In certain circumstances, such as Figures 2, 4 and 5 a two-way ANOVA would be more appropriate, as there are two changing variables- the timeline of days post breeding and the wt vs dtr mice.

Data for Figure 2 (now Fig 1B) have been reanalyzed and text revised to indicate two-way ANOVA. Figures 4 and 5 data (now Figs 3 and 5) were for a single variable related to effect of DT treatment.

Some additional minor editorial suggestions:

The authors are grateful for the recommended corrections

lines 176-179 figure 4 [now Fig 3] *shows that cell nuclei density was in fact decreased by macrophage depletion.*

Good point. Sentence revised to specify cross-linked collagen (now lines 186-87)

- *lines 224-225 an extra word must be removed, either reading 'with the human DT receptor linked to CD11b myeloid cells' or 'When the human DT receptor is linked to CD11b myeloid cells'.*

Sentence revised (now lines 252-53)

- *line 248 - 'These findings' not 'These finding'.*

Corrected (now line 253)

- *line 271 - missing a period after citations 45, 46* [now 45, 46]

Period added (now line 279)

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

Short title: Macrophage depletion and cervix remodeling

Summary sentence: ~~Depletion~~Conditional depletion of macrophages in CD11b *dtr* mice during the critical period for cervix remodeling interfered with ripening and the progress of pregnancy.

Keywords: parturition, diphtheria toxin, collagen, monocytes, ripening, preterm birth

S.M. Yellon[1,2], E. Greaves[3], A.C. Heuerman[1], A.E. Dobyns[1], J.E Norman[3]

[1] Longo Center for Perinatal Biology, [2] Division of Physiology, Departments of Basic Sciences, and Pediatrics,
Loma Linda University School of Medicine, Loma Linda, CA 92350, and
[3] MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh, Scotland EH16 4TJ United Kingdom

Correspondence: Steven M. Yellon, Ph.D., Longo Center for Perinatal Biology, MRW A572, Loma Linda University School of Medicine, Loma Linda, CA 92350. PHONE: 909-558-4325; FAX: 909-558-4029; e-mail: syellon@LLU.edu

Grant support: NIH HD954931, 2013-14 UK-US Fulbright Commission, All Disciplines Scholar award, and the Department of Pediatrics Research Fund

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. ~~Macrophages~~On day 15 postbreeding, macrophages (F4/80+) were depleted in cervix, kidney, but not liver, ovary, or other non-reproductive tissues in DT- compared to saline-treated ~~CD11b-~~ *dtr* mice or ~~WT~~wildtype controls~~.~~ given DT or saline. Within 24h of DT-treatment, the density of cell nuclei and ~~F4/80-~~macrophages ~~were reduced~~declined in cervix stroma in ~~CD11b-~~ *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 ~~appeared~~repopulated the cervix in DT-treated *dtr* mice so numbers were comparable to ~~repopulate the cervix.~~that in controls. However~~, a significant pregnancy loss~~ at term, evidence of fetal mortality without ~~birth~~cervix ripening occurred in ~~DT-treated CD11b-~~most *dtr* mice~~,~~ given DT- a possible consequence of ~~altered morphology in the trophoblast layers of the placenta and fetal mortality. The~~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.

Introduction

More than 10% of all pregnancies world-wide 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 limit 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 occurs 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 ~~macrophages and their products for~~ 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. ~~Although mice are typically insensitive to DT~~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) ~~[26], in transgenic CD11b *dtr* (*dtr*) mice, injection of small amounts of diphtheria toxin (DT) temporarily depletes systemic and, to an extent, resident macrophages in specific organs~~ [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 nonpregnant or male *dtr* mice selectively and acutely ~~eliminates~~ induces apoptosis in some myeloid cells and macrophages in particular that express ~~the~~ CD11b ~~receptor~~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 nonpregnant mice. For the ovary, DT-treatment of nonpregnant *dtr* mice ~~demonstrated~~established an essential role for macrophages to maintain ovarian vascularity and corpus luteum function [30, 33]. ~~Focus on other aspects~~Use of ~~reproductive function including parturition in particular, using~~ this model for the study inflammatory processes that are associated with cervix remodeling as pregnancy nears term and parturition is lacking. Given the heterogeneity of ~~function~~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 ~~associated with~~ of ripening ~~of the prepartum cervix~~that occur before birth in ~~DT-treated pregnant *dtr* mice.~~controls. In addition ~~to this impact on the process of parturition~~, adverse consequences of DT treatment on fetal viability have broader implications for the importance of macrophages to sustain pregnancy.

Materials and Methods

Experiment 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]. ~~Briefly, this transgenic mouse strain has the human DT receptor linked to the macrophage lineage-specific CD11b receptor promoter region in the mouse genome [33, 36]. As in~~In previous reports ~~with use of this model, there were~~ , no signs of diminished well-being were found following ~~saline or~~ 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 ~~performed~~ in compliance with UK Home Office guidelines under approved Project licenses and ~~Veterinary~~Veterinarian supervision.

For the 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, 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 ~~the~~this *dtr* transgenic model, were similarly treated with saline or DT ~~to control for off target effects of DT. Thus~~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 0.1ml vehicle i.p.; D0564; Sigma Aldrich). ~~Mice were euthanized on days 15, 18, and 19 postbreeding (Figure 1 Treatment schema).~~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 response 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 x 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 understand 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 structure [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's 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 ~~and preterm birth~~, pregnancy, and parturition

Serum progesterone ~~was not affected by DT treatment. Progesterone~~ concentrations were ~~the same~~not significantly different in ~~Saline- versus DT-~~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~~, one after the initial injection~~ was not affected by DT treatment given 24h earlier on day 14~~, and~~ of pregnancy. Serum progesterone concentrations were also equivalent in CD11b *dtr* mice given saline or DT on the morning of day 18~~, 2 days~~ postbreeding, i.e., 96h and 48h after the first and second treatment with saline or DT. Thus, DT had no acute or more long-term effect on ~~day 16 (Figure 2).~~systemic concentrations of progesterone as compared to saline treated controls.

In pregnant *dtr* mice on day 15 postbreeding (D15), 24h 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 ~~3A~~2A top panel). By comparison~~, the uterus of~~ in 3 of 11 DT-treated *dtr* mice ~~had~~, the uterus contained fewer ~~distinct~~ segments. ~~In these mice, multiple~~By example, 24h after DT injection, the 2 segments in each uterine horn of this *dtr* mouse each contained two fetal compartments ~~were compacted and~~(see arrows in Figure 2B bottom panel) separated by a ~~line of~~ vascular-dense zone (presumably fetal membranes from post-mortem observation). Based upon shape and firmness to touch, the uterus seemed contracted. The reproductive tract and uterine contents in the other 8 of DT-treated *dtr* mice were similar to that in saline controls. For all *dtr* mice, irrespective of 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 ~~3B). By contrast, 5 of 7 *dtr* mice injected with DT 4 and 2 days earlier, i.e., days 14 and 16 postbreeding, had compacted uterus that contained remnants of resorbing fetal tissues (dark haemorrhagic mass). The remaining 2 DT-treated *dtr* mice had gross uterine morphology that~~2B top panel). By contrast, 5 of 7 *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 7 DT-treated mice was compact and contained resorbing tissue (presumably ~~foetuses). To assess viability of pregnancy, observation of each fetus for size, color (pink indicated oxygenated vascular supply), and anatomical development, less than 30% of~~fetuses). Of the 3 DT-treated ~~CD11b *dtr* dams sustained pregnancy past day 18 postbreeding (Figure 3C). Of the DT-treated~~ 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 post-partum 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, 24h 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 4A).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 in *dtr* mice after DT injection, macrophages were monomorphic and sparse with reduced brown stain for each cell.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 (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 4B). However3B). 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 24h 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 ~~was~~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 3 subregions, the density of macrophages~~/CN~~ were reduced in DT- versus saline-treated ~~CD11b~~ *dtr* mice (~~p<0.05 ectocervix (~~Supplement Figure ~~1~~2).

For cross-linked collagen fibers in the extracellular stroma, DT injection had no effect on picrosirius red stain birefringence (Figure ~~5~~4). Optical density was not different 24h after DT or saline treatment in WT controls or *dtr* mice ~~compared to that after saline treatment~~. 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 ~~structural aspects of cervix stroma with respect to cell nuclei density or~~ cross-linked collagen in the extracellular matrix.

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 ~~6A~~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 ~~6B~~5B). For collagen as well, optical density 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's 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 nonpregnant or male mice, fewer macrophages were found in the kidney within 24h of DT injection in *dtr* mice compared to saline controls on day 15 postbreeding (Figure ~~7~~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 the 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 ~~in~~of saline *dtr* mice (Figure ~~8).~~7). Other effects of DT treatment were apparent in the subset of *dtr* mice with evidence of fetal demise, i.e., ~~intrauterine hemorrhage~~dark deoxygenated blood, or ~~compact~~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). ~~Moreover, neutrophils~~ Neutrophils were also ~~rare and~~ 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 ~~characteristics of~~ a change in placental morphology that ~~suggests~~may reflect impaired function ~~in trophoblast and possibly other regions~~.

Discussion

~~The hypothesis that macrophages function to promote remodeling of the cervix was tested by conditional depletion of systemic and resident CD11b macrophages in mutant *dtr* mice. With the human DT receptor is linked to CD11b myeloid cells, treatment with DT depleted F4/80 stained differentiated macrophages in the cervix. By comparison in WT mice lacking the DT receptor, no~~

~~effects of DT treatment were evident in the cervix, other tissues, or in pregnancy. These findings are the first to demonstrate effects of DT treatment to reduce the presence and alter the morphology of macrophages in cervix stroma in pregnant CD11b-*dtr* mice. The decline of resident macrophages was associated with decreased cell nuclei density, but not, within 24h, associated with further degradation of cross-linked collagen in *dtr* mice. Thus, loss of the CD11b-related F4/80 macrophage phenotype affected cervix structure in a way that differs from preterm ripening where inflammation in the cervix at term and with preterm birth is characterized by both reduced stromal cell nuclei density and collagen cross-linking [17, 19].~~

~~This conclusion is complicated by other~~
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 24h 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 increased in density of macrophages that is proposed to 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 24h 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~~. Due to the mechanism of DT action in *dtr* mice, apoptotic remnants of macrophages are likely to have induced an inflammatory reaction that may not mimic processes that drive the shift from a soft to ripe cervix at term or with preterm birth. By example, in *dtr* mice with atherosclerosis, DT reduced and impaired macrophages and their function [36]. The resulting apoptotic debris was followed by recovery of macrophage density in vasculature. Moreover, depletions of the CD11b macrophage phenotype was associated with aberrant vascularity between the placental trophoblast giant cell and spongiotrophoblast layers. Whether~~ in this ~~caused pregnancy loss and compaction of the uterus in 30% *dtr* mice by day 15 of pregnancy is not known. CD11b monocytes have been proposed as communicators of sprouting vessels in decidua, and depletion of this cell may have had unintended consequences [40]. The presumed~~ model complicate interpretation of findings. Evidence for repopulation of macrophages in the cervix of ~~CD11b~~ *dtr* mice, 2 days after the second DT injection on day 16 postbreeding, ~~appeared to prevent~~may interfere with the ripening process and account for delayed ~~birth~~parturition beyond that in controls in 70% of ~~treated mice. These finding cannot distinguish between the possibility that CD11b macrophage phenotype may be essential to sustain an unripe cervix or whether~~ DT treated mice. Specifically, the phenotype of repopulated ~~macrophages impair ripening or other critical placental functions that maintain pregnancy.~~

~~Although macrophage depletion was associated with reduced cell nuclei/area, a characteristic typical of inflammation, preterm birth did not occur. Inflammation induced by infection is known to reduce~~

~~cell nuclei density in gut smooth muscle [41]. Rather~~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, ~~there was little evidence of~~ premature cervix ripening clearly did not occur even though ~~54% (15/28) of DT-treated *dtr* mice had compact fetal sacks and a uterine morphology that was suggestive of preterm labor.~~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. ~~Understanding the importance of macrophages for ripening and labor would likely benefit from studies of the diversity of resident macrophages phenotypes and their function to regulate inflammation that results from apoptotic signals or stimuli that promote extracellular matrix degradation, as in found in other tissue during remodeling processes [42-44]~~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 ~~not only is~~ the cervix a separate and distinct component of the reproductive tract during pregnancy~~, but~~. 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 ~~the heterogeneous~~a heterogeneity in structure ~~of this tissue, there may be more~~, 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). ~~As a separate structure, the cervix is highly innervated compared to the uterus [45, 46] The~~ Subregional differences in collagen organization and smooth muscle ~~described in a review of cervix remodeling by Leppert's group and others [47, 48], preceded studies of cell nuclei or macrophage densities, but~~content in the cervix were well-recognized [50] and are consistent with findings ~~by Yoshida et al. did find decreased~~ that cross-linked collagen ~~by 4 days~~is decreased before labor and birth in mice ~~[~~[4, 13~~]~~, 41]. ~~These findings raise the possibility that remodeling of the prepartum cervix may~~. This period when the cervix transitions from soft to ripening coincides with reduced cell density and increase macrophage density, evidence of a inflammatory process that is proposed to reflect a uniformity of ~~ripening~~prepartum remodeling that ~~transitions~~

~~into~~precedes dilation, effacement, and a transformation into the lower uterine segment, a term that lacks any reported structural identity across species. ~~Although a role for distinct macrophage populations has yet to be defined, the maturation of phenotypes related to CD11b^{+} in the present study and F4/80^{+}~~ These latter changes in peripartum cervix morphology have been associated with an increased presence of neutrophils [17, 51]~~are implicated as important for remodeling and parturition. However for neutrophils,~~, but little or no change in residency in the present study ~~coupled with evidence for a later increase in the peripartum cervix [17, 50] does~~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 post-term 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 ~~in preparation for labor and delivery focuses on the importance of resident macrophages and their interactions with stromal fibroblasts and the extracellular matrix. This transition occurs~~ 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 ~~[21]. Understanding how~~and extends from Csapo's progesterone block hypothesis that progesterone becomes unable to sustain an unripe cervix ~~is a question that extends from Csapo's progesterone block hypothesis [21, 24]. The loss of these pregnancy sustaining actions must involve a reduced efficacy by genomic progesterone receptors, which are found in stroma cells, but absent in resident macrophages [17].~~ [23]. The present findings advance the importance of ~~F4/80~~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

Formatted: Right: -0.1"

macrophages ~~as local producers of~~may be effector cells in the ripening process because of known capabilities to produce molecules~~,~~ including prostaglandins, vasodilators, inflammatory cytokines, and collagen degrading enzymes ~~for the ripening process.~~. These actions may be guided, in part, by the convergence of ~~contributing~~local factors that regulate differentiate 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.

Acknowledgments

We are thankful for the assistance in conduct of experiments by the technical staff at the University of Edinburgh Centres for Reproductive Health and Inflammation Research (Jean Wade, Spike Clay, Gary Bothwick). Professor Forbes Howie helped generate the serum progesterone data and the SuRF Core Staff (Michael Millar, Ruth Hamblin, Laura Johnstone, Melanie McMillan, and Robert Morris) contributed to process of tissues for histology. The help of Victoria Magloire to evaluate resident macrophages in non-reproductive tissues and of Brigitte Vazquez in manuscript preparation was appreciated. We are grateful for the help of Jeremy Hughes for procurement of the ~~CD11b~~ *dtr* mice (University of Edinburgh MRC Centre for Inflammation Research, Queen's Medical Research Institute), Professor Graham Burton for the consult about the placenta (Cambridge University, Director of the Centre for Trophoblast Research), and to Donald R. Chase, M.D. at Loma Linda University (past Director of the California Tumor Tissue Registry for help with microscopy.

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 Suppl 1: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 (1-17).

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 (Lausanne) 2017; 8:196 (1-7).
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: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; 144 Suppl 1:S82-89.
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:R525-545.

21. Larsen B, Hwang J. Progesterone interactions with the cervix: translational implications for term and preterm birth. Infect Dis Obstet Gynecol 2011; 2011:353297 (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 Suppl 1:S1-S7.

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; 1-3.

42. Croy BA, Yamada AT, Adamson SL, DeMayo FJ. The Guide to Investigation of Mouse Pregnancy. Academic Press; 2014: Chapter 1 Fig 13 p18.

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 U S A 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:G1041-1046.

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. Curr Protein Pept Sci 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. Reproductive Sciences (in press 2018).

Figure Legends

Figure 1. **A** Timeline of injection of saline (Sal) or diphtheria toxin (DT) administered intraperitoneal on days 14 and 16 postbreeding into homozygous ~~CD11b~~ *dtr* mice. X indicated day of euthanasia when blood and tissues were collected for study (n= 4-10/group.

B. ~~**Figure 2.**~~ Serum progesterone concentrations on days 15 and 18 postbreeding in Saline- or DT-treated ~~CD11b~~ *dtr* mice. Treatments described in Methods. p>0.05 ~~Saline-treated day 15~~two-way ANOVA (n=4~~) vs D18 (n=~~ 5~~)~~ mice ~~or p>0.05 DT-treated day 15 (n=5) vs 18 (n=4) mice.~~/group).

~~**Figure 3.**~~**Figure 2.** **A.** Representative photographs of the reproductive tract from *dtr* mice on day 15 (D15) postbreeding that had been injected 24h 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 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 ~~estimates~~of the % of viable pregnancy in DT-treated *dtr* mice based upon ~~morphological characteristics~~morphology and firmness of uterus ~~and~~, 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 4pm in the afternoon when the study concluded.

Figure 43. A. Photomicrographs of cervix sections on day 15 (D15) from wild-type (WT) or CD11b *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 CD11b *dtr* mice (right) injected 24h 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's t-test, n=4-9/group).

Figure 54. Photograph of a picrosirius red-stained section of cervix from a CD11b *dtr* mice on day 15 postbreeding, 24h after injection of Saline (Sal) or DT. The 9 non-overlapping boxes represents the area analyzed for optical density (9 photomicrographs in each of 3 sections/cervix). Scale bar is =50 μm. Histogram 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/group).

Figure 65. A. Top panels. Photomicrographs of cervix sections stained for cell nuclei and macrophages from a day 18 (D18) postbreeding saline (Sal)- or DT-treated CD11b *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/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 (Students t-test).

Figure 76. 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 diminished density of macrophages in kidney, but not liver or ovary.

Figure ~~8~~7. Photomicrographs of ~~D15~~placenta sections from CD11b *dtr* mice on D 15 postbreeding ~~*dtr* mice~~ treated 24h earlier with ~~saline~~DT without or ~~DT.~~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.

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.

Formatted: Font: Bold

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 ~~CD11b~~ *dtr* mice on day 15 postbreeding that had been injected 24h 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 ~~4~~3. p>0.05 DT vs Sal group within each strain (Students t-test).

Figure 1

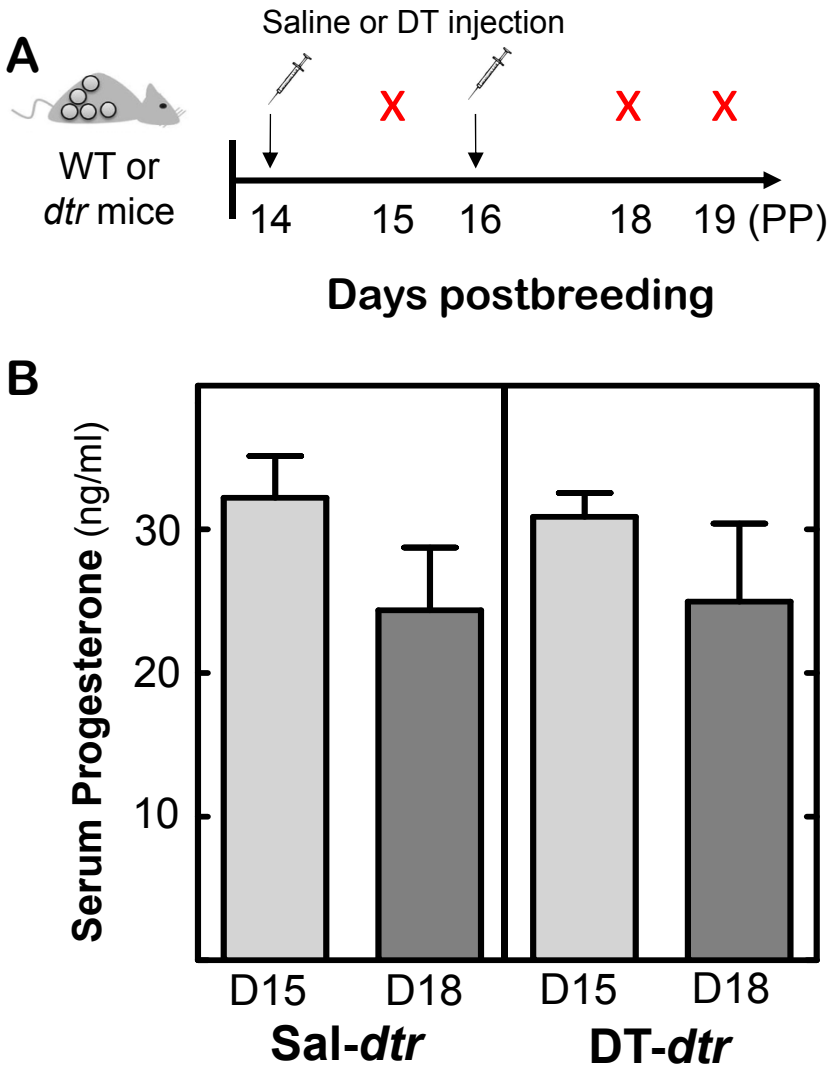


Figure 2

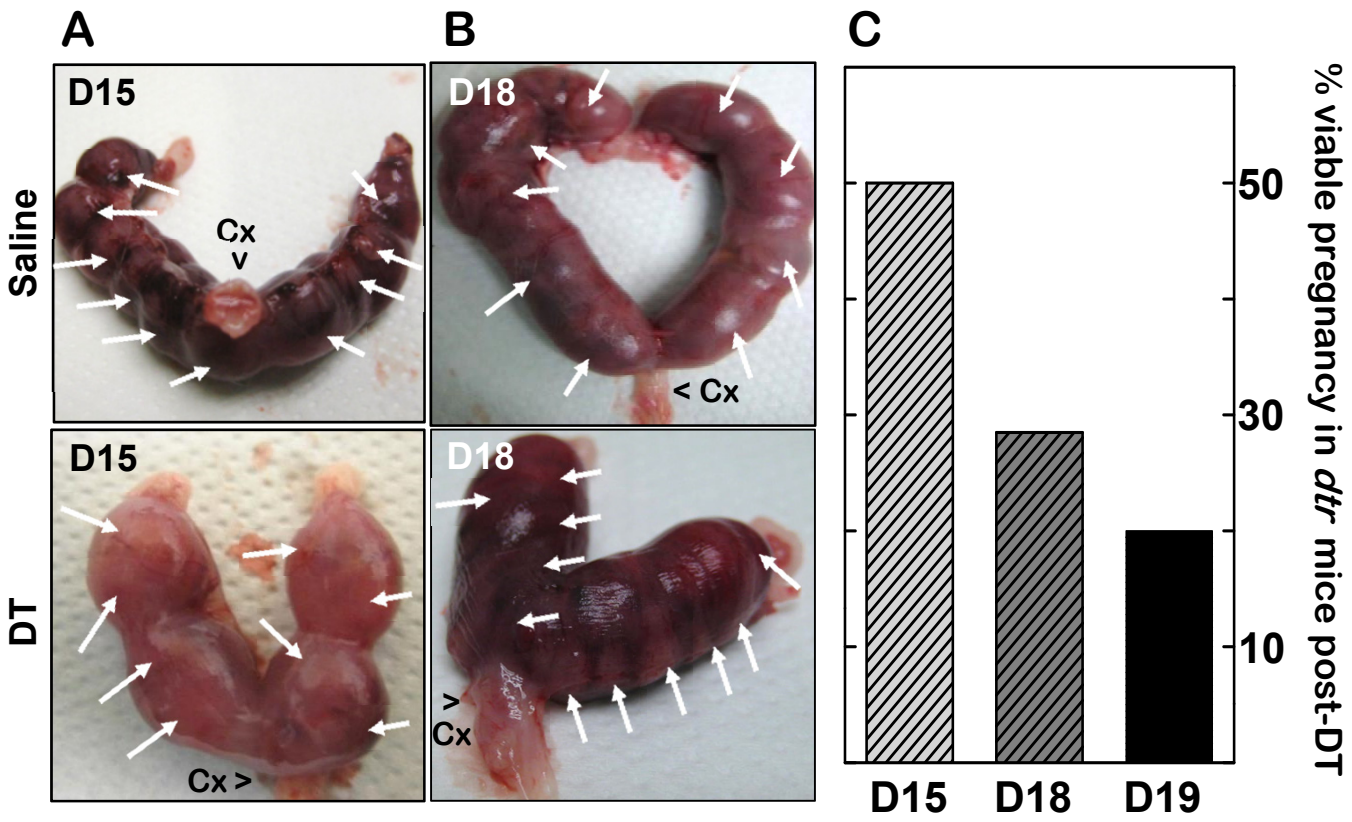


Figure 3

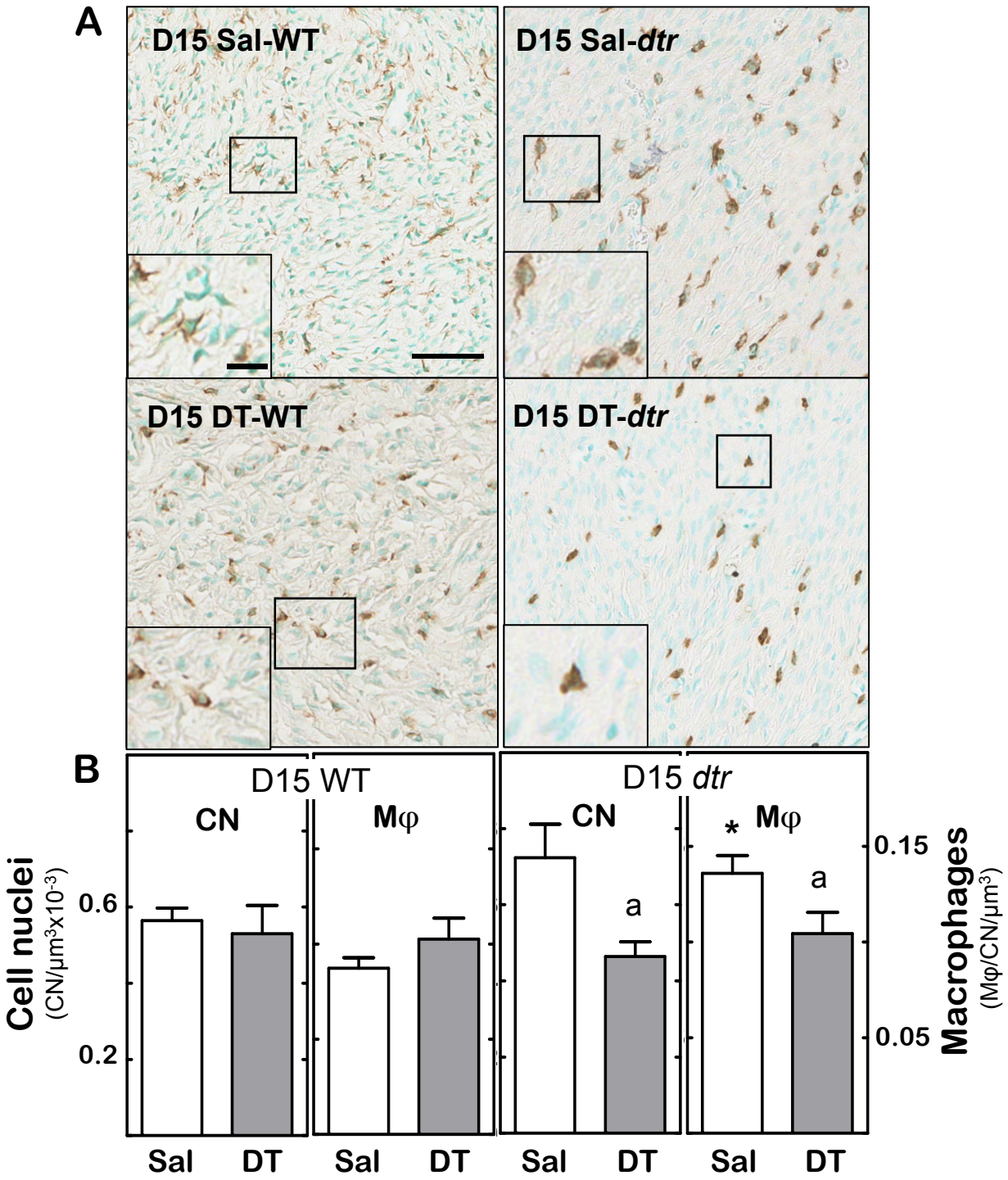


Figure 4

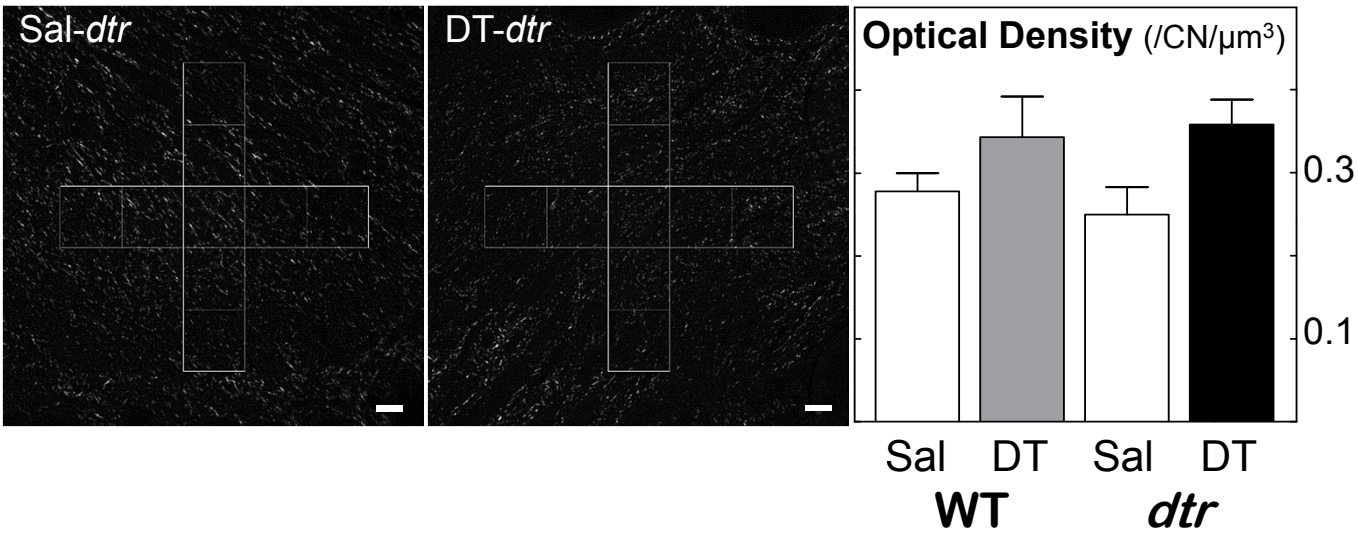


Figure 5

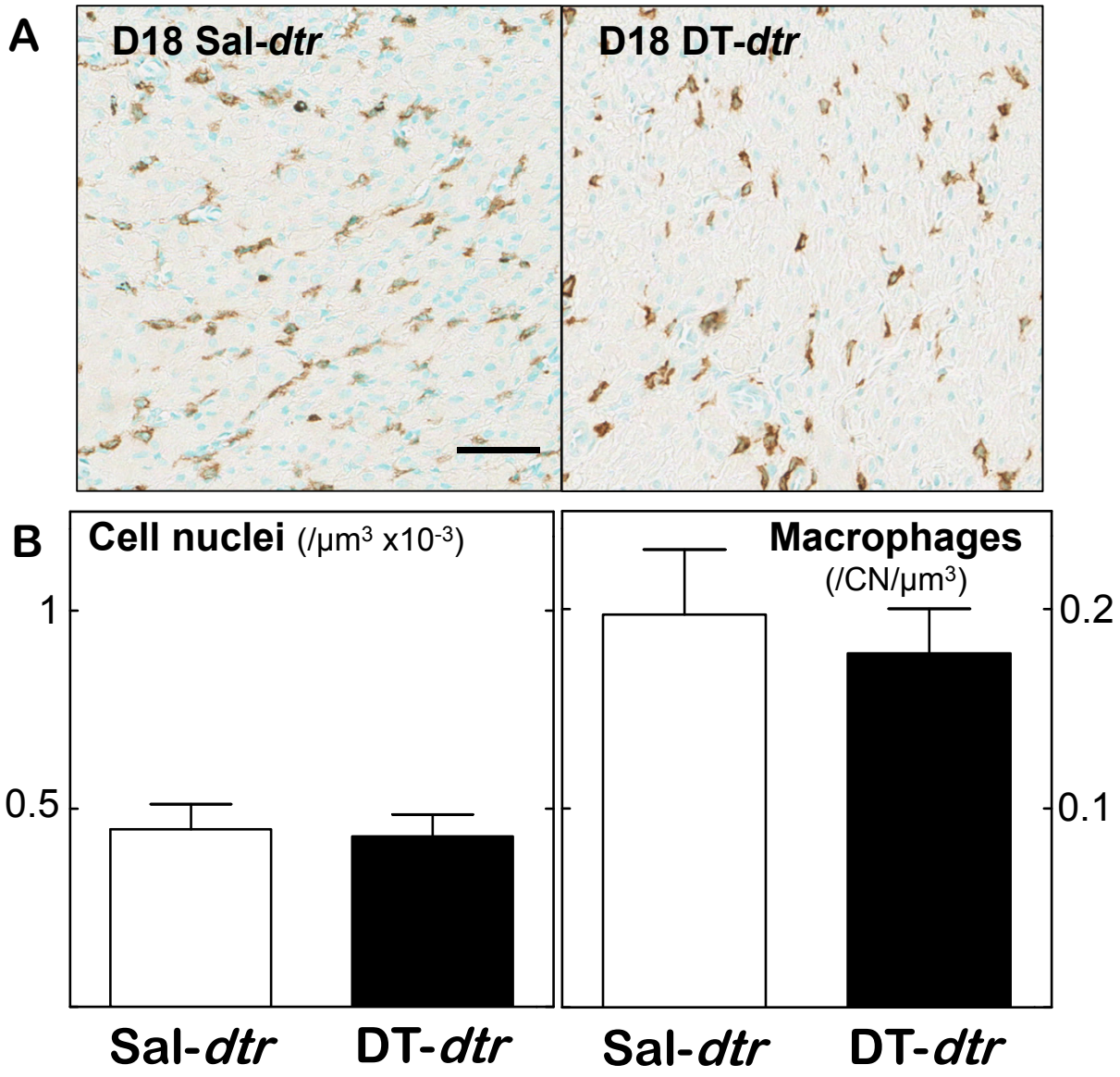


Figure 6

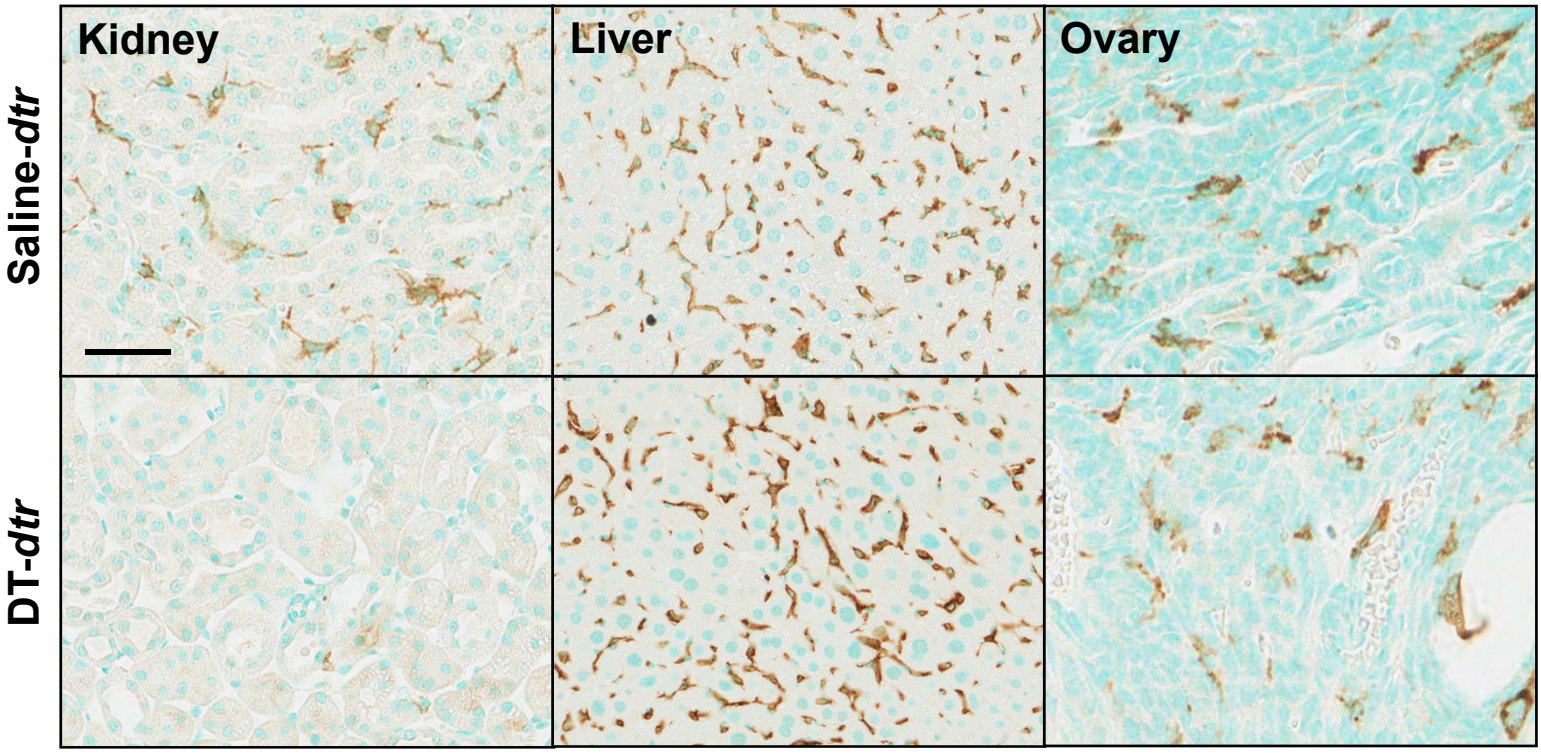
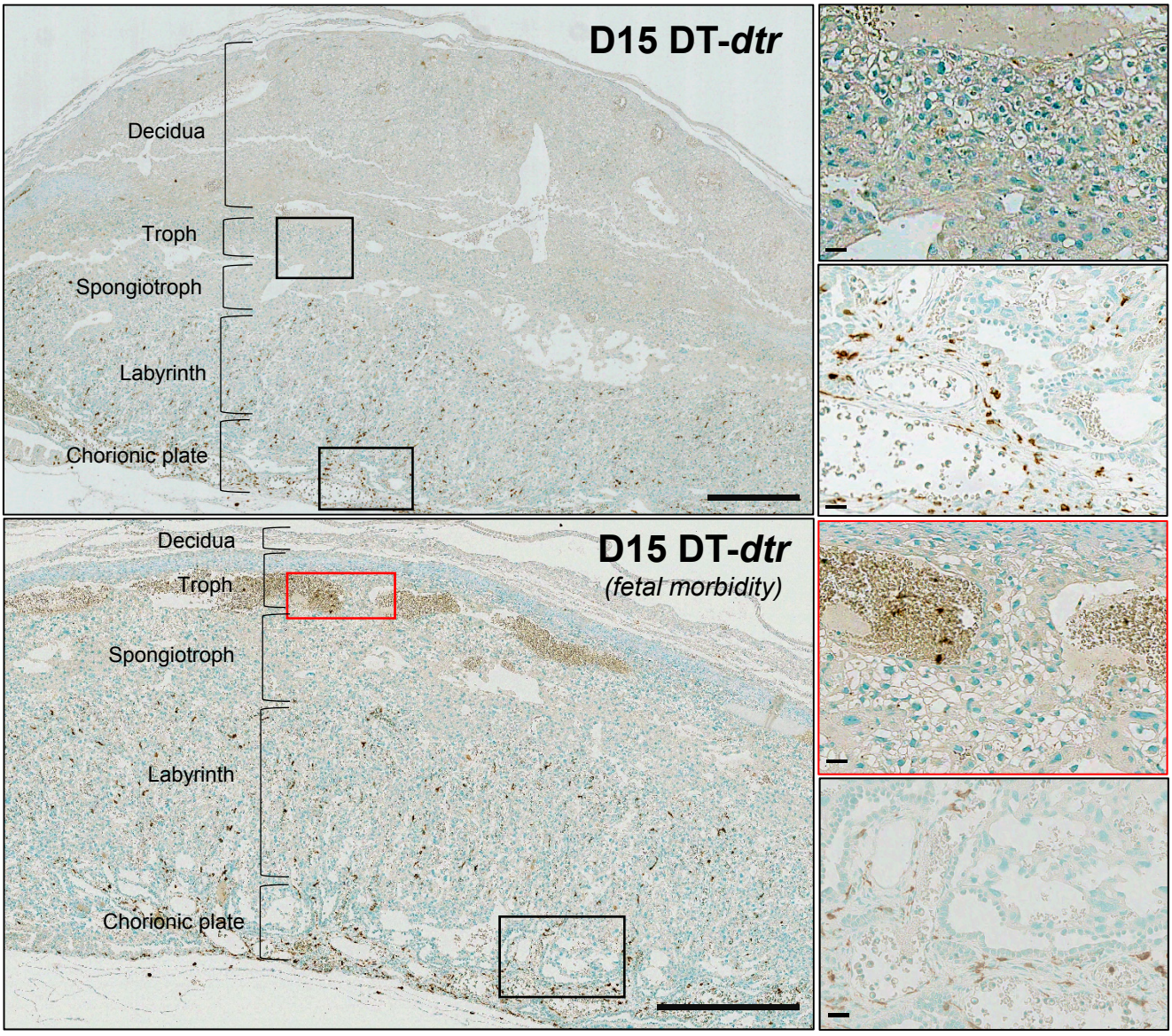


Figure 7



Supplemental Figure 1

Supplemental Figure 2